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THE UNIVERSITY OF ALBERTA

S-2-AMINOETHYL CYSTEINE RESISTANCE AND ANTIBIOTIC OVERPRODUCTION IN *STREPTOMYCES CLAVULIGERUS*

BY

CLARE GALLAGHER

A THESIS

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(Student's signature)

Student's permanent address:

1473 2nd Ave. N.E.

Medicine Hat, Alberta

T1A 6A7

Date: December 15 1988

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled S-2-AMINOETHYL CYSTEINE RESISTANCE AND ANTIBIOTIC OVERPRODUCTION IN *STREPTOMYCES CLAVULIGERUS* submitted by Clare Gallagher in partial fufilment of the degree of Master of Science.

.....
(Dr. S.E. Jensen - Supervisor)

.....
(Dr. M. R. Gray)

.....
(Dr. M. A. Pickard)

.....
(Dr. R. vonTigerstrom)

.....
(Dr. D. W. S. Westlake)

Date: December 8 1988

Dedicated to my Mother,

without whose support and encouragement,

this thesis would not have been completed

ABSTRACT

Studies of mutagenesis of *Streptomyces clavuligerus* were initiated as part of a long term effort to study S-2-aminoethyl cysteine (AEC) resistance in this organism. The three mutagenic treatments investigated were irradiation, treatment with N'-methyl-N'-nitro-N-guanidine (NTG) and transposon mutagenesis. Ultraviolet light was found to be more efficient than NTG in the production of mutants. Ultraviolet light and NTG mutagenized germinated spores at a greater rate then were ungerminated spores. In an effort to assess the feasibility of the third mutagenic treatment, insertional mutagenesis, the transposon Tn4560 was introduced into *S. clavuligerus*.

Mutants resistant to the lysine analog AEC were isolated after a variety of mutagenic procedures. Antibiotic production in these mutants was found to vary depending on type of growth medium used. Overall production of antibiotics was higher in complex medium and lower in defined medium. AEC resistant strains showed variable antibiotic production with some overproducing and others underproducing.

Aspartokinase production in wildtype and AEC resistant strains was examined. It was found that all the mutant strains overproduced the enzyme. The effects of lysine and threonine on aspartokinase production was variable with respect to the wildtype. The only consistent response among the mutants was a drastically reduced production of aspartokinase when AEC was present in the growth medium.

The regulatory characteristics of the aspartokinase in these mutants was

investigatedfor evidence of altered patterns of feedback inhibition by lysine and threonine, but these effects were also variable. Only one mutant tested appeared to show deregulation of aspartokinase, all others showed regulation properties similar to the wildtype.

It was concluded that AEC resistance in *S. clavuligerus* may not be due to deregulation of aspartokinase but rather to an overproduction of the enzyme.

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LIST OF ABBREVIATIONS

αAA	α-aminoadipate
AEC	S-2-aminoethyl cysteine
ACV	α-aminoadipyl-cysteinyl-valine
BSA	Bovine serum albumin
DAP	Diaminopimelate
DDPS	Dihydrodipicolinic acid synthase
EDTA	Ethylenediamintetraacetic acid
HSD	Homoserine dehydrogenase
L	Lysozyme medium
LB	Luria-Bertani medium
MC	Mitomycin C
MM	Minimal medium
MOP	8-methoxypsoralen
MOPS	3-[N-Morpholino]propanesulfonic acid
NQO	4-nitroquinoline-1-oxide
NRRL	Northern Regional Research Laboratory
NTG	N'-methyl-N'-nitro-N-nitrosoguanidine
P	Protoplasting medium
PEG	Polyethylene glycol
rpm	revolutions per minute
R2YE	Regeneration medium

SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
T	Transformation medium
TCS	Trypticase soy broth
TCSS	Trypticase soy broth-starch
TE	Tris-EDTA buffer
TEA	Tris-EDTA-acetate buffer
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

I. INTRODUCTION

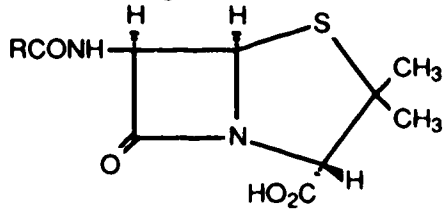
I.1. HISTORY OF PENICILLIN

It has now been 60 years since Fleming noticed partial lysis of *Staphylococci* on a plate contaminated with *Penicillium notatum* . After culturing this fungus, Fleming called the active "mould broth filtrate" penicillin (Abraham, 1982). However, Fleming did not pursue this important discovery and it was not until 1940 when Florey demonstrated the ability of penicillins to cure systemic infections in mice, that it became of medical importance (Abraham, 1982). To expand the availability of penicillin, a joint Anglo-American effort was created. Three major advances were made by the Northern Regional Research Laboratory (NRRL). The first of these was the addition of corn steep liquor to the fermentation (Moyer and Coghill, 1947), the second was the development of submerged culturing techniques rather than the surface cultures being used, and the third was the isolation of new strains of *Penicillium chrysogenum* that produced higher levels of antibiotic (Raper et al., 1944). Although samples of *Penicillium* were sent to NRRL from all over the world, the strain ultimately used to select for overproducing strains came from a mouldy cantaloupe in Peoria, Illinois. With these advances by 1946 penicillin was being produced at such high levels by British industry that 370 million units could be exported monthly. Production in the U.S. was even higher (Queener, 1986)

Penicillium chrysogenum was found to produce different penicillins depending on the medium used for the cultures as determined by structural studies. American workers had added corn-steep liquor to the medium as developed by NRRL (Moyer and Coghill, 1947), however the English workers had not. Acid hydrolysis of penicillin from these two different sources yielded different products.

Hydrolysis of the American product yielded phenylacetic acid, yet this product was not obtained from hydrolysis of the English penicillin. The American penicillin was benzylpenicillin (penicillin G), while the English were producing 2-pentenylpenicillin (penicillin F). Both structures contained the ß-lactam ring.

In 1949, six naturally occurring forms of penicillin were known. The basic structure of these penicillins are given below:



R = 1. 2 - pentenyl
2. 3 - pentenyl
3. p-hydroxybenzyl
4. benzyl
5. n - amyl
6. n - heptyl

The corn-steep liquor added to the American fermentation contained phenylacetic acid which could be incorporated onto the penicillin nucleus to produce penicillin G. Initial experiments were done to determine if degradation products, proposed intermediates or other compounds could be used to increase the production of penicillin. The NRRL had shown that small amounts of phenylacetic acid stimulated production of penicillin, but they could not demonstrate that the type of penicillin produced was affected. They thought perhaps the action of phenylacetic acid was like that of a plant hormone. However Behrens et al. (1948a) were able to show that addition of compounds containing the phenylacetyl group, or a compound which could biologically be converted to contain this group,

stimulated production of benzylpenicillin. That these compounds were incorporated into the penicillin was demonstrated by using precursor compounds which had labelled phenylacetyl groups (Behrens et al., 1948b). Benzylpenicillin was subjected to deuterium analysis which showed that the phenylacetyl moiety was incorporated into the penicillin. This method of adding precursors to the fermentation broth was seen as a way to produce new penicillins. By adding compounds to the fermentations, novel penicillins would be formed if the compounds could act as precursors. A wide variety of acyl compounds were prepared and used as precursors. Some produced good yields of penicillin with 60-100% of the total penicillin produced incorporating the precursor. Over 30 new penicillins were isolated and identified (Behrens et al., 1948d). It was in this way that penicillin V (phenoxymethylpenicillin) was first produced in 1948 (Behrens et al., 1948c). Although overlooked at the time, its stability in acid made it more useful than penicillin G for oral use. However, this method of producing new penicillins was not used extensively for two reasons: 1. the precursor molecules were toxic in many cases and 2. the rate of incorporation could also be very low (Behrens et al., 1948c).

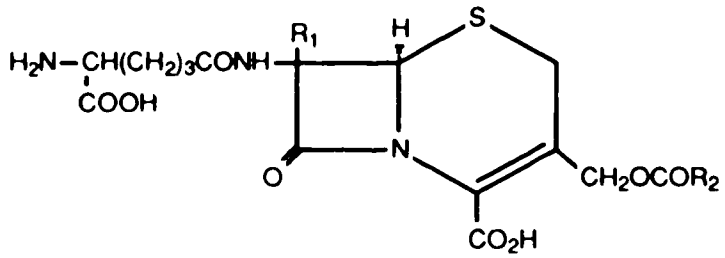
The penicillin nucleus (6-aminopenicillanic acid, 6-APA) was first synthesized by Sheehan who then was able to produce penicillin G by acylation with phenylacetyl chloride. Penicillin V was produced in an analogous reaction by a phenoxyacetylation reaction (Sheehan and Henery-Logan, 1959). This opened the possibility of adding unusual side chains through chemical reactions to form a whole group of new compounds. Although this was a breakthrough allowing for the production of synthetic penicillins, totally synthetic penicillins are not as common as semisynthetics. Semisynthetic penicillins are produced by adding side

chains to 6-APA that has been produced as a fermentation product. Batchelor et al. (1959) were able to isolate 6-APA from a fermentation broth of *P. chrysogenum*. This was shown to be readily converted to penicillin V and penicillin G in reactions similar to those above. They suggested that this would be useful for the preparation of new penicillins (Batchelor et al., 1959). Semisynthetics have been produced continuously since this time. However 6-APA is a minor fermentation product. Most 6-APA is produced by enzymatic deacylation of penicillin G, which is produced in much greater quantities.

I.2. ISOLATION OF CEPHALOSPORINS AND CEPHAMYCINS

In 1956 Newton and Abraham discovered a ß-lactam compound from *Cephalosporium acremonium* called cephalosporin C. It was first isolated during purification of cephalosporin N (later found to be penicillin N). The nucleus of this molecule (7-aminocephalosporanic acid, 7-ACA) was synthesized and reacylated to form a whole new family of synthetic ß-lactam antibiotics based on the cephalosporin nucleus (7-ACA is produced by chemical deacylation of Cephalosporin C). This discovery led to the screening of fermentations uncovering several related compounds which also had antibacterial activity. One of the organisms found to produce a ß-lactam antibiotic was *Streptomyces clavuligerus*. This organism is a member of the Order Actinomycetales, a filamentous gram positive bacterium unlike *Penicillium* and *Cephalosporium* which are both eukaryotic organisms. *Streptomyces clavuligerus* was first described by Higgens and Kastner in 1971 and was also found to produce ß-lactams by Nagarajan et al. in 1971. Two antibiotic compounds were isolated and identified by Nagarajan et al. (1971) as a) 7-(5-amino-5-carboxyvaleramido)-3- carbamoyloxymethyl-3-cephem -4-carboxylic acid and b) 7-(5-amino-5-carboxyvaleramido)-7-methoxy-3-

carbamoyloxymethyl- 3-cephem -4-carboxylic acid (cephamycin C).



a) $R_1 = H$; $R_2 = NH_2$

b) $R_1 = OCH_3$; $R_2 = NH_2$

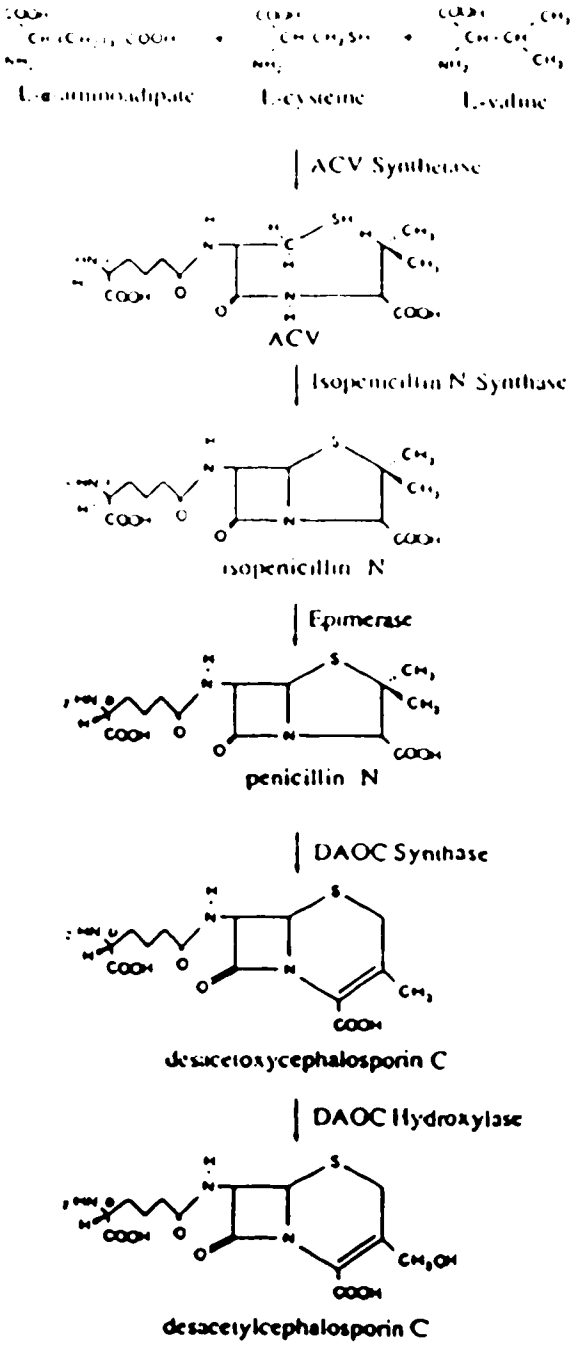
I.3. BIOSYNTHESIS OF BETA-LACTAMS

While the chemistry of the ß-lactams has undergone intensive research, relatively little is known concerning the biosynthesis of these compounds. The first conclusive studies of the origin of penicillin used isotopically labelled amino acids to determine if they were incorporated into penicillin (Arnstein and Crawhall, 1957). These showed that both cysteine and valine were part of the ring system of penicillin. It was shown by Trown et al. (1963) that these amino acids were also precursors of Cephalosporin C. To determine if cysteine and valine were modified before being incorporated into penicillin, modified peptides containing both cysteine and valine were looked for. A peptide called α-aminoadipyl-cysteinyl-valine (ACV) was discovered (Arnstein and Morris, 1960). It was not until a cell-free system for studying this biosynthesis was developed that this was proved to be a precursor of penicillin. Using cell free systems it has been shown that ACV is converted to penicillin in cell free extracts of both *C. acremonium* and *S.*

clavuligerus. In all penicillin producing organisms studied, the first product in the pathway to ß-lactam antibiotics appears to be ACV. However each differs in the final endproduct that this leads to. *Penicillium chrysogenum* produces a penicillin product. *C. acremonium* also produces a penicillin but then uses this to produce cephalosporin (Warren et al., 1967). Finally, *S. clavuligerus* also produces penicillins and then uses them to produce cephalosporins and cephamycin (Jensen et al., 1982a and 1982b). The first reaction in this biosynthetic pathway involves the three precursor amino acids, L-α-aminoadipic acid, L-cysteine and L-valine, condensing to form ACV (See figure 1). This is accomplished by a single multifunctional enzyme in both *C. acremonium* and *S. clavuligerus* (Banko et al., 1987 and Jensen et al., 1988). See Figure 1. The ACV molecule is then cyclized to form isopenicillin N. The reaction involves the closure of 2 rings to form the fused ß-lactam thiazolidine ring structure, while removing four H^+ (Jensen et al., 1982a). This is a very important reaction in that this is the first appearance of the ß-lactam structure and was reported for both *C. acremonium* and *S. clavuligerus* (Abraham, 1978, Fawcett et al., 1976, and Konomi et al., 1979).

Conversion of the L-α-aminoadipyl side chain of isopenicillin N to the D configuration of the side chain in penicillin N is the point of divergence between organisms which produce only penicillins and those which produce both penicillins and cephalosporins. Organisms which produce only penicillin do not have this epimerase enzyme while cephalosporin producers do (Jensen, 1986). Epimerase enzymes from *C. acremonium* and *S. clavuligerus* appear to be quite different in terms of lability. The enzyme was first demonstrated in *C. acremonium* and its study was quite difficult due to extreme lability (Baldwin et al., 1981, Jayatilake et

Figure 1. The biosynthetic pathway of penicillin and desacetylcephalosporin C production in *S. clavuligerus*. Cephamycin C is formed after three further reactions.


L-α-aminoadipate
L-cysteine
L-valine
ACV Synthetase
ACV
Isopenicillin N Synthase
isopenicillin N
Epimerase
penicillin N
DAOC Synthase
desacetoxycephalosporin C
DAOC Hydroxylase
desacetylcephalosporin C

al., 1981 and Konomi et al., 1979). This enzyme was also demonstrated in *S. clavuligerus* but found to be much more stable and was used for purification studies (Jensen et al., 1982b and Jensen et al., 1983).

The ß-lactam-thiazolidine structure of penicillin N is expanded to the ß-lactam-dihydrothiazine ring to form desacetoxycephalosporin C. The enzyme ("expandase"), which carries out this reaction, has requirements for ferrous ion, ascorbate and α-ketoglutarate. Jensen et al. (1982a) have shown that the expandase from *S. clavuligerus* has similarities to that of the *C. acremonium* enzyme including the above cofactor requirements, although differences in stability, and perhaps structure and composition, are indicated (Kupka et al., 1983; Jensen, 1986).

Desacetoxycephalosporin C undergoes hydroxylation to yield desacetylcephalosporin C. The hydroxyl group is added to the methyl group of C-3 by a hydroxylase which has the same cofactor requirements as the expandase. This reaction has been demonstrated in both *C. acremonium* (Felix et al., 1980) and *S. clavuligerus* (Turner et al., 1978). Figure 1 shows the biosynthetic pathway to this point.

It is at this point where *C. acremonium* and *S. clavuligerus* differ in their pathway to their respective antibiotics. *C. acremonium* acetylates the hydroxyl group formed in the previous reaction producing cephalosporin C (Felix et al., 1980). Cephamycin production in *S. clavuligerus* follows a different route with more reactions. Although the mechanism for cephamycin production is not fully understood it is believed that the initial reaction is addition of a carbamoyl group to the hydroxymethyl group of C-3 (Brewer et al., 1980). A hydroxyl group is then added to C-7 and this is methylated to produce cephamycin C.

I.4. REGULATION OF SECONDARY METABOLISM

Antibiotic biosynthesis is an example of secondary metabolism and therefore the enzymes in the antibiotic biosynthetic pathways require the products of primary metabolism as substrates. To control the synthesis of antibiotics, regulatory mechanisms act on the primary metabolic pathways (Drew and Demain, 1977; Martin and Demain, 1980).

Secondary metabolites have a variety of chemical structures including the unusual ß-lactam ring structure. The products of secondary metabolism are dependent on both genetic and environmental factors. This is probably due to the low specificity that secondary metabolism enzymes have. Primary metabolism has high specificity however and only one product is formed as mutations or errors may be lethal to the cell. Secondary metabolites are formed during stationary growth of batch culture and so errors or lack of specificity in forming these metabolites are not lethal to the cell.

The regulation of secondary metabolism seems to occur in two ways. These metabolites are produced at low growth rates and so they have regulatory controls which respond to growth rates. Also, the individual biosynthetic pathways are subject to specific mechanisms including induction, catabolite regulation and endproduct regulation (Martin and Demain, 1980).

Wildtype strains of antibiotic producers typically yield low levels of antibiotic as a result of the regulatory systems which control secondary metabolism. Most high producing strains which are used for the commercial production of antibiotics have been isolated by screening for high antibiotic production after random mutagenesis.

I.5. MUTAGENESIS OF ANTIBIOTIC PRODUCING ORGANISMS

A mutation is an important biological phenomenon. Mutants which have defects in complex biochemical pathways (so called 'blocked mutants') can be used to understand both the genetic and biochemical aspects of metabolism. Mutations may be induced or may occur apparently spontaneously. There are three different ways in which a mutagen can cause mutagenesis. The mutagen itself may substitute for a normally occurring base causing mispairing (eg. 2-aminopurine). It could also alter the bases in the DNA causing mispairing (e.g. N'-methyl-N'-nitro-N-nitrosoguanidine, NTG) and finally it may damage the DNA so that base pairing is interfered with (eg. UV light)(Miller, 1983).

I.5.1. Repair Systems for Mutagenesis

Since DNA damage could have lethal consequences, organisms have developed a variety of systems to repair DNA damage. Three different repair systems are known to operate in *Escherichia coli*. The simplest one is photoreactivation. Ultraviolet light irradiation produces thymidine dimers in DNA. An enzyme binds to the dimers but requires light of between 310 and 400 nm before the dimer can be split. The second method involves excision repair. The uvr system in *E. coli* excises DNA damaged by UV light as well as bulky lesions caused by other agents (4-nitroquinoline-1-oxide (NQO) and mitomycin C (MC)). There are three proteins produced as the products of the *uvrA*, *uvrB* and *uvrC* genes. The endonucleases produced cause two breaks in the DNA on either side of a pyrimidine dimer. Not only dimers are recognized but also other damage which causes structural distortion. This oligomer may then be excised by another system involved in repair (Yueng et al.,1983; Sancar and Rupp, 1983). This type of repair is also referred to as "short patch repair". The third type of repair involves the recA

protein and is referred to as postreplication repair. The pyrimidine dimers which are not repaired by either of the preceding repair mechanisms block replication of the DNA. The daughter strands which are being produced have gaps of approximately 1000 nucleotides long opposite to the dimers. Recombination occurs between the parental strands of DNA and the daughter strands to close these gaps. The parental strands are repaired and ligated. The pyrimidine dimers are still present in the DNA however replication of the DNA is allowed to proceed.

I.5.2. Ultraviolet Light Mutagenesis

Ultraviolet light induces all types of base pair substitutions and frameshift mutations (Miller, 1983). It is probably the most studied mechanism of mutagenesis in prokaryotes. Although UV light produces a variety of effects, the most common lethal and mutagenic effect is the production of cyclobutane-type dimers of pyrimidines. Dimers can be formed between any adjacent pyrimidines, however thymine-thymine dimers are formed at a higher rate than either cytosine-thymine or thymine-cytosine (Witkin, 1969).

UV mutagenesis is thought to occur via errors in the repair of the dimers. Evidence for this comes from recA and lexA mutants of *E. coli* which are not mutated by UV light. Neither the photoreactivation nor excision repair processes are thought to be sources of error. Photoreactivation is considered to be accurate, as exposure to visible light decreases the numbers of mutants after exposure to UV light. RecA and lexA mutants which have excision repair do not exhibit UV mutagenesis and therefore excision repair must be error free. The mutants produced by UV light must be due to errors in repair using the postreplicative repair system. A comparison of mutability between uvr^+ and uvr^- strains of *E. coli*

shows that the mutants deficient in excision repair are extremely sensitive to UV and those that do survive are mutated at a much higher frequency. This would suggest that postreplicative repair is much more error-prone. Witkin (1976) suggests that errors are caused when daughter strands are joined during the recombination stage of post-replication repair.

Both *Streptomyces coelicolor* and *S. clavuligerus* have been shown to be mutated by UV light (Clarke and Hopwood, 1976; Saunders and Holt, 1982; Saunders et al., 1982). Saunders and Holt (1982) isolated five different UV sensitive strains of *S. clavuligerus*. Several mutants exhibited hypermutability with UV light, resembling excision-deficient mutants of *E. coli* (Witkin, 1976). One mutant was thought to be deficient in the incision step of that repair. These mutants are insensitive to the effects of caffeine giving further evidence that the mutants are polymerase or incision deficient. Caffeine has been shown to be an inhibitor of pyrimidine dimer excision (Fong and Bockrath, 1979). It has been suggested that these hypermutable strains might be useful for producing mutants but it was not clear whether the ratio of mutational hits to lethal hi ıctually increased (as would be required for a truly hypermutable strain). Other mutants were found which did not show any level of mutagenesis when treated with UV. Saunders and Holt (1982) suggest that these mutants are similar to lexA and recA mutants of *E. coli*.

Ultraviolet light mutagenesis has also been used by Hara and Beppu (1982) to produce mutants of *Streptomyces griseus* which were blocked in streptomycin production. Two different classes of mutants were obtained with one class complementing the other. In this case spores were irradiated to produce mutants. UV irradiation has also been used to produce chloramphenicol

non-producing mutants of *Streptomyces venezulae* (Akagawa et al., 1979). Townsend et al. (1971) have used UV irradiation, on *S. coelicolor* and *S. scabies*, however they used it in conjunction with 8-methoxypsoralen (MOP), a compound which photosensitizes the cell to long wave ultraviolet light. In this case, auxotrophic mutants were selected at fairly high frequencies (3.4% mutants at 3% survival). They noticed wide variation in the susceptibility of strains to mutagenesis and suggested a preliminary survival curve be done for each strain used. Auxotrophic mutants were isolated in *S. clavuligerus* by using both UV irradiation and UV irradiation in the presence of MOP (Kirby, 1978). The percentage of auxotrophic mutants was much lower than that obtained with *S. coelicolor*. No photoreactivation was found with *S. clavuligerus* after exposure to UV. Kirby used both spores and aerial mycelium in mutagenesis procedures as opposed to just spores as used by Townsend et al. (1971). He did not report differences in mutation rates between UV and UV + MOP mutagenesis.

I.5.3. N'-methyl-N'-nitro-N-nitrosoguanidine (NTG) Mutagenesis

NTG causes mutations by altering the bases of DNA leading to mispairing. Although many positions on the bases can be alkylated, the initial reaction involved in producing a mutation seems to be alkylation of the O^6 position of guanine. The O^6-alkylguanine mispairs with thymine causing a transition from G:C---A:T (Miller, 1983).

NTG has been used as a mutagen in many *Streptomyces* species and tends to make multiple mutations at high frequency. The replication fork has been shown to be the site for NTG mutagenesis in *E. coli* causing multiple closely linked mutations and this may be explained as a preference for single stranded

DNA (Miller, 1983). This was found to be the case in *S. lipmanii* by Godfrey (1974). Cells which were undergoing DNA replication were found to be mutated at a rate 6 times higher than cells which were not. Mutations are very often clustered and this property of NTG has been used in *S. coelicolor* to map regions of the chromosome (Randazzo et al., 1976). Large numbers of linked nitrate reduction mutants have been isolated by this method in *S. coelicolor* (Ortali et al., 1980). Mutagenesis using NTG has been used in a number of *Streptomyces* species to produce a variety of mutants. As part of the same study reported earlier for UV mutagenesis, Akagawa et al., (1979) also used NTG mutagenesis to produce chloramphenicol non-producing mutants of *S. venezulae* (Akagawa et al., 1979). The effects of NTG were found to be different when protoplasts were mutagenized rather than spores (Vyskocil et al., 1987). Mutagenesis was used to select for improved strains of chlorotetracycline producing *Streptomyces aureofaciens*. When NTG was used on protoplasts there was high mutagenic activity even when there was little effect on survival. Mutagenesis with NTG was found to be most effective when treated for long periods at low doses.

I.5.4. Repair Systems in *Streptomyces*

Baltz and Stonesifer (Baltz and Stonesifer, 1985a; Stonesifer and Baltz, 1985) have suggested that base pair substitution in *Streptomyces fradiae* required either error-prone DNA repair or replication and is under genetic control. They produced mutants by NTG mutagenesis of *S. fradiae* M1 which had characteristics such as sensitivity to UV light, NTG and other mutagens suggesting that they may be similar to recA mutants of *E. coli*. RecA mutants showed only 1/10 of the mutant frequencies of wildtype when NTG is the mutagen. Mutagenesis was almost completely absent with UV. As an explanation for this, they suggested that

the recA protein could be involved in error-prone DNA repair which is required for UV mutagenesis and is required for only 90% of NTG mutagenesis.

They also suggested that these mutants may actually be closer to *Saccharomyces cerevisiae* nad6 mutants than to *E. coli* recA mutants. Rad 6 mutants differ from recA mutants in that they are proficient in recombination and show reduced but not abolished response to UV and 4-nitroquinoline-1-oxide (NQO). This indicates that *Streptomyces* has repair systems intermediate between prokaryotes and eukaryotes.

I.6. GENETIC INSTABILITY IN *STREPTOMYCES*

Streptomyces have a very high level of spontaneous mutation which alters colony morphology. Wainwright (1956) has found spontaneous frequencies of morphological mutations of 0.5-1.0% in stored cultures of *Streptomyces*. Wainwright was not able to determine the nature of the mutational events although she was able to show that they occurred under conditions where no nuclear division was taking place. She suggested two possibilities. Mutations may have occurred during the storage in the absence of nuclear division, or perhaps events taking place during storage increased the probability that mutations will occur in the early stages of cell division following storage. These mutations were probably caused by macrolesions such as, deletions, transpositions, rearrangements or DNA amplifications (Baltz, 1986).

Mutations can be classed into 2 basic types: 1) microlesions, such as base pair substitutions and 2) macrolesions including deletions, duplications transpositions rearrangement and amplifications (Baltz, 1986). Macrole- quite common in *Streptomyces* and have been reported in a number of - may occur spontaneously or can be induced in several ways including

environmental stress or treatment with agents including intercalating dyes. Protoplast formation appears to be a major contributor to genetic instability. It has been shown to cause loss of plasmid DNA. Hopwood et al. (1983) were able to use this technique of protoplast formation and regeneration of *S. lividans* to obtain plasmid free strains. Furumai et al. (1982), in looking for an improved method of protoplast regeneration using *S. kasugaensis* , found that protoplast regeneration caused loss of plasmid or variation in plasmid copy number. Mutants of *S. fradiae* were isolated by Baltz and Stonesifer (1985b) using protoplast formation and regeneration, which through this process had lost several phenotypic markers including tylosin production. Since the formation and regeneration of protoplasts can cause loss of plasmid this was investigated as a possible mechanism for the loss of these markers in these mutants. There are however substantial differences between these strains which could not be explained by plasmid curing and which suggests that chromosomal changes are occurring. A spontaneous spectinomycin resistant derivative of the above mutants was obtained by Fishman and Hershberger (1983) and was found to have amplified DNA with tandem repeats of an amplifiable unit of DNA (aud). This unit appeared in a single copy in the wildtype strain and was found to be a DNA fragment with a size of 10.5 kb. Hybridization kinetics showed there were approximately 500 copies/genome arranged in tandem repeats with a sequential head to tail arrangement in the amplified mutant. In the wildtype strain the single copy of the aud was bounded by direct repeats characteristic of insertion sequences (IS)(Fishman et al., 1985).

The spectinomycin resistant mutant could be restored to wildtype by conjugation with wildtype *S. fradiae*. When this occurred DNA amplification was lost (Baltz, 1986). There was only one copy of the amplified region in all the

recombinants tested bounded by the direct repeats and 2 additional copies of the repeats in the chromosome (Fishman et al., 1985).

Baltz and Stonesifer (1985b) and Fishman et al. (1985) were also able to isolate a tylosin sensitive, nonproducing mutant which was found to have deletions in the aud. The formation of protoplasts and regeneration seems to exert stress which causes both amplification and deletion in *S. fradiae*. Whether this is due to transposition or IS elements is not clear but the fact that the amplified region is bounded by repeats would indicate this (Fishman et al., 1985). They suggest that these families of repeated sequences could serve as sites for DNA rearrangements, amplifications and deletions and may contribute to genetic instability in *Streptomyces*.

Schrempf (1982) was able to show amplification to be associated with unstable mutations. Mutants of *Streptomyces reticuli* , which were defective in melanin production were found to have amplified DNA. In addition, a melanin producer which was highly unstable was found. This mutant gave rise to melanin non-producing variants at a rate of 10-20% during growth. Producers had no amplified DNA whereas the non-producers did have amplified DNA (Schrempf, 1983). Therefore amplification correlated with non-production. There was variability within the amplified regions although all had common shared sequences. The amplified DNA hybridized weakly to several other places in the chromosome. This suggested to Schrempf that there were IS-like elements (Schrempf, 1983) associated with the amplification.

Frequent and extreme DNA amplification seems to occur in *Streptomyces* even without selection. It would seem to be the only prokaryote which undergoes this (Baltz, 1986).

I.7. TRANSPOSITION IN *STREPTOMYCES*

A transposable element has been identified from a neomycin producing strain of *S. fradiae* (Chung, 1987). The transposon was discovered when a plasmid was isolated and found to be 6.8 kb larger than would be expected from its construction. Hybridization studies have shown that the 6.8 kb transposon (Tn4556) to have originated in the *S. fradiae* chromosome. Chung then constructed a temperature sensitive plasmid which contained Tn4556 with a viomycin resistance marker inserted within Tn4556 (pMT660::Tn4556::vph). He was able to show that the transposon Tn4556::vph (Tn4560) did transpose to the chromosome of *S. fradiae* randomly. The ends of the transposon have been sequenced and show 70% homology to Tn3 (Olson and Chung, 1988). The insertion of Tn4556 caused duplication of bases at the target site. Chung has also been able to show transposition of Tn4556 in other species of *Streptomyces* .

The use of Tn4560 as a mutagen gives a significant advantage over the use of UV and NTG for mutagenesis. By inserting within a gene, the transposon inactivates this gene and produces a mutant. The gene can then be localized as it is marked by the presence of the transposon. A fragment of DNA can then be cloned from the mutant which has the transposon sequences. The Tn4560 encodes for viomycin resistance and by this its presence is recognized. The mutations induced by the transposon are single hit and are due to a single mutational event rather then a multiple event which could be caused by UV or NTG mutagenesis. A high mutation rate can be gained without killing the organism.

This method of mutagenesis has not previously been reported in *Streptomyces* since Tn4556 is the first *Streptomyces* transposon isolated, but transposon mutagenesis has been used in a variety of other organisms. Although

most work has been done in *E. coli* , transposon mutagenesis techniques have been developed in such diverse organisms as *Rhizobium meliloti* (Selvaraj and Iyer, 1983), *Myxococcus xanthus* (Blackhart and Zusman, 1986), and the eukaryotic organism *Saccharomyces cerevisiae* (Seifert et al., 1986).

In the past, mutagenesis to produce high producers has relied on screening large numbers of mutagenized cells to isolate the overproducers. By using the information available on biosynthesis of antibiotics, it should be possible using selective media to isolate specifically mutants which are deregulated in the primary metabolic pathways which form antibiotic precursors. By increasing precursor availability this may lead to antibiotic overproduction.

I.8. REGULATION OF PENICILLIN PRODUCTION IN *PENICILLIUM CHRYSOGENUM*

Penicillin production in *Penicillium chrysogenum* is regulated by lysine (Mendelovitz and Aharonowitz, 1983). Penicillin production in *P. chrysogenum* is part of a branched pathway starting with α-ketoglutarate. The pathway branches with the production of the intermediate α-aminoadipic acid (αAA) and leads to either lysine or penicillin. It has been suggested by Demain and Masurekar (1974) that lysine exerts feedback on homocitrate synthase the first enzyme in this pathway and in this way inhibits the production of αAA and therefore the endproduct of the second branch, penicillin. The addition of αAA reverses this effect and stimulates penicillin synthesis (Drew and Demain, 1977). In this way the regulation of secondary metabolism is actually exerted by primary metabolites on primary metabolite pathways. Valine is also used in the production of penicillin by *P. chrysogenum*. The first enzyme in the pathway leading to valine, acetohydroxy acid synthase has been found to be less sensitive to feedback inhibition in high

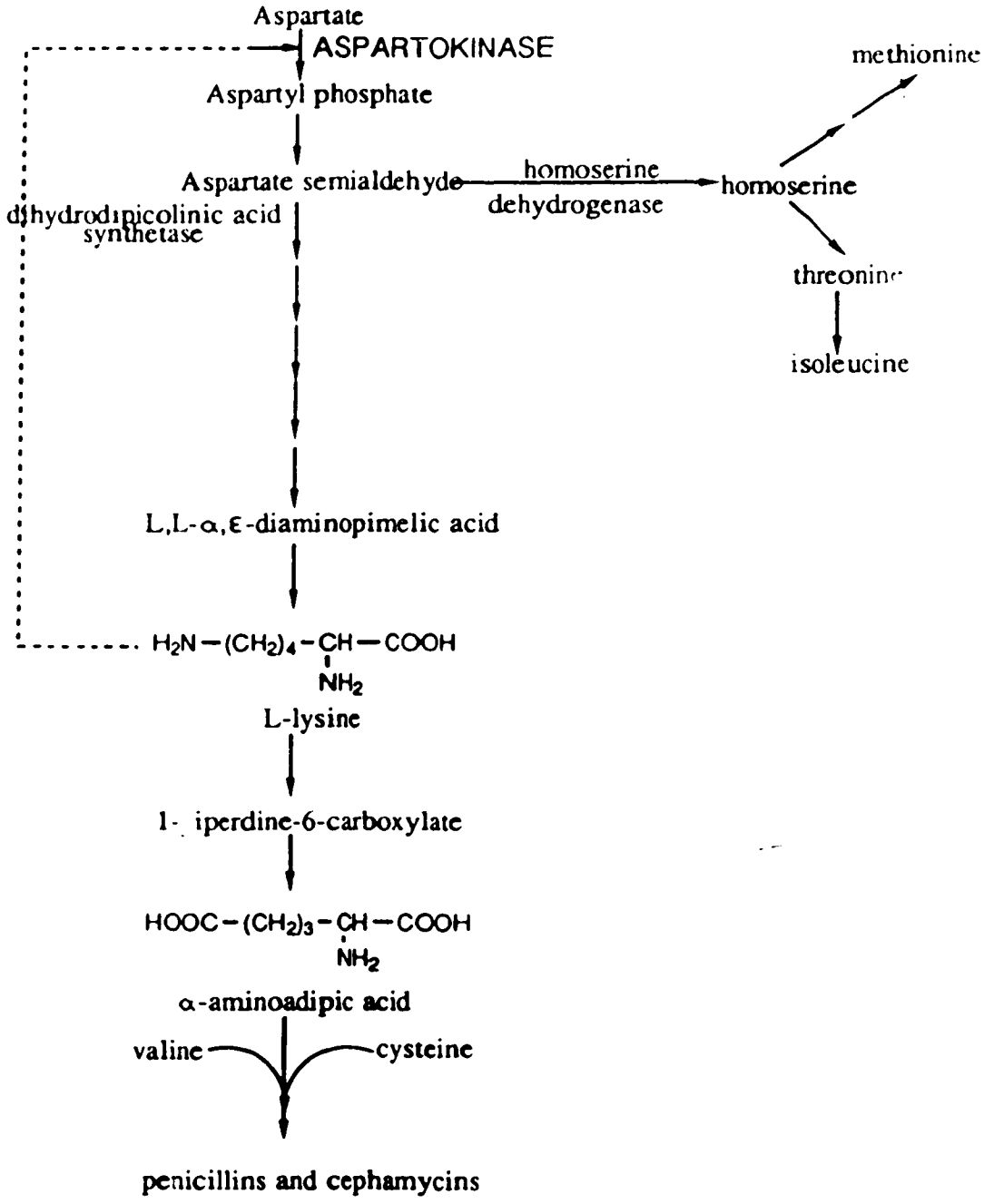
producing mutants than in strains which produce less penicillin (Goulden and Chattaway, 1968). The enzyme was also overproduced to a level twice that of the lower producers.

I.9. REGULATION OF CEPHAMYCIN PRODUCTION IN *S. CLAVULIGERUS*

The pathway leading to production of the aspartic acid family of amino acids has been suggested as the site of control for the production of cephamycin C in *S.clavuligerus* (Mendelovitz and Aharonowitz, 1982; Mendelovitz and Aharonowitz, 1983; Whitney et al., 1972) The pathways of antibiotic production and the aspartic acid family of amino acids interact at four different places: 1) α-aminoadipate side chain of cephamycin C originates from this pathway, 2) The methyl group on C-7 of the antibiotic comes from the aspartic acid pathway, 3) cysteine, which shares intermediats with this pathway, is incorporated into the nucleus of the antibiotic molecule and 4) the enzymes of valine biosynthesis are shared with those of the aspartic acid family and valine is incorporated into the cephamycin C molecule (Aharonowitz et al., 1984)

In *Streptomyces* the synthesis of lysine is thought to be carried out via the diaminopimelic acid pathway outlined in Figure 2. Aharonowitz et al. (1984) suggested that the flow of carbon through this pathway is the rate limiting step in the formation of cephamycin C. It has been demonstrated in *S. lactamdurans* (Kern et al., 1980) that L-lysine is converted to L-α -aminoadipic acid (αAA) in extracts (L-lysine aminotransferase activity). This reaction was demonstrated using a cell free system in which intermediates were identified in the conversion to αAA. The product of the first reaction in this conversion was found to be

Figure 2. The diaminopimelic acid pathway of lysine production in bacteria. The dashed line indicates feedback inhibition by lysine on aspartokinase. Lysine is then converted to α-aminoadipic acid and this is condensed with valine and cysteine to form ACV.


Aspartate
ASPARTOKINASE
Aspartyl phosphate
Aspartate semialdehyde
homoserine dehydrogenase
homoserine
methionine
threonine
isoleucine
dihydrodipicolinic acid synthetase
L,L-α,ε-diaminopimelic acid
H2N—(CH2)4—CH—COOH
NH2
L-lysine
1- iperdine-6-carboxylate
HOOC—(CH2)3—CH—COOH
NH2
α-aminoadipic acid
valine
cysteine
penicillins and cephamycins

l -1-piperdine-6-carboxylate. This is an epsilon deamination reaction which previously was only known in *Flavobacterium*. The same reaction was thought to occur in *S. lipmanii* but was not conclusively shown (Kern et al., 1980). The oxidation of 1-piperidine-6-carboxylate is presumed to be the next reaction. This activity has been demonstrated in Pseudomonas but has not yet been identified in Streptomyces (Calvert and Rodwell, 1966). This pathway differs from the fungal pathway in that αAA is not an intermediate in the formation of lysine. In Streptomyces αAA is produced from lysine.

Kirkpatrick et al. (1973) have showed that the formation of diaminopimelate (DAP) is a necessary step for the production of lysine in *S. lipmanii*. They were able to isolate mutants defective in DAP decarboxylase which could not grow without the addition of lysine and DAP accumulated under lysine starvation conditions. They also have shown that αAA or antibiotic can only be produced if lysine is added. This could only be explained if αAA was being produced from lysine and not that it was an intermediate.

Mendelovitz and Aharonowitz (1982) have demonstrated that L-lysine and DL-meso DAP when supplied to *S. clavuligerus* increased cephamycin C production. There are several places in the lysine pathway (and therefore the pathway to the α-aminoadipyl side chain of cephamycin C) where regulation could occur. Unlike the situation in *P. chrysogenum*, antibiotic production is not a branch point from a common pathway leading to both lysine and penicillin, but rather αAA is produced from lysine. Mendelovitz and Aharonowitz (1982) looked at three points in the lysine pathway which would be expected to exert some regulatory effects: 1) Aspartokinase activity; the first step in the pathway converting aspartate to aspartyl-phosphate. 2) dihydrodipicolinic acid synthase

(DDPS); the first step in the pathway that leads to lysine specifically, condensing pyruvate with aspartate semialdehyde to yield 2,3-dihyrodipicolinic acid. 3) homoserine dehydrogenase (HSD); the first enzyme in the branch of the pathway leading to the formation of threonine, isoleucine and methionine. Mendelovitz and Aharonowitz (1982) found that both DAP and lysine increased antibiotic production by 75% when added to the growth medium of *S. clavuligerus*. They found that by adding both compounds the specific antibiotic production could be raised four-fold and increased further by adding valine. Threonine when added to cultures, inhibited antibiotic production by 41%. The inhibitory effect of threonine could be reversed by addition of lysine. Aspartokinase activity was found to be subject to feedback inhibition by both lysine and threonine. This is unusual as there is only one aspartokinase in *S. clavuligerus* and inhibition by both these amino acids would reduce the amount of methionine and isoleucine produced. Mendelovitz and Aharonowitz (1982) suggest that threonine feedback inhibition causes lysine to be in short supply and this decreases antibiotic production. Adding lysine to the cultures relieved the shortage allowing antibiotic to be produced at higher levels.

In in vitro studies aspartokinase activity was stimulated by lysine. No individual members of the aspartic acid family of amino acids caused inhibition of aspartokinase, although at high concentration (>10mM) biosynthesis of antibiotic was repressed by both methionine and isoleucine. Methionine also decreased cephamycin production by 27%. The second enzyme studied by Mendelovitz and Aharonowitz (1982), DDPS, was found to be unaffected by lysine but was partially inhibited by both high concentrations of DAP and αAA and was slightly repressed by DAP. Homoserine dehydrogenase, the first step in the pathway leading to threonine, isoleucine and methionine, was slightly repressed by isoleucine and

inhibited by threonine.

They suggested that aspartokinase is the key step in carbon flow to αAA and therefore cephamycin C. In view of the stimulatory effects of DAP and lysine on antibiotic production, Mendelovitz and Aharonowitz (1982) decided to see if it was possible to alter the lysine pool within the cell by genetic means and thereby increase antibiotic production. One way of obtaining lysine-regulatory mutants is to use lysine antimetabolites as a selective pressure. Masurekar and Demain (1974) were able to obtain lysine overproducers by selecting for *P. chrysogenum* colonies which were resistant to S-2-aminoethyl cysteine, α-hyroxylysine or 4,5-dehydrolysine. They found that some of these lysine overproducing mutants were defective in penicillin production. When the mutants defective in penicillin production reverted to normal levels of lysine production antibiotic levels also returned to normal.

Brevibacterium flavum mutants which are resistant to AEC are used to produce lysine commercially. Mutants of *B. flavum* which were threonine auxotrophs were isolated by Sano and Shiio (1970). They found that maximal lysine production can only occur under starvation threonine. This is explained by concerted feedback inhibition of aspartokinase by threonine and lysine. Overproduction of lysine may occur due to a decrease in feedback inhibition of aspartokinase. By generating mutants which were resistant to feedback inhibition these mutants will overproduce lysine.

I.10. AEC RESISTANT MUTANTS OF *S. CLAVULIGERUS* AND OVERPRODUCTION OF CEPHAMYCIN C

Mendelovitz and Aharonowitz (1983) isolated *S. clavuligerus* mutants

which were resistant to AEC. A significant proportion of the mutants which they isolated were antibiotic overproducers. They paid particular attention to the regulation of aspartokinase to see if altered regulation was responsible for both AEC resistance and antibiotic overproduction. Approximately 70% of the AEC resistant mutants isolated were shown to have aspartokinase which was insensitive to feedback inhibition by lysine and threonine. Seventy percent of these mutants (app oximately 50% of the total) had increased antibiotic production. Lysine had been shown to stimulate aspartokinase activity in wildtype cultures; AEC had the opposite effect. The inhibitory effect of AEC on wildtype cultures could be relieved by threonine at high concentrations. It has been reported that two uptake systems exist for lysine, arginine and their analogs in *S. hydrogenans* (Gross and Burkhardt, 1973). The first system is used when lysine levels are low and another system when lysine and arginine are in higher concentration. The second system is used for neutral amino acids and so has a lower affinity for lysine and arginine but a higher overall capacity than the specific system for lysine and arginine. Mendelovitz and Aharonowitz (1983) suggest that when AEC and threonine are added to the medium the second transport system is working for AEC as it is at a high concentration. By increasing threonine concentration the threonine is transported at a much higher rate then the AEC. As the rate of threonine transport increases the rate of AEC transport decreases and its inhibitory effects are lessened.

As reported (Mendelovitz and Aharonowitz, 1982), L-lysine and DL-meso DAP stimulated antibiotic production in *S. clavuligerus*, while lysine and threonine in combination caused feedback inhibition of aspartokinase. Mendelovitz and Aharonowitz (1983) assumed that aspartokinase was the rate limiting step in antibiotic biosynthesis. If AEC resistant mutants are deregulated in aspartokinase

activity they should produce higher levels of antibiotics. This was found with about 50% of all AEC resistant mutants overproducing antibiotics (Mendelovitz and Aharonowitz, 1983). Other types of AEC resistant mutants were also found. Twenty percent of mutants were deregulated but did not overproduce antibiotics, 6% overproduced antibiotics but were not deregulated in aspartokinase, 20% had unaffected aspartokinase activity and produced normal levels of antibiotic. Mendelovitz and Aharonowitz (1983) suggested that the last two types of mutants could be due to mutations in AEC transport or AEC modification. However, for those classes of AEC resistant mutants which do not show deregulation of aspartokinase, other explanations are possible. Simple overproduction of aspartokinase without deregulation could also give rise to AEC resistance. Mendelovitz and Aharonowitz did not determine levels of aspartokinase only regulation. Overproduction could be due to mutations in transcriptional control sequences for aspartokinase or alternatively to amplification of the gene coding for aspartokinase. This latter alternative is of particular interest in view of the previously mentioned ability of *Streptomyces* sp. to amplify large segments of the chromosome at high frequency. This also provides a possible explanation for that small class of antibiotic overproducing mutants which were not deregulated in aspartokinase. If other genes involved in αAA production and/or antibiotic production were clustered near the aspartokinase gene, amplification could increase the production of these other gene products as well and increase antibiotic production.

This study investigates independently isolated AEC resistant mutants of *S. clavuligerus* and characterizes them with respect to antibiotic production, aspartokinase production and regulation.

II. MATERIALS AND METHODS

II.1. MATERIALS

Restriction endonucleases were purchased from either Boehringer Mannheim or Bethesda Research Laboratories, Inc. *Escherichia coli* DNA polymerase I large fragment was purchased from Pharmacia. All enzymes were used according to the specifications supplied by the manufacturer.

Deoxynucleoside triphosphates were purchased from Pharmacia.

Thiostrepton was a gift from S. Lucania, Squibb & Sons, Inc., and viomycin was a gift from J. Clancy, Pfizer.

All other chemicals were of reagent grade.

II.2 BACTERIAL CULTURES

II.2.1 Sources of Bacterial Cultures and Plasmids

Streptomyces clavuligerus NRRL3585 was obtained from the Northern Regional Research Laboratory, Peoria, Illinois and *Streptomyces lividans* containing pUC1169 was obtained as a gift from S-T. Chung, Upjohn. *Escherichia coli* Ess was used in the bioassay for antibiotic production by *S. clavuligerus*. It was obtained from A.L. Demain of the Massachusetts Institute of Technology. This *E. coli* mutant is extremely sensitive to ß-lactam antibiotics.

II.2.2 Maintenance and Cultivation of Bacterial Cultures

II.2.2.1 Single Spore Isolates and Spore Stocks of *S. clavuligerus*

A 100 µl aliqout of spore suspension of *S. clavuligerus* was plated on Modified Bennetts Agar (1.0 g yeast extract, 1.0 g beef extract, 2.0 g casitone, 10.0g starch, 10.0 mg $CoCl_2$, 20.0 g agar, pH 6.8, about 20 ml per plate) and bated at 28°C for 5 days. Twenty colonies were removed with a 6 mm cork borer. These plugs were placed in a sterile petri plate and incubated in a humid

enviroment at 28°C for 7 days. The plugs were placed on a bioassay plate (23 cm x 23 cm, Nunc, Denmark) containing trypticase soy agar (TCS)(BBL Microbiology Systems, MD) inoculated to 2% (v/v) with a 24 h culture of *E. coli* Ess grown in TCS broth . The plate was incubated at 28°C overnight. The plug with the biggest zone of inhibitionwas removed, homogenized and spread on Sporulation Agar (8 g tomato paste, 8 g oatmeal, 10 g agar per 400 ml pH 6.8; Jensen et al. 1982) plates. After the colonies on the sporulation agar had sporulated they were scraped with a sterile spatula and spores placed in 10 ml of sterile 20% glycerol. This was then vortexed to mix, sonicated in a ultrasonic waterbath (Bransonic 42, Branson Cleaning Equipment Company, Conn.) for 2 min and then filtered through non-absorbant cotton loosely packed in a pasteur pipette. Aliquots were then frozen in screw capped plastic vials (NUNC Laboratories, Denmark) at -75°C at a spore density of approximately 0.5 x 10^8 spores/ml. All studies were carried out using *S. clavuligerus* cultures derived from this single spore isolate.

II.2.2.2. Spore Stocks of *Streptomyces lividans*

Spore stocks of *S. lividans* were prepared by streaking a liquid culture of *S. lividans* onto Sporulation Agar (Jensen et al. 1982) and incubating at 28°C for 10 days. After sporulation the procedure used with *S. clavuligerus* was followed. *S. lividans* spore stocks were not single spore isolates therefore the first part of this procedure was not followed in preparing spores of this organism.

II.2.2.3. Cultivation of *S. clavuligerus* in Liquid Media

Liquid cultures of *S. clavuligerus* are obtained by inoculating 25 ml amounts of TCS + 1% (w/v) soluble starch (TCSS) medium in 125 ml flasks to 2% (v/v) with a spore stock. These cultures were incubated at 28°C on a New Brunswick Scientific Shaker (New Brunswick, NJ) at 250 rpm for 2 days and are

referred to as seed cultures. These cultures were then used to inoculate with a 2% inoculum 100 ml amounts of liquid media in 500 ml flasks. Liquid medium was one of two types: TCSS or Minimal Medium (MM)(50 mM K_2HPO_4, 2.4 mM $MgSO_4$, 1% (v/v) glycerol, 40 mM NH_4Cl, 3.6 µM $FeSO_4$, 5.1 µM $MnCl_2$, 3.5 µM $ZnSO_4$, 8.8 µM $CaCl_2$). TCSS cultures were incubated for 48 h and MM for 96 h at 28°C at 250 rpm.

II.2.2.4. Cultivation of *Streptomyces lividans* in Liquid Media

The same procedure as that for *S. clavuligerus* was followed.

II.3. MUTAGENESIS PROCEDURES

II.3.1. Ultraviolet Light Mutagenesis

Four vials of spore stocks prepared as in II.3.1 were pooled, centrifuged at 11,400 x g for 10 min and resuspended in 10 ml of sterile distilled water. Two ml amounts of this suspension were then dispensed into sterile glass petri plates. Plates were placed 10 cm below a prewarmed 254 nm ultraviolet (UV) lamp (.016 W/m^2). Lids were removed from the plates and the plates were swirled gently to ensure even exposure to UV light. At timed intervals, plates were removed from exposure to the lamp. The samples were then diluted and plated on four 10 ml TCSS plates for each appropriate dilution to determine both the numbers of mutants and number of survivors and incubated at 28°C for 5-6 days. Mutants were scored for the acquisition of rifampicin resistance as in II.4.1.

II.3.2. N'-methyl-N'-nitro-N-nitrosoguanidine Mutagenesis

Four vials of spore stocks were pooled, centrifuged at 11,400 x g for 10 min and resuspended in 10 ml of sterile water. A 1.8 ml aliquot of spore suspension was mixed with 200 µl of 5 mg/ml N'-methyl-N'-nitro-N-

nitrosoguanidine (NTG) dissolved in ethanol. The spores were held at 21°C for time intervals of up to 2 h. At the appropriate time intervals the spores were filtered through a sterile 0.45 µM filter (Type HA, Millipore Corporation, Mass.) and washed with 5 ml of sterile distilled water. The filter paper and gasket were placed in a test tube containing 2 ml of sterile distilled H_2O, vortexed and sonicated to remove spores from the filter paper and gasket, then serially diluted and plated in quadruplicate on 10 ml of TCSS plates. Plates were scored as above for rifampicin resistance.

II.3.3. Transposon Mutagenesis

II.3.3.1. Plasmid Purification

The plasmid pUC1169 containing the transposon Tn4560 was isolated from *S. lividans* and *S. clavuligerus* using the procedure of Kieser (1984) with some modifications as follows. Cells were harvested by centrifugation at 11,400 x g for 20 min and were lysed by addition of lysozyme and alkaline SDS and incubated at 55°C. The aqueous phase was acid phenol/chloroform extracted until the interface was clear. The DNA was then alcohol precipitated and redissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA pH 8.0). Again DNA was phenol/chloroform extracted. After this second set of phenol/chloroform extractions the procedure was modified by transferring the upper aqueous phase to a new eppendorf tube. This was repeatedly extracted with diethyl ether until the interface was clear of lipid. One volume of isopropanol was added, mixed, centrifuged in an eppendorf microcentrifuge (2 min at maximum centrifugation force) and all the supernatant removed with a drawn-out pasteur pipet. The pellet was dried under vacuum for 15 min and redissolved in 500 µl of TE buffer.

The plasmid DNA was furthur purified by cesium-chloride centrifugation

as follows: the volume of the DNA solution was increased to 8 ml with TE buffer. Cesium chloride was added to a final concentration of 1g/ml and mixed gently to dissolve the salt. Ethidium bromide solution (10 mg/ml in H_20) was added at 0.08 volume. The solution was placed in a Beckman Quick Seal centrifuge tube and the top of the tube filled with mineral oil. The samples were then centrifuged at 100,000 x g for 40 h. After centrifugation the DNA bands were visualized using a hand held UV lamp and the lower (closed circular plasmid DNA) band removed with a syringe and needle. The band which was removed was then added to a screw capped tube and extracted with 3 times 1 volume of n-butanol to remove the ethidium bromide. The CsCl was removed by dialysis overnight against 2 times 1 L of TE buffer.

II.3.3.2.1. Protoplast Formation

The procedure of Hopwood et al. (1985) including modifications by Illing (1986) was followed. A seed culture of *S. clavuligerus* growing in TCS+1% (v/v) glycerol was incubated for 44 h after which glycine was added to 0.5% (w/v). This was used to inoculate 100 ml of TCS+1% (v/v) glycerol+ 0.5% glycine after 48 h. The culture was harvested after 24 h of incubation by centrifugation at 1480 x g for 10 min at room temperature. The pellet was washed twice with 50 ml of 10.3% sucrose. The resulting pellet was resuspended in 25 ml of L medium (lysozyme solution consisting of 1 mg/ml of lysozyme dissolved in P medium: 0.5 M sucrose, 0.57 mM K_2SO_4, 48 mM $CaCl_2$, 25 mM MOPS, pH 7.2, 0.59 µM $ZnCl_2$, 1.48 µM $FeCl_3$, 0.12 µM $CuCl_2$, 0.10 µM $MnCl_2$, 0.052 µM $Na_2B_40_7$, 0.016 µM $(NH_4)_6Mo_7O_{24}$, 1% (w/v) bovine serum albumin, (BSA)) and allowed to digest at 28°C with gentle swirling at 50 rpm and checked every 30 min. Protoplasting was

complete when a drop of solution cleared upon addition of a drop of 10% SDS on a microscope slide. The protoplasts were filtered through non-absorbant cotton loosely packed in pasteur pipettes and then centrifuged for 10 min at 1480 x g. Protoplasts were washed twice with 25 mL of P medium. The pellet was resuspended in 5 ml of P medium. Protoplasts were counted in a hemacytometer (AO Scientific Instruments, Bright-Line) and aliquots of 2-4 x 10^9 protoplasts were frozen in screw capped plastic tubes at -75°C.

II.3.3.2.2. Transformation Procedure

The plasmid containing Tn4560 was introduced into *S. clavuligerus* using the following transformation procedure. One tube of *S. clavuligerus* protoplasts produced as above (II.3.3.2.1)was centrifuged at 1480 x g for 10 min and washed with 5 ml of P-medium (II.3.3.2.1). The pellet was redissolved in the small amount of P-medium that remained after the supernatant was removed. The protoplasts were heat shocked by incubation at 44°C for 5 min, and then supplemented with 10 µl of 10.3% sucrose and either 0.6 µg of plasmid DNA or 10 µl of TE buffer. One half millilitre of T medium (25% of PEG 1000 dissolved in P medium without BSA) was immediately added to the tube, mixed gently for 30 sec, and then 5 ml of P medium was added. The protoplasts were pelleted by centrifugation at 1480 x g for 10 min. The pellet was resuspended in 0.5 ml of P medium and counted in a hemacytometer. Fifty microlitres of a ten-fold dilution was plated (as many as possible) on regeneration agar (R2YE)(0.5 M sucrose, 0.58 mM K_2SO_4, 59 mM Na glutamate, 48 mM $CaCl_2$, 25 mM MOPS, pH 7.2, 0.20 mM $MgSO_4$, 0.29 µM $ZnCl_2$, 0.74 µM $FeCl_3$, 0.059 µM $CuCl_2$, 0.051 µM $MnCl_2$, 0.026 µM $Na_2B_4O_7$, 0.008 µM $(NH_4)_6Mo_7O_{24}$, 1% (v/v) glycerol, 0.1%

(w/v) Difco casamino acids, 0.5% (w/v) yeast extract, 2% (w/v) agar). Plates were incubated at room temperature for 24 h and then transferred to 28°C . Each plate had a volume of 20 ml. The plates were overlayed at 40 h with 5 ml R2YE soft agar (0.6% (w/v) agar containing thiostrepton at a final concentration of 5 µg/ml) to select for thiostrepton resistant transformants.

II.3.3.3. Induction of Transposition

Overlayed plates from II.3.3.2 were treated in two ways. The overlay from the plates was removed, homogenized and used to inoculate TCSS containing thiostrepton (5µg/ml) + viomycin (0.3 mg/ml) or the overlay was removed and the underlying plate scraped with a sterile spatula. The cell material removed by scrapping was then used to inoculate TCSS as above. After a 48 h incubation at 28°C the cultures were then subcultured into fresh TCSS containing viomycin only at 2% (v/v) and these cultures were grown for 48 h. The procedure of alternating subcultures between TCSS and TCSS + viomycin was repeated four times. At each subculture from TCSS the resistance of the culture to thiostrepton was determined. When the cultures appeared to be sensitive to thiostrepton the cultures were frozen in 1 ml aliquots.

II.4. SCORING FOR MUTANTS

II.4.1. Rifampicin Resistance

Mutants were scored for the acquistion of rifampicin resistance after they had grown to give visible colonies on 10 ml TCSS plates after mutagenesis (approx. 40 h). The plates were then overlaid with 10 ml of molten TCSS agar containing 2 µg/ml of rifampicin giving a final co . ntration of 1 µg/ml. The plates were then incubated for a further 5-6 days and those colonies which continued to grow further were counted.

II.4.2. S-2-Aminoethyl-L-cysteine resistance

To obtain spontaneous mutants which were S-2-Aminoethyl-L-cysteine (AEC) resistant, glycerol spore stocks were washed and plated on MM agar plates containing 1 mg/ml of AEC. Induced mutants were isolated by plating directly onto Difco ISP4 Medium containing 1 mg/ml AEC after mutagenesis.

II.5. ANALYSIS OF TRANSPOSON MUTANTS

II.5.1. Genomic DNA Preparation

Genomic DNA was prepared by the method of Hopwood (1985). Glycerol stocks of *S. clavuligerus* were used to inoculate 25 ml of TCSS. The culture was incubated at 28°C and 250 rpm for 48 h and then used to inoculate 100 ml of TCSS culture to 2% (v/v). Cells were harvested after 48 h by centrifugation at 25,700 x g for 15 min. The procedure of Hopwood (1985) was followed as stated until the DNA was precipitated with PEG 6000. Instead of being spooled on a glass rod the DNA was kept at 4°C for 30 min and then centrifuged at 11,400 x g for 10 min. The supernatant was decanted and the tubes allowed to drain dry for 15 min. The DNA pellet was dissolved in 5 ml of TE buffer and when dissolved (this could take up to 2 days) 0.6 ml of 3.0 M sodium acetate and 12 ml of 98% ethanol were added and mixed. This precipitated DNA suspension was kept at 4°C for 30 min and then centrifuged at 3020 x g for 10 min. The supernatant was decanted and the DNA pellet was washed with 70% ethanol. The pellet was again dissolved in 5 ml TE buffer and stored at 4°C.

II.5.2. Restriction Endonuclease Digestion

All restriction endonuclease digestions were carried out using the buffers and temperatures recommended by the manufacturer.

II.5.3. Nick Translation of Plasmids

The procedure of Maniatis et al. (1982) was used. A reaction mixture consisting of: nick translation buffer (NT buffer: 20 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.02 µg/µl BSA), ~ 1 µg of plasmid, 10 µCi α-^{32}P-ATP, 0.011 mM each of CTP, GTP and TTP was chilled to 4° C. To this mixture was added 1.1 ng/ml DNase and 3.5 units DNA polymerase I and the mixture was then incubated at 16° C for 60 min. After incubation the labelled DNA was separated from unincorporated, labelled ATP by passing through a Sephadex G25 (Pharmacia Fine Chemicals, Sweden) column.

II.5.4. DNA Transfer

DNA was separated by agarose gel electrophoresis as described in II.5 after digestion with restriction endonucleases. The DNA was transferred to nylon filters (Hybond N, Amersham) by the method of Southern (1975) as described by Maniatis et al. (1982).

II.5.5. Hybridization

Hybridization was performed by placing the nylon filter obtained after II.5.4 in a heat-sealable, plastic bag (Dazey, Corp.) together with 15 ml of prehybridization buffer (0.6 M NaCl, 0.06 M sodium citrate (6X SSC), 0.5% SDS, 15 ml 5X Denhardts solution (Denhardt, 1966). The bag was sealed after the air was removed and 500 mg of denatured salmon sperm DNA was added. The sealed bag was incubated horizontally for 1 h at 65°C. Hybridization was carried out by adding to the bag containing the prehybridizing solution about 10 µCi of ^{32}P labelled probe. The filter was incubated overnight at 65°C. The hybridized filter was washed 2X with 2X SSC containing 0.1% SDS and 2X with 0.1X SSC at 65°C for 30 min. The filter was removed from the bag, air dried and bound

radioactivity was located by autoradiography as in II.5.6.

II.5.6. Autoradiography

Hybridized nylon filters were analyzed by autoradiography by placing the filter between sheets of Saran Wrap (Dow Chemicals) and exposing X-ray film to them. The filter was placed in a X-ray film cassette and two X-ray films (Kodak X-Omat AR film) were placed on either side. Two intensifying screens (DuPont Cronex Lightening Plus) were also placed in the cassette, one on the outside of either film. The sealed cassette was placed at -75°C for 1-7 days, and then the film was developed according to the manufacturers directions.

II.6. ASSAYS

II.6.1. Preparation of Cell Free Extracts

Depending on the type of media used cultures of *S. clavuligerus* were grown for 48-96 h in 100 mL of media in 500 ml flasks, and harvested by centrifugation at 25,700 x g for 15 min at 4°C. The resulting pellets were washed with 50 ml of 0.2 M potassium phosphate buffer, pH 7.0, containing 0.3 M ß-mercaptoethanol (referred to as phosphate buffer) and resuspended in 10 ml of phosphate buffer in a 30 ml beaker. Cells were disrupted by sonication at setting 7 (Sonifer Cell Disrupter 350, 0.75 inch diameter probe, Branson Sonic Power Co.) in 15 sec bursts for a total of 90 sec at 4°C. Cells were centrifuged to remove cell debris at 25,700 x g for 10 min. The extracts were stored at -75°C.

II.6.2. Aspartokinase Assay

Aspartokinase activity of the cell free extracts prepared as described above was concentrated by precipitation with ammonium sulfate. Ammonium sulfate was added to 30% saturation and kept at 4°C for 30 min before centrifugation at 25,700 x g for 10 min. Supernatant was recovered and the ammonium sulfate

concentration increased to 65% saturation. The extract was again kept at 4°C for 30 min and then centrifuged at 25,700 x g for 10 min. The resulting pellet was resuspended in 1 or 5 ml of phosphate buffer.

Aspartokinase activity was measured by using the procedure of Truffa-Bachi and Cohen with some modifications (Truffa-Bachi and Cohen, 1970). Activity is measured as the formation of ß-hydroxamate. The assay mixture consisted of: 0.05 M Tris-HCl (pH7.6), 0.02 M ATP, 0.01 M $MgSO_4$, 0.02 M L-aspartic acid (potassium salt), 0.4 M NH_2OH-HCl (stock solution of 8 M was neutralized immediately before use with an equal volume of 8 M KOH) and 100-300 µl of enzyme. Distilled water was used to bring the final volume to 1 ml. This mixture was incubated for 40 min at 30°C. The reaction was stopped by the addition of 1 ml of 10% $FeCl_3$, 3.3% trichloroacetic acid in 0.7 N HCl. The mixture was centrifuged at 100 x g for 10 min to remove the precipitate and the absorbance of the supernatant was measured at 540 nm. A blank was made using the above mixture without aspartic acid. A standard curve using L-aspartic acid ß-hydroxamate was used to determine the amount of product produced.

II.6.3. Protein Assay

All protein assays were used the dye binding procedure of Bradford (1976) using the reagents supplied with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories). A standard curve was determined using bovine gamma globulin (Bio-Rad Laboratories).

II.6.4. Bioassay for Antibiotic Production

The bioassay for antibiotic production was completed with TCS agar inoculated to 2% (v/v) with an overnight culture of *E. coli Ess* . One hundred ml

of inoculated TCS agar was dispensed into a bioassay plate. Discs of filter paper (.25 inch diameter Analytical Paper, Schleicher and Schuell) were placed on the agar and saturated with 20 µl samples of filtered supernatant from *S. clavuligerus*. The plates were then incubated at 37°C for 24 h. The diameters of the zones of inhibition were then measured and compared. *E. coli Ess* is sensitive to penicillins and cephamycins at the same level (A.L. Demain, personal communication).

II.6.5. Antibiotic Production By Mutant and Wildtype Cultures of *S. clavuligerus*

Seed cultures of wildtype and AEC resistant mutant strains of *S. clavuligerus* were used to inoculate 100 ml of TCSS and MM with or without 1.5 mg/ml of AEC in 500 ml flasks. These flasks were then incubated at 28°C and shaken at 250 rpm. At 8 h intervals for a total of 136 h, 1 ml samples were removed and the optical densities at 600 nm (OD_{600}) were determined. The samples were then centrifuged in an eppendorf microcentrifuge for 5 min at maximum speed. The supernatants were used to determine the total antibiotic content using the bioassay procedure outlined in II.6.4.

II.6.6. Aspartokinase Production by Mutant and Wildtype Cultures of *S. clavuligerus*

Seed cultures of wildtype and AEC resistant mutant strains of *S. clavuligerus* were used to inoculate 100ml of TCSS and MM with or without AEC to 2% (v/v). At 8 h intervals for a total of 96 h, 500 ml flasks containing 100 ml of each culture were removed. The cultures were centrifuged at 30,100 x g for 20 min. The loose cell pellet was then transferred to a funnel containing Whatman #1 filter paper and washed with phosphate buffer and stored at -75° C until all samples had been collected. Cells were thawed and cell free extracts prepared as in II.6.1 and assayed for aspartokinase activity (II.6.2).

II.7. EFFECTS OF AMINO ACIDS ON ASPARTOKINASE

II.7.1. Effect of Amino Acids on Production of Aspartokinase

Seed cultures of both wildtype and AEC resistant mutant strains of *S. clavuligerus* were used to inoculate various media. One hundred ml amounts of MM containing: no additional amino acids; 10 mM lysine; 10 mM threonine; 10 mM lysine + 10 mM threonine; and 1.5 mg/ml AEC (7 mM) were inoculated as well as TCSS. After 48 h these cultures were harvested and cell free extracts produced as in II.6.1.

II.7.2. Effect of Amino Acids on Aspartokinase Activity

Cell free extracts of both wildtype and AEC resistant mutant strains of *S. clavuligerus* were prepared using cells grown for 48 h on MM as in II.6.1. These cell free extracts were used to assay aspartokinase activity under varying conditions. Both lysine and threonine were added at concentration of 5 mM to the assay solution and assayed as in II.6.2.

II.8. ELECTROPHORESIS AND PHOTOGRAPHY

Agarose gel electrophoresis was used to separate DNA fragments resulting from restriction endonuclease digestion. One percent agarose (SeaKem Type LE) gels were prepared by dissolving the agarose in TEA buffer (20 mM Tris-HCl (pH8.0), 2 mM EDTA, and 50 mM sodium acetate) with the addition of 0.0001 volume of ethidium bromide (10 mg/ml). Molecular weight markers were prepared by BstEII digestion of lambda phage DNA. The gel was placed in a Max Submarine Agarose Gel Unit (Model HE 99, Hoefer Scientific Instruments, San Francisco) covered with TEA buffer and a voltage of up to 6 V per cm was placed across the gel. When electrophoresis was completed the gel was photographed using Polaroid type 667 or 665 film, using a Kodak Wratten gelatin filter number 23A and an ultraviolet light transilluminator at 300 nm.

III. RESULTS

III.1. AEC RESISTANCE IN *S. CLAVULIGERUS*

III.1.1. Sensitivity to AEC and Appearance of Spontaneous Mutants to AEC

In previous studies, Mendelovitz and Aharonowitz (1983) found that some AEC resistant mutants of *S. clavuligerus* produce higher levels of antibiotics than do wild type strains. To isolate and study these types of mutants it was first necessary to determine the normal sensitivity of wildtype *S. clavuligerus* to AEC. To determine the level of *S. clavuligerus* sensitivity to AEC a series of plates with different concentrations ranging from 1 µg/ml to 1 mg/ml of AEC in MM was prepared. Spore stocks were spread onto these plates. After 48 h there was good growth on the 1 µg/ml plates with gradually less growth as the concentration of AEC increased. However there was still some growth at 1 mg/ml. The AEC appeared to limit growth but not to inhibit growth completely at these concentrations. To eliminate any carryover of amino acids from the spore stocks that could be used for growth, the spore stocks were pregrown in MM broth for 48 h. These cultures were then used to repeat the above experiment.

The cultures pregrown in liquid MM showed inhibition at 1 mg/ml AEC. The lawn of growth that had been observed at 1 mg/ml AEC with spores that were not pregrown was not present. However with prolonged incubation isolated colonies began to appear. After 4 days incubation, 2 colonies appeared on the 1 mg/ml AEC plate: after further incubation (1 week) 6 more colonies grew. These colonies were streaked on the same plate to obtain sufficient inoculum for future experiments, and allowed to grow. Further growth occurred around the streaks after approximately 1 week. It was decided to use 1 mg/ml of AEC in MM plates to

select for AEC resistant mutants in future experiments. When sensitivity to AEC was tested in liquid medium using the same increasing concentration of AEC, cultures appeared somewhat less sensitive to AEC in liquid medium. From these findings it was decided to use 1.5 mg/ml of AEC in liquid medium to distinguish AEC resistant from AEC sensitive isolates. Although there is not total inhibition, the amount of growth is substantially less than at lower concentrations in both solid and liquid medium.

III.1.2. Isolation of Spontaneous AEC Resistant Strains

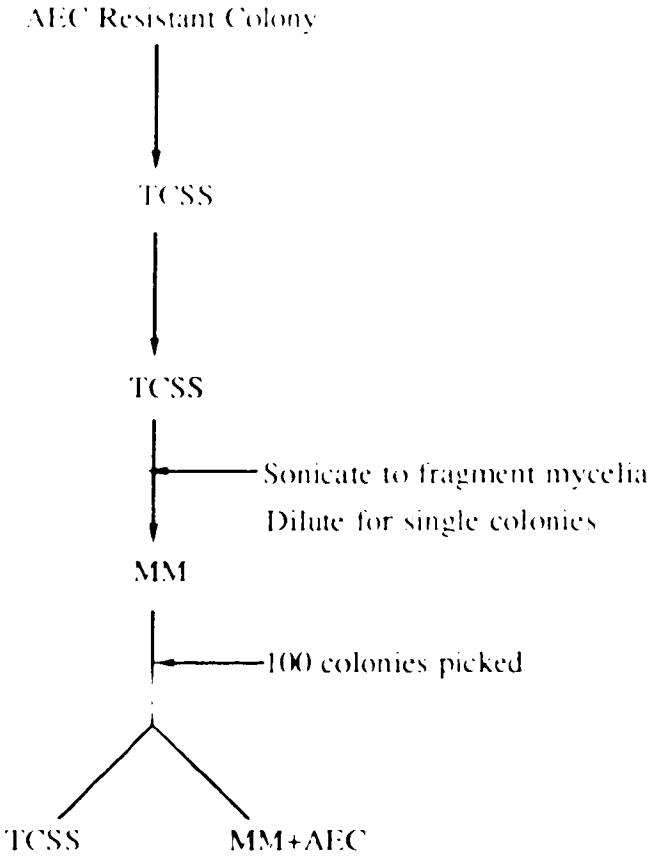
To quantitate the number of AEC resistant mutants which occur spontaneously, spores from a single spore isolate were plated onto MM containing AEC at 1 mg/ml and the number of colonies which resulted were determined. Glycerol spore stocks were washed, serially diluted and plated on MM to determine the number of spores/ml. Undiluted samples were plated on MM+AEC to determine the number of spontaneous mutants. The spontaneous frequency of mutation was found to be 2.6×10^{-7}.

III.2. Investigation of the Mechanism of AEC resistance

III.2.1. DNA Amplification

To determine if the spontaneous resistance to AEC was due to an amplification of the genes which produce AEC sensitive enzymes or to some different mechanism, reversion tests were done. If gene amplification had occurred, a high frequency of reversion to wildtype would be expected upon removal of the AEC. The experiment followed the scheme outlined in Fig. 3. Spontaneous AEC resistant mutants isolated in III.1.1 were used to inoculate plates of MM+AEC. At 24 h, growth from plates was scraped and used to inoculate 10

Figure 3. Experimental procedure used to investigate amplification in AEC resistant mutants. An AEC resistant colony was grown in liquid TCSS for 48 h, and subcultured into TCSS. The mycelia/cells were then sonicated, serially diluted and plated onto MM. One hundred colonies were then picked and inoculated onto both TCSS and MM+AEC.


AEC Resistant Colony
TCSS
TCSS
Sonicate to fragment mycelia
Dilute for single colonies
MM
100 colonies picked
TCSS
MM+AEC

ml amounts of TCSS liquid medium. Cultures were aseptically homogenized after 24 h of incubation to disrupt pelletized cell masses and allow smooth filamentous growth. The cultures were allowed to grow for a further 48 h after homogenization. The cultures were then subcultured using a 1% inoculum into TCSS and grown for a further 48 h. The TCSS cultures were then sonicated for 70 sec at power setting 1 on the Branson Sonicator. The fragmented mycelia were serially diluted and spread on MM and incubated for 1 week at 28°C. One hundred colonies were picked onto both MM+AEC and then TCSS. The results are shown in Table 1. The spontaneous AEC resistant isolates were found to be quite stable, as reversion to wild type was low in most cases. The resistant isolate designated as mutant 4 had the highest reversion rate at 42%. In order to determine whether this relatively high reversion rate was indicative of gene amplification the isolate was studied further.

To further investigate amplification, DNA was purified from mutant 4, digested with restriction endonucleases and analyzed by agarose gel electrophoresis Plate 1. When DNA from mutant 4 was digested with restriction endonucleases and analyzed by agarose gel electrophoresis, it gave a diffuse pattern of many faintly stained bands. No apparent differences were seen between the wildtype and AEC resistant mutant cultures. If amplification had occurred intense bands representing amplified DNA would be seen (Demuyter et al., 1988).

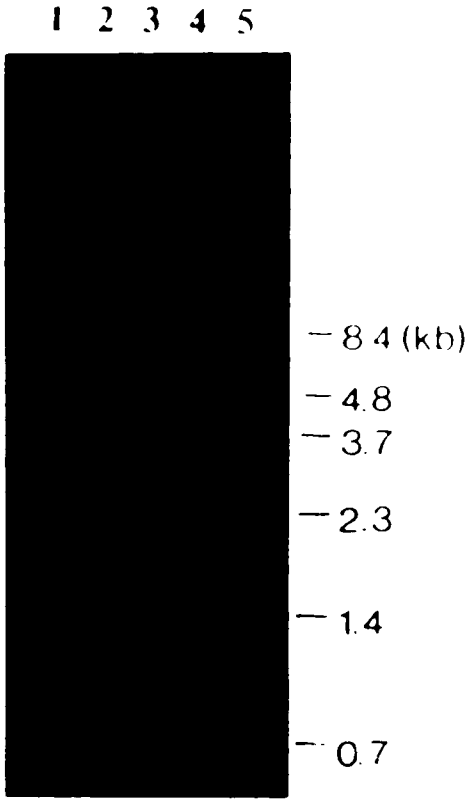
III.2.2.Scavenger Colonies

When colonies resistant to AEC were first isolated, continued incubation allowed more colonies to grow up surrounding the original mutant colonies (refer to III.1.1). The additional colonies which grew were thought to be scavenger colonies rather than true AEC resistant colonies in that their growth seemed to be

Table 1: The rate of reversion of AEC resistant mutants to wildtype.

Mutant	Growth on TCSS	Growth on MM+AEC	% Reversion
1	100	93	7
3	100	73	27
4	100	58	42
5	100	99	1
6	100	100	0

Plate 1. Agarose gel electrophoresis of restriction endonuclease digests of wildtype and AEC resistant mutant 4 chromosomal DNA. Chromosomal DNA was digested separately with Bgl II and Bcl, and then subjected to electrophoresis on a 1% agarose gel. Lane 1 contained Bgl II digested wildtype DNA , lane 3 contained λ DNA digested with BstE II. Lanes 4 and5 contained Bgl II digests of mutant 4 DNA.


1 2 3 4 5
8.4 (kb)
4.8
3.7
2.3
1.4
0.7

dependent on some effect caused by the original AEC resistant colonies. To determine if these were scavenger colonies the mutant and wild type strains were streaked on MM+AEC with the wildtype streak crossing into the mutant streak. All of the mutant strains grew well along the streak. Wildtype streaks also eventually grew on the plate but they grew more slowly than the AEC resistant mutants. There did not appear to be any scavenger colonies as wildtype grew as well near to the AEC resistant streak as it did at other regions of the streak.

III.3. ISOLATION OF INDUCED AEC RESISTANT STRAINS

In the above studies spontaneous AEC resistant mutants were used. Preliminary studies on antibiotic production by these spontaneous AEC resistant isolates gave results which did not agree with the findings of Mendelovitz and Aharonowitz (1983). Overproduction of antibiotics was found in some experiments and underproduction in others, while using the same mutant. Mendelovitz and Aharonowitz (1983) used induced mutants for their studies. To conform more closely to the studies of Mendelovitz and Aharonowitz (1983), AEC resistant isolates arising as a result of mutagenic treatments were sought. Many different methods are available to generate mutants. In order to produce mutants of *S. clavuligerus* a study of the factors affecting mutagenesis was needed. Various mutagenic treatments known to be effective for mutagenizing *Streptomyces sp.* were tried to determine the optimum method for production of mutants as well as the optimum stage in the life cycle of *S. clavuligerus* for mutagenic treatments. In initial studies aimed at evaluating the effectiveness of various mutagenic procedures mutants were scored for the acquistion of resistance to rifampicin at 1 µg/ml. Rifampicin resistance was used in preference to AEC resistance for these studies because of the lower cost of the rifampicin and the clearly defined difference

between sensitive and resistant isolates.

III.3.1. Determining Optimum Conditions for Mutagenesis of *Streptomyces clavuligerus*

III.3.1.1. Ultraviolet Light Mutagenesis Using Ungerminated Spores

Ungerminated spores were placed under UV light for timed intervals to induce mutations. These spores were then plated to determine the number of viable spores and the number of mutants. The number of viable cells was determined by plating on TCSS and the number of rifampicin resistant mutants determined by plating on TCSS and overlaying with TCSS+2 µg/ml rifampicin (refer to II.3.1). The effects of UV irradiation on spore survival and production of rifampicin resistant mutants are shown in Table 2. The survival rate of the spores decreased exponentially with increasing time of exposure to UV light from 0 to 80 sec. The frequency of mutants producedwas very low. There were no detectable mutants at 0 sec and the highest frequency occurring at 80 sec with only 1.84×10^{-5} of the survivors resistant to rifampicin.

III.3.1.2. Ultraviolet Light Mutagenesis Using Germinated Spores

The same procedure as above was used here except that spores were allowed to germinate by growth in TCSS for 6-8 h before mutagenesis as described in II.3.1. After UV treatment the germinated spores were scored for survival rate and aquisition of resistance to rifampicin (Table 2). The survival rate closely matched that of the ungerminated spores as seen in Table 2. However the frequency of mutant isolation was much greater than that seen with ungerminated spores. After 20 sec of exposure to UV light the rate was already 10 times that of the ungerminated spores and the rate after 80 sec of exposure is 100 times greater.

Table 2: Ultraviolet Light Mutagenesis of Spores

Spore Type	Time (sec)	% [illegible]	Frequency of Mutants
Ungerminated	0	[illegible]	0
	20	56.5	1.1×10^{-7}
	40	34.7	3.3×10^{-7}
	60	18.3	1.5×10^{-7}
	80	5.3	1.8×10^{-5}
Germinated	0	100	0
	20	60.7	1.2×10^{-4}
	40	14.3	2.0×10^{-4}
	60	13.1	2.4×10^{-4}
	80	6.5	1.3×10^{-3}

Obviously the use of germinated spores for mutagenesis was much more efficient than treatment of ungerminated spores

III.3.1.3. NTG Mutagenesis Using Ungerminated Spores

Spores were incubated with NTG at a final concentration of 500 µg/ml for up to 2 h. The spores were then filtered, washed with distilled water and serially diluted. As described above, the number of viable cells and the number of rifampicin resistant mutants were determined (Table 3). The survival rate of ungerminated spores upon NTG mutagenesis was higher in UV mutagenesis. However the frequency of mutants produced was approximately the same as in the UV experiments. The mutation frequency was slightly higher than in UV. However the total number of mutants produced was higher, as the survival rate was higher and mutants are expressed as a rate of the number of survivors.

III.3.1.4. NTG Mutagenesis Using Germinated Spores

The procedure used above was repeated using germinated spores (refer to II.3.2). Results of these experiments are shown in Table 3. As was seen above in the experiments on UV mutagenesis, the survival rate for ungerminated and germinated spores is very similar. While the mutant production rate was somewhat higher than that of ungerminated spores, the difference was not as great as was seen in UV mutagenesis between ungerminated and germinated spores. The rate at 120 min in the germinated spores experiments . only 30% higher than in the ungerminated spore results. However the initial rate of mutation showed a much greater difference. Because mutagenesis procedures using NTG were not significantly better than UV treatment for the production of rifampicin resistant mutants, and because of the greater inherent danger in working with NTG, UV light was used to produce AEC resistant mutants for further study.

Table 3: NTG mutagenesis of spores

Spore Type	Time (min)	% Survival	Frequency of Mutants
Ungerminated	0	100.0	3.5×10^{-7}
	30	42.9	1.0×10^{-6}
	60	44.7	4.4×10^{-5}
	120	26.6	9.6×10^{-5}
Germinated	0	100	0
	30	58.8	4.2×10^{-5}
	60	45.0	1.0×10^{-4}
	120	20.3	1.4×10^{-4}

III.3.2. Isolation of AEC Resistant Strains by Mutagenesis of Germinated Spores with Ultraviolet Light:

To obtain AEC resistant mutants by UV mutagenesis, germinated spores were exposed to UV light for 40 sec. The spores were then serially diluted and plated directly onto Difco ISP4 medium containing 1 mg/ml of AEC. For further investigation 6 colonies which appeared to have the best growth were chosen. These colonies were then used to produce spore stocks as in II.2.2.1.

III.3.3. Isolation of AEC Resistant Strains by Transposon Mutagenesis

Another method of mutagenesis which became available during the course of this study was transposon mutagenesis. The transposon was obtained as a freeze dried culture of *S. lividans* containing the plasmid pUC1169. This plasmid contains the transposon Tn4560 which has a viomycin phosphotransferase gene as a selectable marker. In addition the plasmid itself carries the thiostrepton resistance gene as a selectable marker. See Figure 4.

III.3.3.1 Plasmid Purification

The *S. lividans* culture carrying the plasmid pUC1169 was inoculated into TCSS. Plasmid preparations were carried out according to the method outlined in II.3.3.1. After purification by CsCl density gradient centrifugation, the plasmid preparation was digested with the restriction endonucleases Pst I and Cla I to determine if the correct plasmid was present. The fragments were analyzed by agarose gel electrophoresis as seen in Plate 2. The plasmid fragments seen corresponded to those expected as calculated from the restriction map in Figure 8 in the Pst 1 digests. The DNA fragment of approximately 2 kb is seen faintly. The 400 bp DNA fragment that should be seen in the Cla I digest, however may have been eluted from the gel. A stock of pUC1169 was now available for use as a tool

Figure 4. Restriction enzyme map of pMT660 containing the transposon Tn4560. This map was reproduced from a map provided by Dr. Chung (Chung, personal communication, 1987).

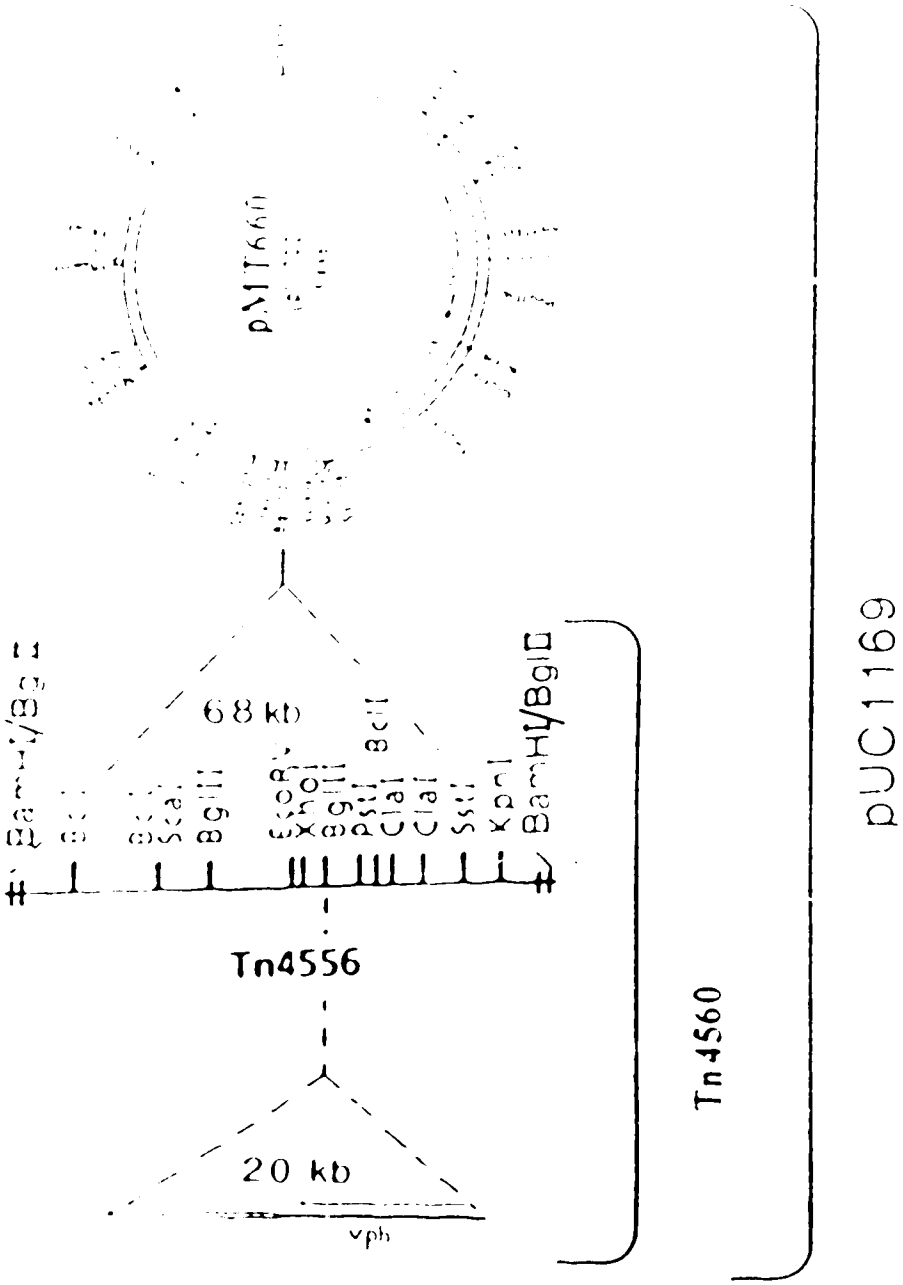
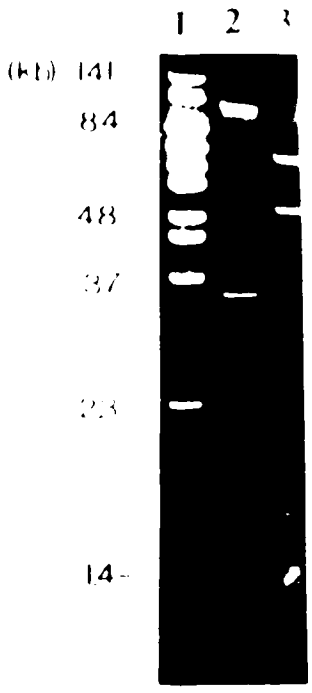

pUC1169
Tn4560
68 kb
Tn4556
20 kb
vph
BamHI/BglII
BglII
ClaI
SstI
KpnI
BamHI/BglII

Plate 2. Agarose gel electrophoresis of restriction endonuclease digests of purified pUC1169 isolated from *S. lividans*. Aliquots of plasmid DNA were digested separately with Pst I and Cla I. Lane 2 contained plasmid DNA digested with Cla I and lane 3 plasmid DNA digested with Pst I. Lane 1 contained λ size markers as described in plate 1.


1 2 3
(kD) 141
84
48
37
14

for mutagenesis.

III.3.3.2. Transformation of *S. clavuligerus* with pUC1169

Initially the plasmid was transformed into protoplasts prepared from a restriction deficient mutant of *S. clavuligerus* . *S. clavuligerus* possesses a restriction-modification system which would ordinarily recognize the plasmid from *S. lividans* as foreign and it would be digested by restriction endonucleases. To overcome this, the plasmid is initially transformed into a restriction deficient mutant of *S. clavuligerus*. The transformation procedure outlined in II.3.3.2. was followed. Fourteen transformants were picked and grown in TCSS + 5 µg/ml of thiostrepton. From these transformants four were randomly picked and used to prepare plasmid. The plasmids were analysed as above. Plasmid from one of the transformants was further purified and digested with Pst I again as seen in Plate 3. Purified pUC1169 was now available from *S. clavuligerus* (and therefore appropriately modified for *S. clavuligerus*) and could be used to transform the wildtype single spore isolates of *S. clavuligerus* at a higher frequency. Protoplasts were prepared from the single spore isolate, for the purpose of inducing transposon mutants according to II.3.3.2.1. The purified plasmid pUC1169 obtained from *S. clavuligerus* was used to transform these protoplasts without the restriction barrier which would prevent high efficiency transformation with plasmid from *S.lividans*. Transformants were again selected for acquisition of thiostrepton resistance.

III.3.3.3. Induction of Transposition

The scheme outlined in Fig. 5 and described in more detail in II.3.3.3. was used to induce transposition of Tn4560. The resultant cultures from this procedure were viomycin resistant and thiostrepton sensitive suggesting that the transposon had moved from the plasmid to the chromosome. Loss of resistance to

Plate 3. Agarose gel electrophoresis of restriction endonuclease digests of purified pUC1169 isolated from *S. clavuligerus*. Aliquots of plasmid DNA were digested with Pst I. Lanes 1, 3, and 4 contained plasmid DNA. Lane 2 contained λ size markers as described in plate 1.

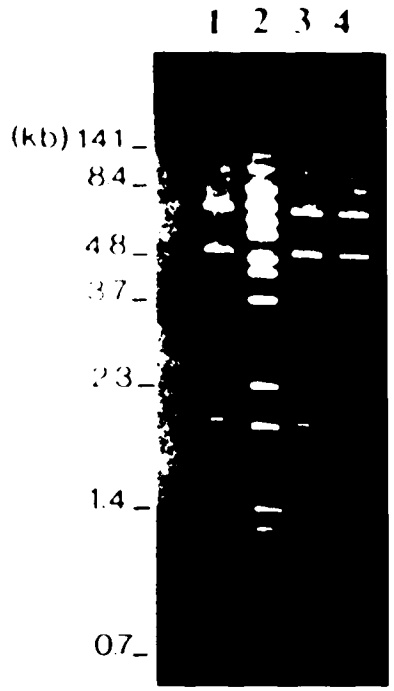
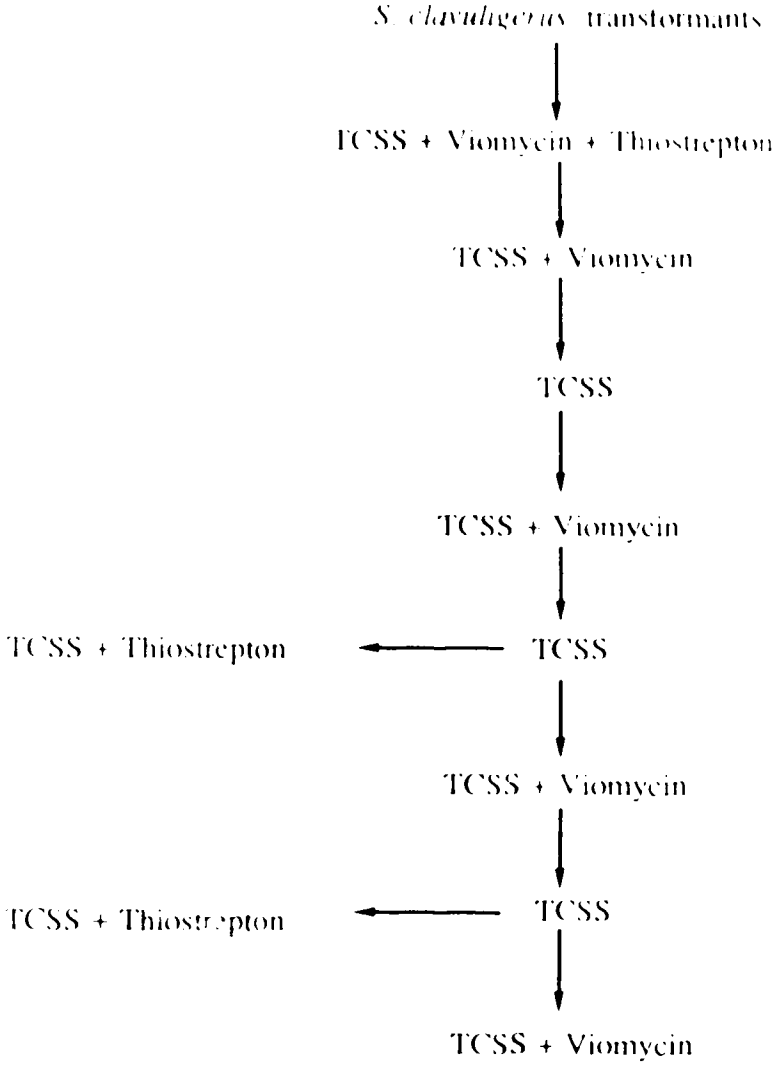

1 2 3 4
(kb) 14.1
8.4
4.8
3.7
2.3
1.4
0.7

Figure 5. Experimental procedure used for induction of transposition. *Streptomyces clavuligerus* transformants containing the plasmid pMT660 were scraped from a plate homogenized and used to inoculate TCSS + Viomycin + Thiostrepton. The cultures were then subcultured repeatedly into TCSS + Viomycin and then TCSS. Cultures were tested for thiostrepton sensitivity when subcultured into TCSS.


S. clavuligerus transformants
TCSS + Viomycin + Thiostrepton
TCSS + Viomycin
TCSS
TCSS + Viomycin
TCSS + Thiostrepton
TCSS
TCSS + Viomycin
TCSS + Thiostrepton
TCSS
TCSS + Viomycin

thiostrepton indicated that the plasmid was not present. The viomycin resistance marker on the transposon is not very useful in *S. clavuligerus*, since the intrinsic level of resistance in wild type cultures is 300 µg/ml. However, the presence of the transposon was detectable as it allowed growth above this level.

III.3.3.4. Isolation of AEC Resistant Mutants Containing Tn4560

Cultures obtained from above were serially diluted and plated onto Difco ISP4 medium + 1 mg/ml of AEC. Resistant colonies were then picked and analysed for the presence of Tn4560. Because viomycin resistance was not a clear cut property, mutants were analyzed for the presence of transposon by hybridization of chromosomal DNA with radioactively labelled transposon DNA. Four AEC resistant mutants which grew well were picked and total DNA was prepared as described in II.5.1. The total DNA was digested with Bgl I and Sal I and separated using agarose gel electrophoresis (Plate 4). The wildtype DNA digests are seen faintly in lanes 2 and 8. Although the wildtype DNA was seen on the negative for this plate it does not appear on the plate to the same brightness. There is less wildtype DNA present than mutant strain DNA. Also present on the agarose gel was Bgl II and Sal I digested pUC1169 and wildtype *S. clavuligerus* chromosomal DNA. The DNA was then transferred from this gel to a nylon filter as described in II.5.4. The plasmid was labelled as in II.5.3 and was used to hybridize to the total DNA digests (see II.5.5). The plasmid pIJ702 was also used in the same procedure as a negative control to check that any hybridization was due to transposon only. There was no hybridization of pIJ702 to wildtype or AEC resistant strain chromosomal DNA (results not shown). After autoradiography (see II.5.6) two predominant bands were seen in the Bgl II digests of all mutants (Plate 5). The Sal I digests showed no discrete bands but a smear of hybridization

Plate 4. Agarose gel electrophoresis of restriction endonuclease digests of total DNA from wildtype and transposon containing AEC resistant mutants. Total DNA was prepared from mutants T1, T2, T3, T4 and wildtype. The DNA was digested separately with Bgl II and Sal I. Lane 1 and 13 contained pUC1169 digested with Bgl II and Sal I respectively. Lanes 2-6 contained Bgl II digests of wildtype, T4, T3, T2, and T1. Lane 7 contained λ size markers as described in plate 1. Lanes 8-12 contained Sal I digests of wildtype, T4, T3, T2, and T1.

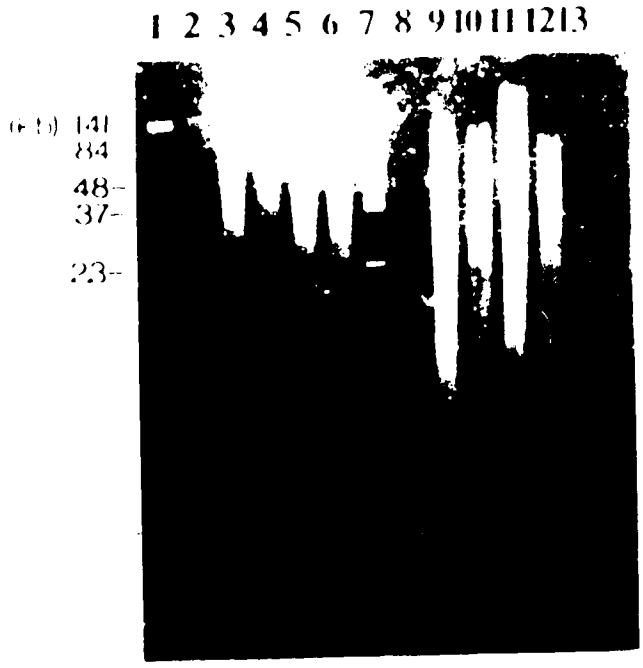
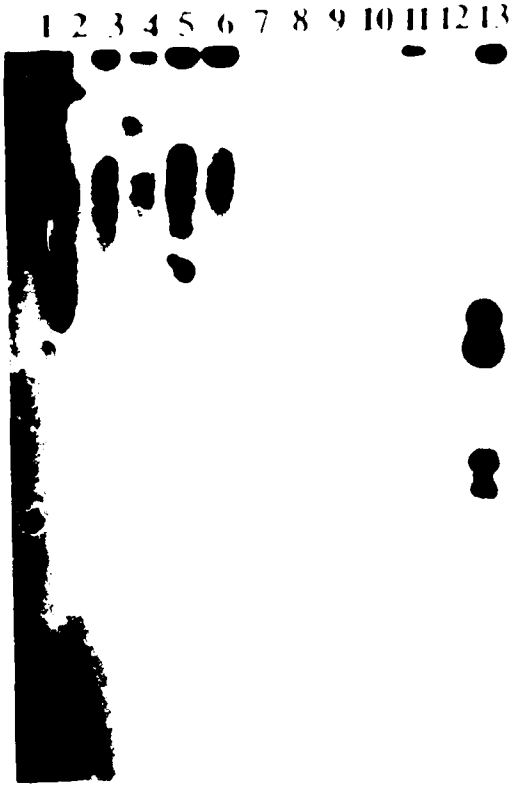

1 2 3 4 5 6 7 8 9 10 11 12 13
(kD) 141
84
48
37
23

Plate 5. Hybridization of restriction endonuclease digests of total DNA from wildtype and transposon-containing AEC resistant mutants, with the ^{32}P-labelled pUC1169 probe. Following electrophoresis, as described in plate 4, the DNA fragments were transferred to a nylon filter and the filter exposed to the ^{32}P-labelled probe.


1 2 3 4 5 6 7 8 9 10 11 12 13

from each mutant. No hybridization was seen to wildtype digests. This would indicate that the transposon is present in mutant isolates. Although the bands in the mutant DNA lanes are fainter than with pUC1169 this is expected as the copy number is also lower.

III.4. CLONING OF A REC-A LIKE GENE IN *S.CLAVULIGERUS*

In addition to investigating AEC resistant mutants it was decided to try and find a recA⁻ mutant from *S.clavuligerus* . Recent papers (Kokjohn and Miller,1987, Owttrein and Coleman, 1987, and Goldberg and McKalonos 1987) had described the cloning of recA like genes from *Pseudomonas aeruginosa* PAO, *Anabena variabilis* and *Vibrio cholerae* using the same method as these species were quite diverse and the method of cloning relatively easy it was decided to try and isolate this gene in *S. clavuligerus*. A recA⁻ mutant would be particularly useful for studies on transposition since recA⁻ mutants should be unable to carryout recombination this would allow for the recognition of true transposition rather than recombination as the mode of action for acquistion of viomycin resistance. It would also be advantageous to have this mutant for any further cloning work in which complementation is involved.

A library of *S. clavuligerus* chromosomal DNA fragments carried on the plasmid vector pUC119 (a gift from J. Vieria, Waksman Institue of Microbiology, Rutgers University) in *E. coli* JM109 (a rec⁻ mutant) was available for use. The sensitivity of *E. coli* JM109 to nitrofurantoin (NFT) and methylmethanesulfonate (MMS) was determined and used to select for *E. coli* JM109 transformants with the *S. clavuligerus* rec-A like gene. Rec⁻ cells are sensitive to NFT and MMS at concentrations of 4 µg/ml and 0.04% respectively, while rec⁺ cells are not. Colonies of JM109 carrying the library of *S. clavuligerus* DNA fragments were

picked onto LB broth + ampicillin, to select for the plasmid vector pUC119 which confers ampicillin resistance, and LB + NFT or MMS to identify putative rec$^+$ isolates. Those colonies which contained a plasmid but were sensitive to NFT and MMS were further characterized. Plasmids were isolated from the putative rec$^+$ strains according to Maniatis et al. (1982). Plasmids were digested using Kpn I and Sal I and the DNA fragments separated by agarose gel electrophoresis (Plate 6). There were no bands which were common to all of the plasmids or to a majority of the plasmids, indicating that no common DNA fragment was isolated. The UV sensitivity of *E. coli* JM109 and wildtype was determined. Rec$^-$ strains are UV sensitive; this criterion was used to determine if any of the colonies picked had become rec$^+$. All the colonies picked exhibited UV sensitivity characteristics which were indistinguishable from *E. coli* JM109 (results not shown), and were therefore not apparently rec$^+$. This approach to cloning the rec$^+$ gene relies upon the ability of *E. coli* JM109 to express the *S. clavuligerus* rec gene. Since *E. coli* has been found by other workers to be unable to express many genes of *Streptomyces* genes this line of investigation was not pursued further.

III.5. ANALYSIS OF AEC RESISTANT MUTANTS

III.5.1. Antibiotic Production In AEC Resistant Mutants

As stated above Mendelovitz and Aharonowitz had found that a large proportion of their AEC resistant mutants showed higher levels of ß-lactam antibiotic production than did wildtype cultures (Mendelovitz and Aharonowitz, 1983). In my initial experiments with spontaneous AEC resistant mutants, conflicting results were obtained in the levels of antibiotic production. Some mutants had produced higher levels of antibiotic than wildtype in some experiments and lower in others (results not shown). In order to determine if this was due to a

Plate 6. Agarose gel electrophoresis of restriction endonuclease digests of plasmids from library colonies which showed rec$^+$ characteristics. Aliquots of plasmid were digested separately with Kpn I and Sal I, and then subjected to electrophoresis on a 1% agarose gel. Lanes 1-13, 15 and 1[illegible] contained plasmids digested with Kpn I. Lanes 17-25, and 27-3[illegible] contained plasmid digested with Sal I. Lanes 14 and 26 contained λ size markers as described in plate 1. Sixteen samples were electrophoresed on the top half of the gel and sixteen additional samples were electrophoresed on the bottom half of the gel.

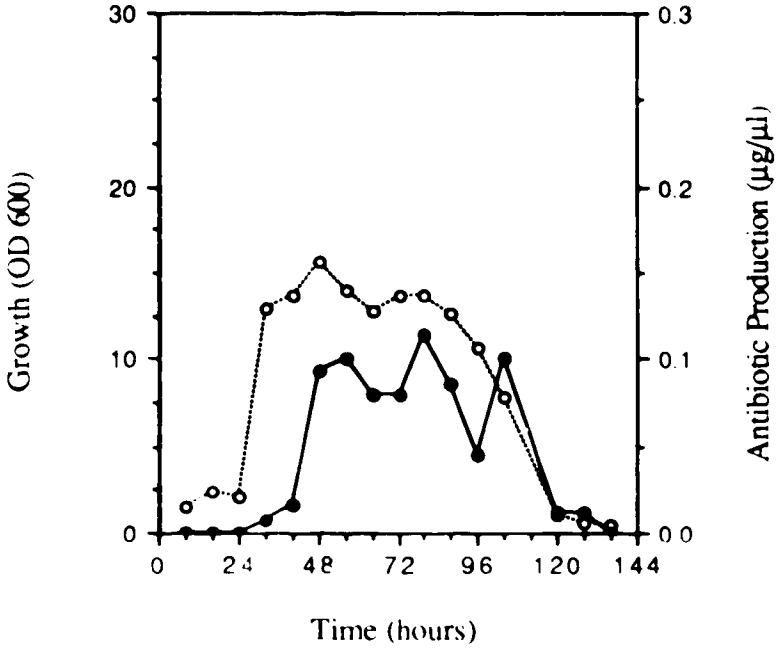

1
14
16
17
26
32

difference in type of mutants studied (Mendelovitz and Aharonowitz, 1983) had used induced AEC resistant mutants whereas the ones used in these initial studies were AEC resistant spontaneous mutants), antibiotic production was determined for the various different types of AEC resistant mutants which were isolated during this study, including both spontaneous and induced mutants. For each mutant examined, antibiotic production was followed throughout the growth cycle to take into account differences which might arise due to the age or growth stage of the culture. Mutants used in this experiment were spontaneous (5 and 8), UV mutagenized (UV1 and UV3), and transposon containing mutants (T2 and T4). Wildtype strains and mutants isolates were grown in TCSS and MM. Mutants were also grown in MM+AEC. As TCSS is a complex medium, AEC (a lysine analog) was not added to this medium. Data from this set of experiments are shown in Figures 6-14. The peak antibiotic production appeared to occur at approximately 72 h in TCSS and 96 h in MM in most cases. All mutants appeared to overproduce antibiotics in TCSS compared to wildtype but to varying degrees. No one type of AEC resistant mutant seemed particularly better than any other in terms of antibiotic production. All isolates produced much lower amounts of antibiotics on defined medium than in TCSS but the AEC resistant mutants produced about the same or lesser amounts of antibiotic than did wildtype.

III.5.2. Time Course of Aspartokinase Production in Wildtype and Mutant 5

Mendelovitz and Aharonowitz (1983) had found that AEC resistant mutants which overproduced antibiotics had deregulated aspartokinase production. Spontaneous AEC resistant mutant 5 appeared to overproduce antibiotic on TCSS in

Figure 6. The production of antibiotic by wildtype and AEC resistant mutants grown in TCSS. Seed cultures of wildtype and AEC resistant mutants were grown for 48 h and used to inoculate TCSS. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. Wildtype. b. Spontaneous mutant 5. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))


30
20
10
0
0.3
0.2
0.1
0.0
0
24
48
72
96
120
144
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

b

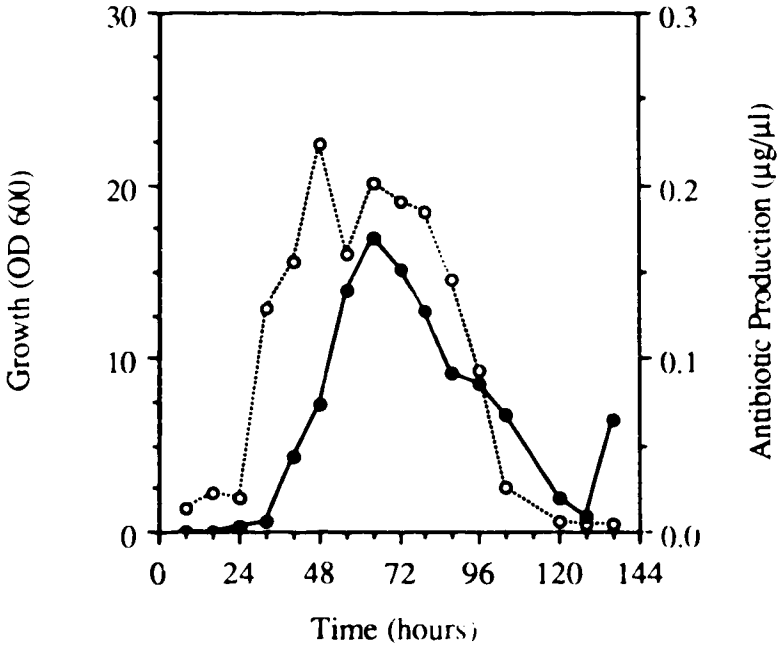
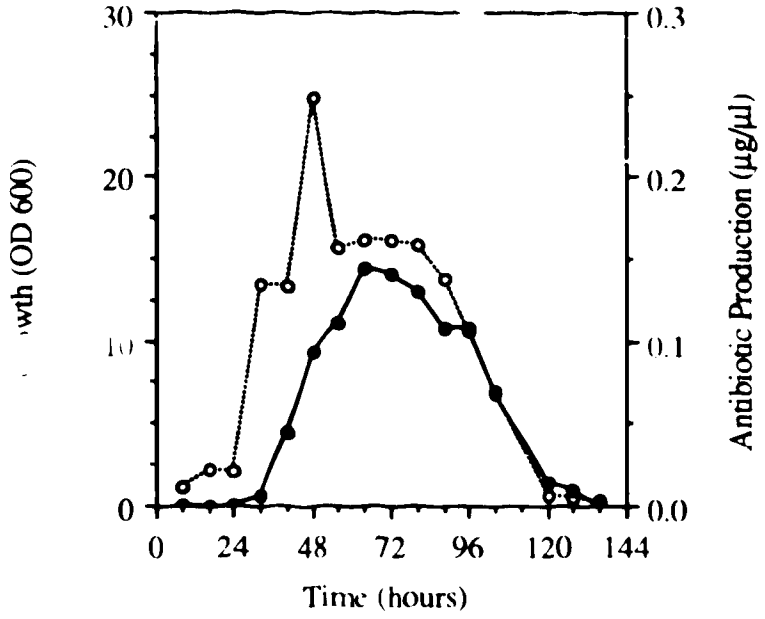

30
20
10
0
0.3
0.2
0.1
0.0
0
24
48
72
96
120
144
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

Figure 7. The production of antibiotic by AEC resistant mutants grown in TCSS. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate TCSS. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. Spontaneous mutant 8. b. UV1(○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


30
20
10
0
0.3
0.2
0.1
0.0
0
24
48
72
96
120
144
Time (hours)
Antibiotic Production (µg/µl)

b

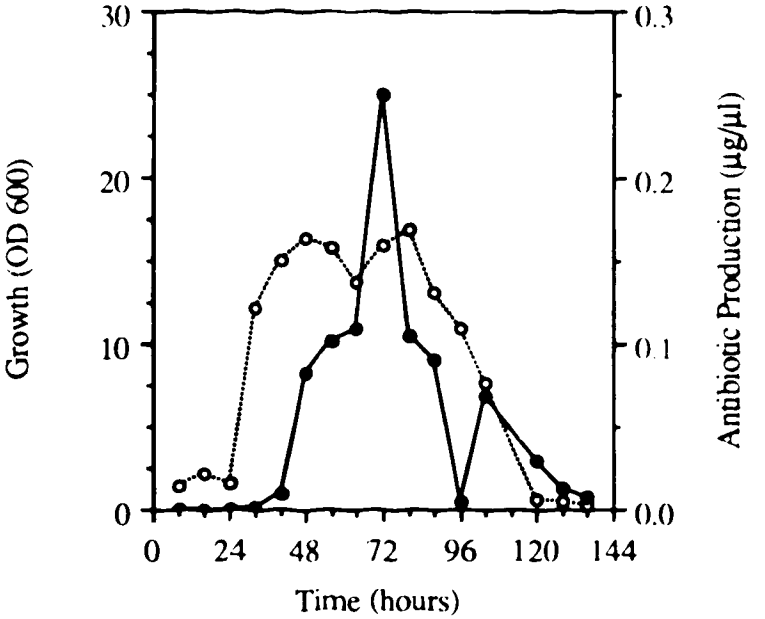
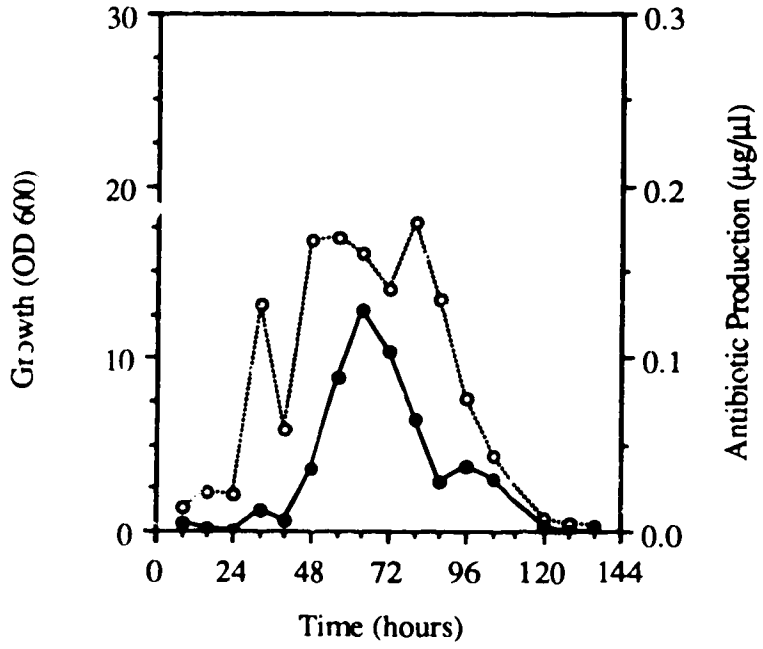

30
20
10
0
0.3
0.2
0.1
0.0
0
24
48
72
96
120
144
Time (hours)
Growth (OD 600)
Antibiotic Production (µg/µl)

Figure 8. The production of antibiotic byAEC resistant mutants grown in TCSS. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate TCSS. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4.

a. T2. b. T4. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


30
20
10
0
0.3
0.2
0.1
0.0
0 24 48 72 96 120 144
Time (hours)
Antibiotic Production (µg/µl)

b

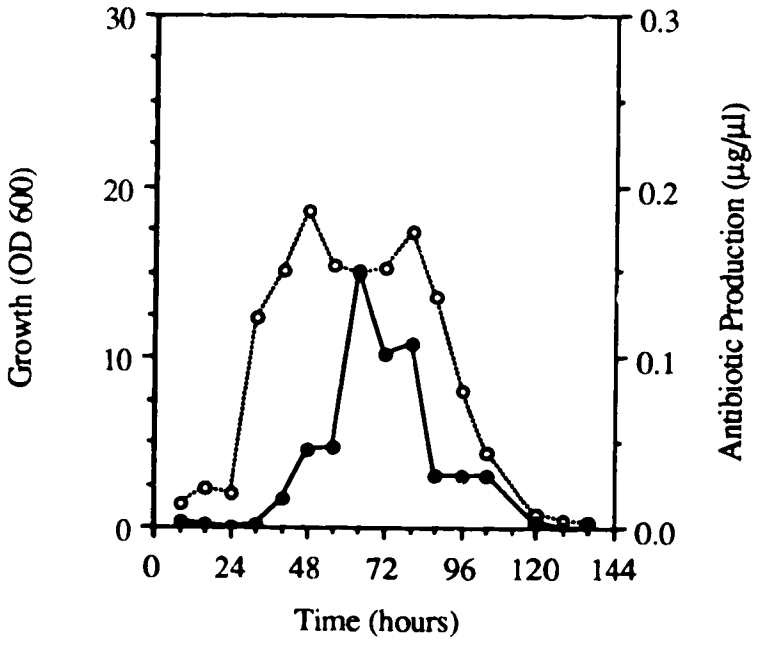
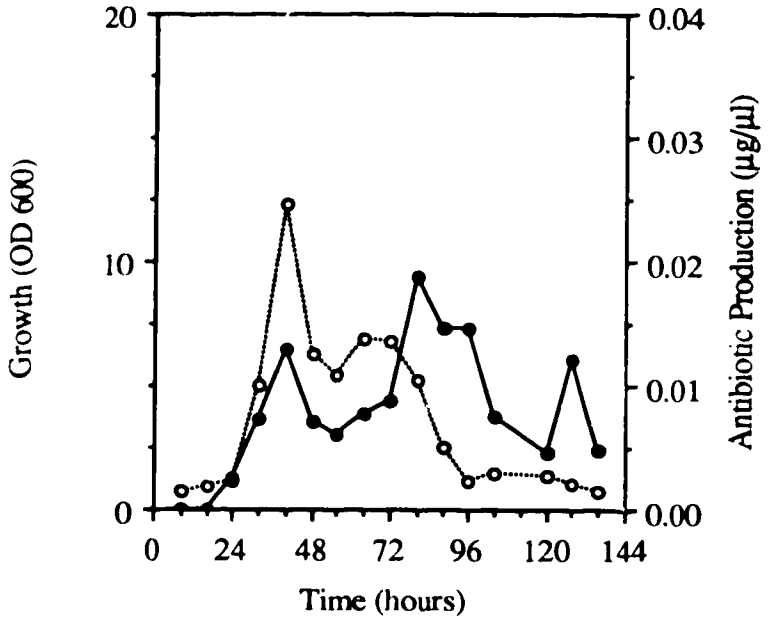

30
20
10
0
0.3
0.2
0.1
0.0
0 24 48 72 96 120 144
Time (hours)
Growth (OD 600)
Antibiotic Production (µg/µl)

Figure 9. The production of antibiotic by wildtype and AEC resistant mutants grown in MM. Seed cultures of wildtype and AEC resistant mutants were grown for 48 h and used to inoculate MM. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. Wildtype. b. Spontaneous mutant 5. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


20
10
0
0.04
0.03
0.02
0.01
0.00
0
24
48
72
96
120
144
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

b

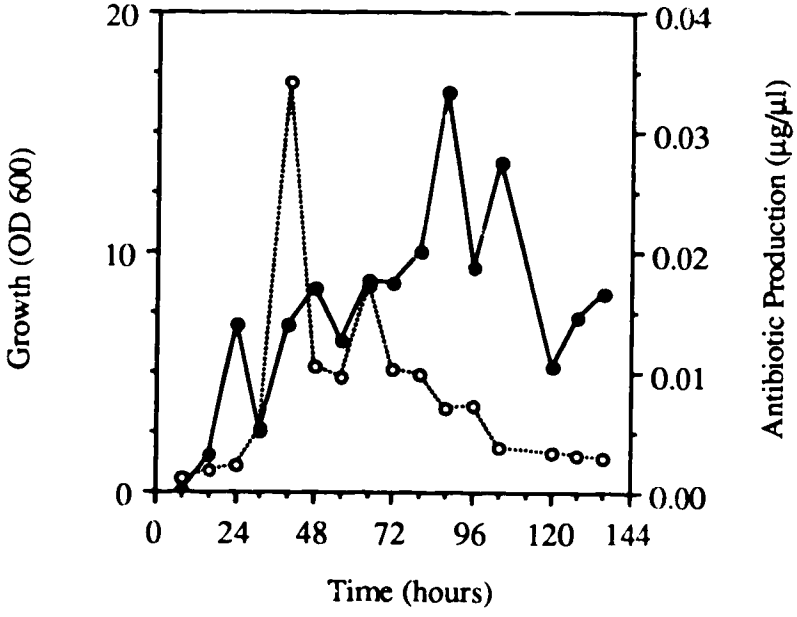
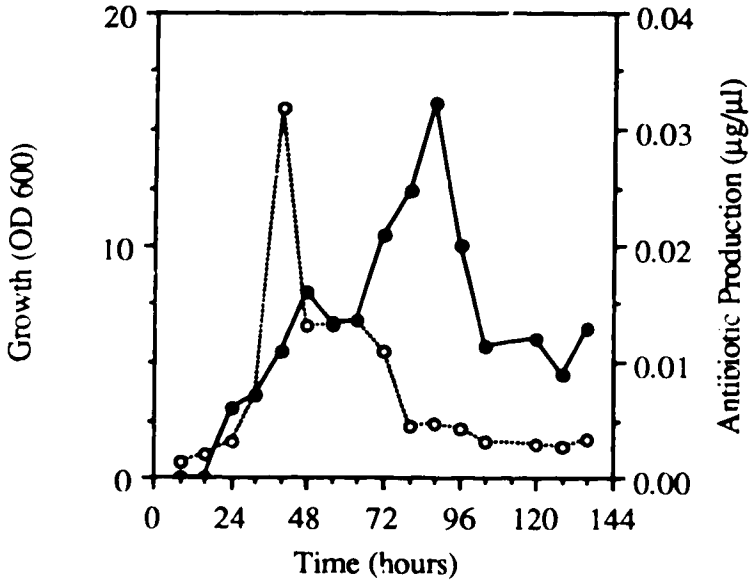

20
10
0
0.04
0.03
0.02
0.01
0.00
0
24
48
72
96
120
144
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

Figure 10. The production of antibiotic by AEC resistant mutants grown in MM. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate MM. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. Spontaneous mutant 8 b. UV1. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


20
10
0
0.04
0.03
0.02
0.01
0.00
0
24
48
72
96
120
144
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

b

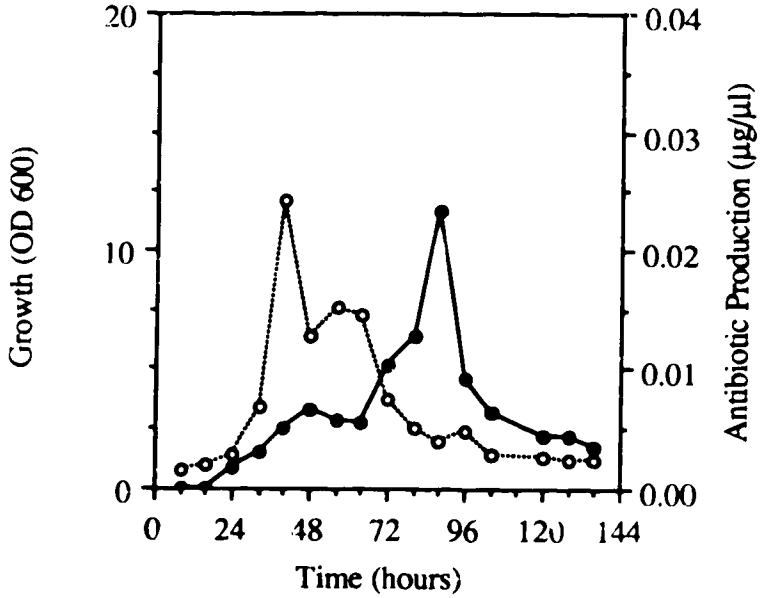
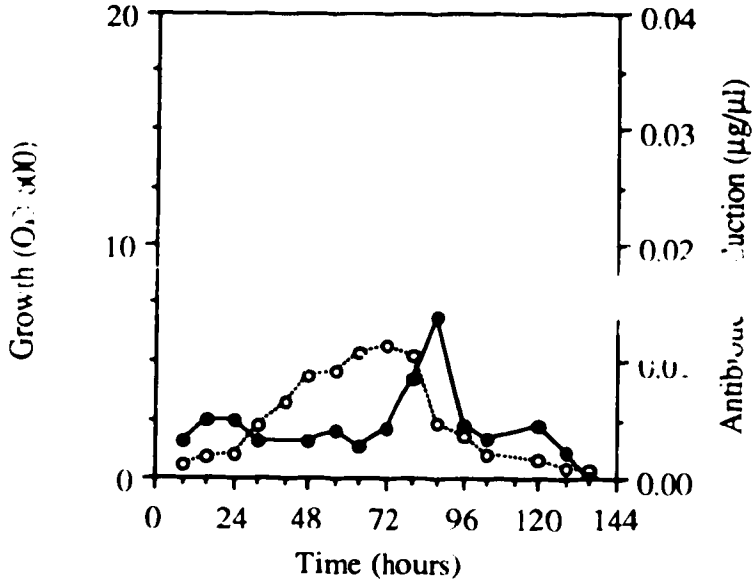

20
10
0
0.04
0.03
0.02
0.01
0.00
0
24
48
72
96
120
144
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

Figure 11. The production of antibiotic by AEC resistant mutants grown in MM. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate MM. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4.

a. T2 h. T4. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


20
10
0
0.04
0.03
0.02
0.00
0 24 48 72 96 120 144
Time (hours)

b

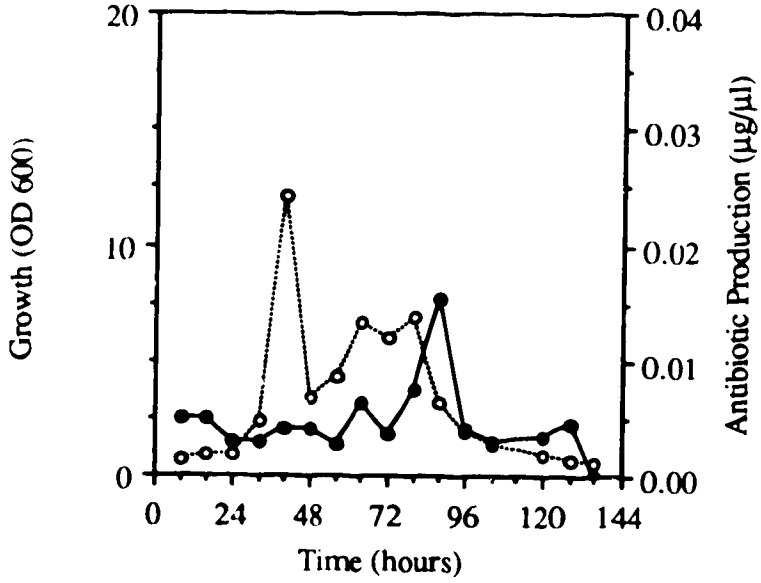
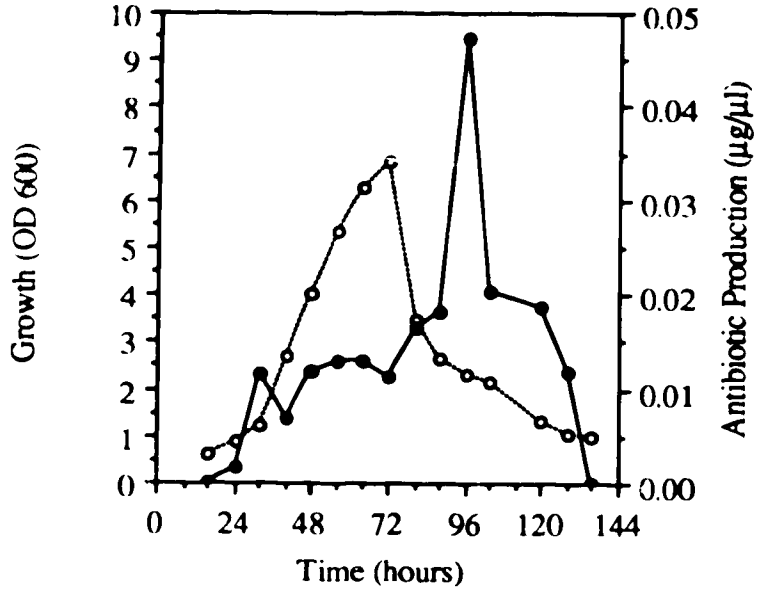

Growth (OD 600)
20
10
0
0.04
0.03
0.02
0.01
0.00
Antibiotic Production (µg/µl)
0 24 48 72 96 120 144
Time (hours)

Figure 12. The production of antibiotic by AEC resistant mutants grown in MM+AEC. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate MM+AEC. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. Spontaneous mutant 5 b.Spontaneous mutant 8.

(○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
0.00
0.01
0.02
0.03
0.04
0.05
0
24
48
72
96
120
144

b

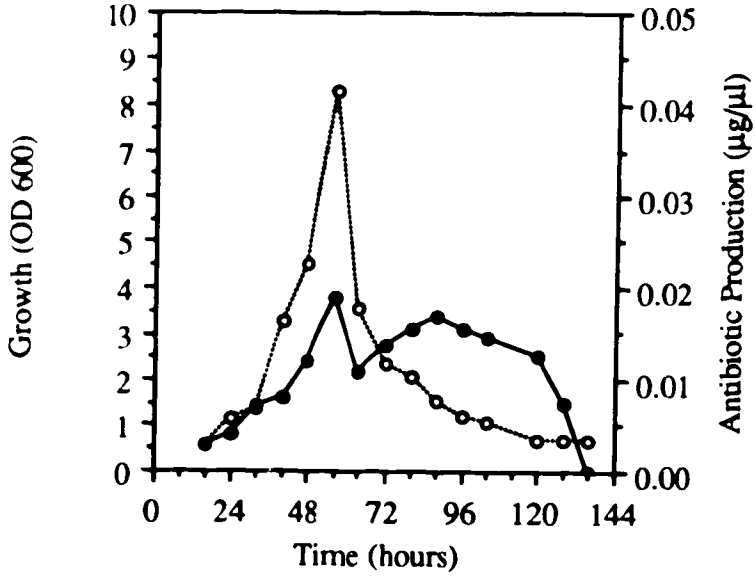

Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
0.00
0.01
0.02
0.03
0.04
0.05
0
24
48
72
96
120
144

Figure 13. The production of antibiotic by AEC resistant mutant (UV1) grown in MM+AEC. Seed cultures of the AEC resistant mutant were grown for 48 h and used to inoculate MM+AEC. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

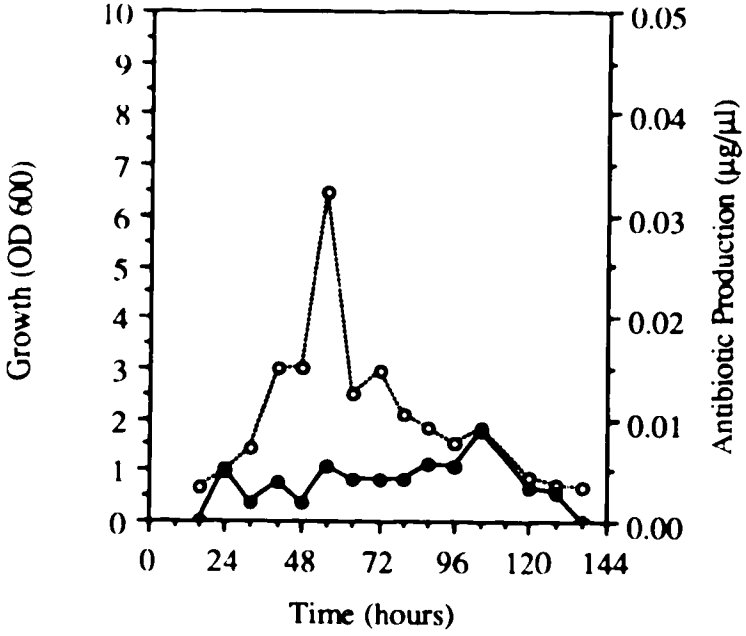
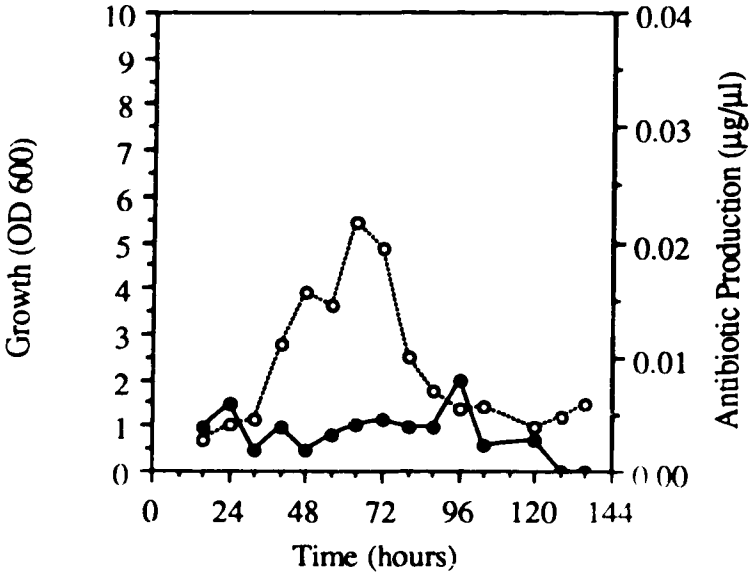
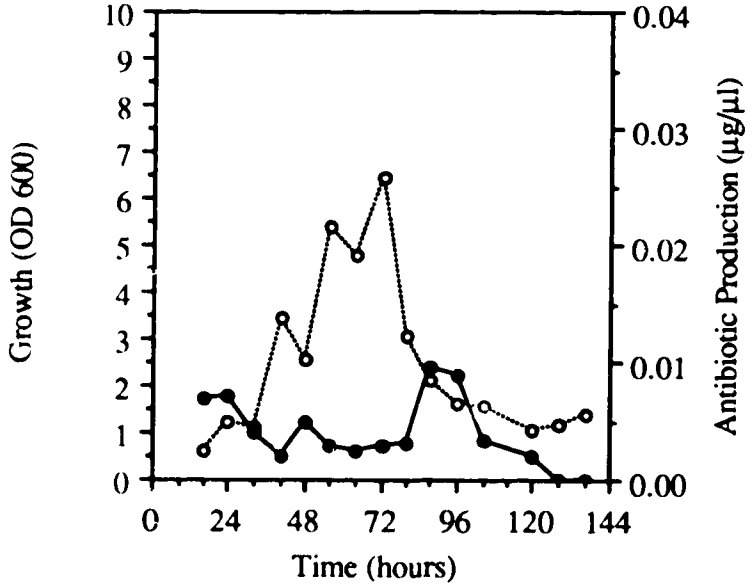

Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
0.00
0.01
0.02
0.03
0.04
0.05
0
24
48
72
96
120
144

Figure 14. The production of antibiotic by AEC resistant mutants grown in MM+AEC. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate MM+AEC. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. T2 b.T4. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
0.00
0.01
0.02
0.03
0.04
0
24
48
72
96
120
144

b


Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
0.00
0.01
0.02
0.03
0.04
0
24
48
72
96
120
144

the above experiment and was therefore used to investigate whether the AEC resistant of this isolate was similarly due to effects on aspartokinase regulation. To determine the optimum time for aspartokinase production a time course experiment was carried out (II.2.3.2) in which aspartokinase content of cells was measured throughout the growth period. Seed cultures of wildtype and spontaneous mutant 5 were used to inoculate TCSS, and MM, and spontaneous mutant 5 was also used to inoculate MM+AEC. Cell free extracts were prepared at 8 h intervals for 96 h and these used to determine aspartokinase activity (refer to II.6.6.). The results are shown in Figures 15 and 16. The time of optimum production appears to be at 48 h. Wildtype and mutant 5 produce about the same amount of aspartokinase in TCSS, however in MM mutant 5 produces more aspartokinase than wildtype and the aspartokinase activity is produced in the early stages of the growth cycle. Mutant 5 produces similar amounts of aspartokinase (in terms of specific activity) in both MM and in MM+AEC.

III.5.3. Regulatory Characteristics of Aspartokinase in AEC Resistant Mutants and Wildtype Isolates

III.5.3.1. Effect of Amino Acids on Production of Aspartokinase

To determine what effect amino acids had on the level of production of aspartokinase, lysine, threonine and AEC were added to the medium. Seed cultures of wildtype and AEC resistant mutants were used to inoculate MM containing no additional amino acids; 10 mM lysine; 10 mM threonine; 10 mM lysine + 10 mM threonine; and 1.5 mg/ml (7 mM) AEC. The cell free extracts prepared from wildtype and AEC resistant mutant strains were used to determine aspartokinase activity. Based on the results of the study described above, cells were harvested at

Figure 15. The production of aspartokinase by wildtype and spontaneous AEC resistant mutant 5 during growth in TCSS. Seed cultures of wildtype and mutant 5 were used to inoculate TCSS. At 8 h intervals 100 ml of culture was removed and cell free extracts prepared. The protein content was assayed and also the aspartokinase activity. Enzyme activity is measued as units/mg protein. One enzyme unit equals 1 nmol/min. (● -Wildtype, ○ -Mutant 5)

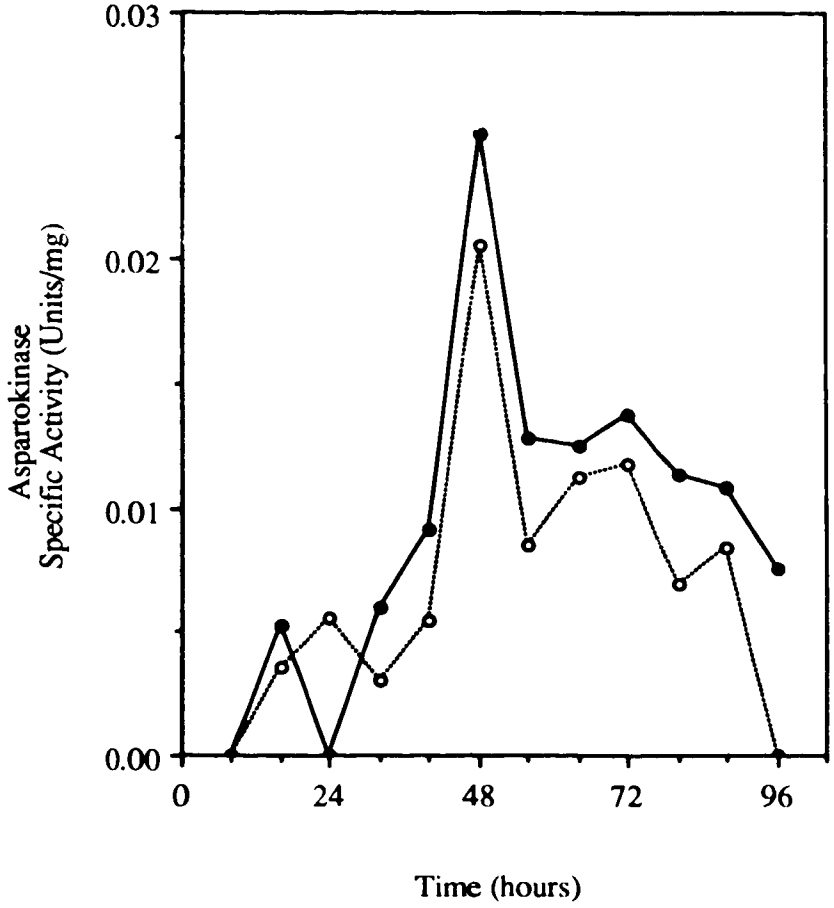
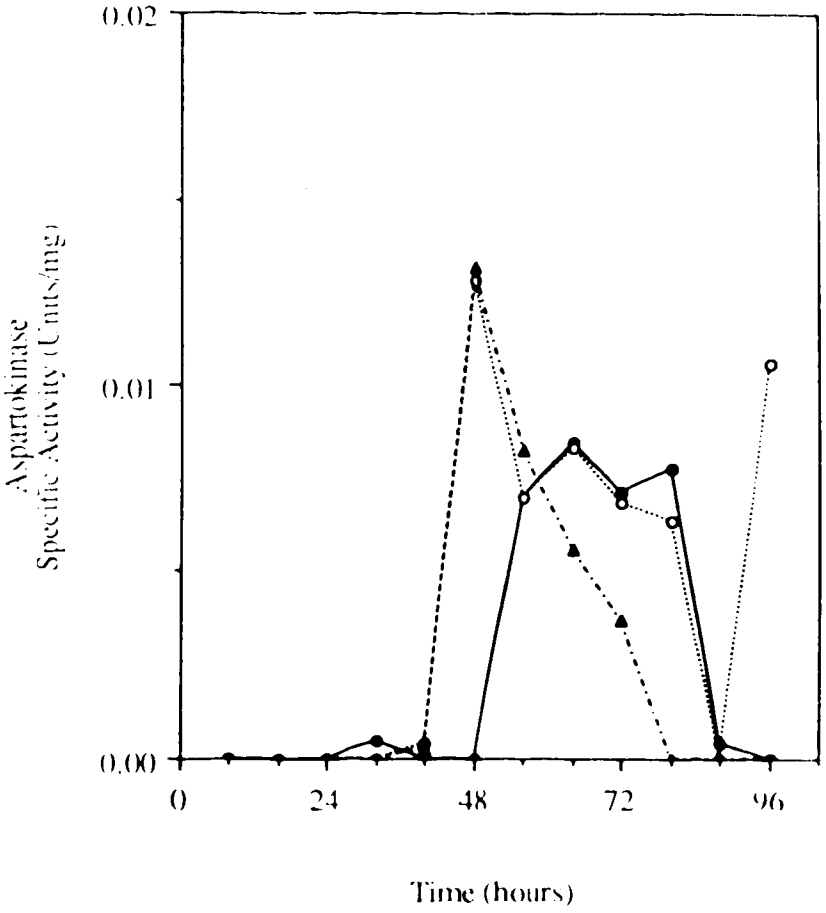

0.03
0.02
0.01
0.00
Aspartokinase
Specific Activity (Units/mg)
0
24
48
72
96
Time (hours)

Figure 16. The production of aspartokinase by wildtype and spontaneous AEC resistant mutant 5 during growth in MM and MM+AEC. Seed cultures of wildtype and mutant 5 were used to inoculate MM, mutant 5 was also used to inoculate MM+AEC. At 8 h intervals 100 ml of culture was removed and cell free extracts prepared. The protein content was assayed and also the aspartokinase activity. Enzyme activity is measued as units/mg protein. One enzyme unit equals 1 nmol/min. (● -Wildtype, ○ -Mutant 5 (MM), ▲ -Mutant 5 (MM+AEC))


0.02
0.01
0.00
Aspartokinase
Specific Activity (Units/mg)
0
24
48
72
96
Time (hours)

56 h for the production of cell extracts for the assay of aspartokinase activity. The results are expressed in Table 4. The aspartokinase activity of each isolate growing in MM was set to 100% and aspartokinase activities of the extracts from the other growth conditions were expressed relative to that. Wildtype *S. clavuligerus* showed increased aspartokinase production when MM was supplemented with L-lysine at 10 mM as well as with L-lysine + L- threonine, each at 10 mM. These results are as expected and compare with those obtained by Mendelovitz and Aharonowitz (1982) as they reported that L-lysine and L-lysine + L-threonine incresed production. Addition of L-threonine by itself inhibited the production of aspartokinase whereas it was reported by Mendelovitz and Aharonowitz (1982) that aspartokinase biosynthesis was not affected by threonine alone. The effect of AEC on biosynthesis of aspartokinase was not reported by Mendelovitz and Aharonowitz (1982) as only wildtype cultures were used in studies on aspartokinase production. Amino acid supplementation of MM had a variable effect on the levels of production of aspartokinase in the different mutant strains. Increasing biosynthesis of aspartokinase was found in mutants 8 and UV2, decreasing biosynthesis in UV1 and T1, and unchanged biosynthesis in mutant T4. Threonine increased production in mutants 8 and T1, decreasing biosynthesis in mutants UV1,UV2 and T4. The combination of lysine and threonine increased production in 8, UV1 and UV2 and lowered it in T1 and T4. Although AEC is a lysine analog it decreased the biosynthesis of aspartokinase to very low levels, in mutants UV2 and T1 to undetectable levels. However all the mutants exhibited higher levels of aspartokinase than did wildtype. The level of aspartokinase biosynthesis in mutant strains compared to wildtype is shown in Table 5. For each growth medium, aspartokinase production of the various mutants is expressed relative to the

Table 1. The effects of amino acid on aspartokinase biosynthesis in wildtype and AEC resistant cultures.

	ASPARTOKINASE ACTIVITY				
	Amino Acid Added to Medium				
Culture	None	Lys	Thr	Lys+Thr	AEC
Wildtype	100	114	85	118	*
8	100	153	146	115	27.9
UV 1	100	93.9	95.8	134	8.9
UV 2	100	123	80.4	161	nd
T1	100	82.2	111	85.3	nd
T4	100	99.8	86.7	87.7	11

a. Activity expressed relative to activity in MM for each culture after addition of amino acids

b. Lys = 10 mM lysine
Thr = 10 mM threonine,
Lys+Thr = 10 mM lysine + 10 mM threonine
AEC = 7 mM AEC

* = not tested

nd = not detectable

Specific activity of the wildtype cultures in MM 6.68 units/mg protein

Table 5. Aspartokinase production of AEC resistant cultures compared to wildtype

	ASPARTOKINASE ACTIVITY			
	Amino Acids Added to Medium			
Culture	None	Lys	Thr	Lys + Thr
8	124	171	213	121
UV 1	154	126	173	175
UV 2	163	176	154	222
T1	157	113	205	114
T4	135	118	128	110

a. Aspartokinase activity is expressed as a percentage of the wildtype activity under the same conditions. Basal medium = minimal medium

b. Lys = 10 mM lysine
Thr = 10 mM threonine,
Lys+Thr = 10 mM lysine + 10 mM threonine
AEC = 7 mM AEC
Specific activity of the wildtype cultures in MM 6.68 units/mg protein

aspartokinase activity of the wildtype culture growing on the same medium. All of the mutants appear to overproduce the enzyme to some extent, compared to the wildtype.

III.5.2. Effects of Amino Acids on Aspartokinase Activity in AEC Resistant Mutants and Wildtype

Mendelovitz and Aharonowitz (1982) have reported that L-lysine increases activity of aspartokinase while L-threonine has no effect on activity. Lysine and threonine in combination decrease activity. Results obtained in Table 5 indicate that both L-lysine and L-threonine individually decrease activity in wildtype and that the combination of L-lysine + L-threonine has an inhibitory effect that is about additive. Mendelovitz and Aharonowitz (1982) suggest that a mutant is deregulated in aspartokinase activity if the addition of L-lysine + L-threonine does not inhibit activity to a level of 50% of wild type. According to this definition only one mutant (spontaneous mutant 1) was deregulated in aspartokinase activity. The levels of aspartokinase activity in the presence of combined L-lysine + L-threonine were unchanged or lowered in all mutants and wild type except for mutant 1 when compared to aspartokinase activity with no amino acids present. The effect of amino acids on activity of the aspartokinase of mutant 1 is similar to that reported for deregulation of aspartokinase activity.

Table 6 The effects of amino acids on the aspartokinase activity of AEC resistant and wildtype cultures

	ASPARTOKINASE ACTIVITY			
	Amino Acid Added to Assay Mixture			
Culture	None	Lys	Thr	Lys+Thr
Wildtype	100	72.1	48.2	28.4
1	100	145	100	85.7
3	100	144	79.2	30.1
5	100	54.2	30.4	15
8	100	54.3	30.3	12.2
UV 1	100	56.3	36.9	13.5
T2	100	56.4	38.6	18.3
T3	100	30.9	22.3	15
T4	100	38.9	21.9	9.6

a. Aspartokinase activity is expressed as a percentage of the activity of which is seen with no amino acids added to the assay mixture

b. Lys = L-lysine at a final concentration of 5 mM
Thr = L-threonine at a final concentration of 5 mM
Lys+Thr = L-lysine and L-threonine both at a final concentration of 5 mM

Specific activity of the wildtype cultures in MM 6.68 units/mg protein

IV. DISCUSSION

Resistance to AEC has been reported to be associated with antibiotic overproduction in *S. clavuligerus* (Mendelovitz and Aharonowitz, 1983). To study this further, and to try and determine the mechanism of AEC resistance in *S. clavuligerus* , spontaneous mutants resistant to AEC were isolated. Initially, the level of sensitivity to this compound had to be determined in *S. clavuligerus*. This proved to have several difficulties associated with it. Inhibition of growth by AEC was found, however there was not total inhibition. Wildtype *S. clavuligerus* grew in the presence of AEC at concentrations of 1 mg/ml. By pregrowing the cultures in MM, growth was substantially limited at AEC concentrations of 1 mg/ml. This concentration was then used to obtain spontaneous mutants for subsequent experiments. When cultures were grown in liquid media wildtype cultures were difficult to distinguish from AEC resistant cultures. Growth of the wildtype cultures was slow to start in comparison to the AEC resistant cultures but seemed to reach the same level of growth. This may have been due to selection for spontaneous mutants in these cultures. To try to overcome this problem the concentration of AEC in liquid cultures was increased to 1.5 mg/ml. At this concentration the wildtype cultures grew poorly and were easily separated from AEC resistant cultures. The AEC resistant cultures did not appear to be effected by this increase in AEC concentration. Mendelovitz and Aharonowitz also reported using higher concentrations of AEC in liquid cultures.

A second problem arose when the AEC resistant colonies were first isolated. After 4 days growth, single resistant colonies appeared on plates of MM+ AEC. When these plates were incubated further, more colonies appeared

surrounding these colonies. The colonies were smaller than the original colonies and were thought to be scavenger colonies. When AEC resistant isolates were streaked out as on MM+AEC and the streaks were crossed by streaks of wildtype culture, no scavenger colonies were found. The wildtype streak grew very slowly on the plate, but grew as well distant from the AEC resistant streak and close to it. It was decided to use just the initially appearing, fast growing colonies when isolating spontaneous mutants and also when isolating induced AEC resistant mutants in subsequent experiments.

As stated earlier, in the introduction, *Streptomyces* are known to have high levels of genetic instability. It was shown by Mevarech (personal communication) that methotrexate resistance in *Halobacterium* was correlated with an amplification of chromosomal DNA. In order to determine if this could also be a mechanism for AEC resistance in *S. clavuligerus* , evidence for DNA amplification was sought. The first experiment carried out to determine if amplification was occurring, was a reversion experiment. If DNA amplification had occurred, this amplified DNA would be expected to be lost rapidly when the selective pressure for it was removed. After several rounds of cultivation in the absence of AEC, none of the spontaneous mutants was found to have reverted to wildtype. Mutant 4 did show a higher level of reversion than the other mutants selected. As mutant 4 had shown the highest reversion, this mutant was used to further investigate amplification. Amplification produces a characteristic pattern of amplified bands when chromosomal DNA is examined by agarose gel electrophoresis after restriction endonuclease digestion. This amplification of bands was not seen with mutant 4. Amplification did not appear to be associated with AEC resistance.

When spontaneous AEC resistant mutants were assayed for antibiotic production, the high levels reported by Mendelovitz and Aharonowitz (1983), of up to 500% higher than wildtype, were not found. This may have been due to the nature of the mutation, as Mendelovitz and Aharonowitz (1983) had used induced mutants. Mutagenesis of *Streptomyces* to produce various mutants has been reported but no consensus exists as to the optimum procedures for generating mutants. The optimum mutagen and the time in the life cycle of the cell were determined for *S. clavuligerus*. *Streptomyces* are filamentous organisms and each mycelial filament will have more than one copy of the chromosome. For mutagenesis it is desirable that there be only one chromosome/cell so that mutations are not complemented. In initial studies, mycelium was fragmented ultrasonically to separate the individual cells. Plate counts done to enumerate the organism after this step were highly variable. This may have been due to only partial breakage of the mycelia and subsequent breakage during plating. To achieve the greatest amount of breakage, survival of the cells had to be sacrificed. Because of this variability, sonicated mycelia were not used in mutagenesis experiments. Comparison of growing and dormant stages in the lifecycle of the cell was achieved by using ungerminated spores and germinated spores. Germinated spores should be equivalent to vegetative cells, however there are relatively few genomes/germinated spore as opposed to mycelium which has many genomes.

Two different mutagens were compared in this study. As stated earlier, these mutagens produce different effects and have both been used in *S. clavuligerus*. Ultraviolet light does not appear to produce significant differences in the survival of ungerminated spores or germinated spores. The frequency of

mutant production is higher in the germinated spores than the ungerminated spores. As there would appear to be the same number of lethal hits produced in both types of spores, the reason for more mutagenic hits in the germinated spores is unclear. Excision repair is error free (Witkin, 1976) while postreplicative repair is error prone. If the ungerminated spores were using only excision repair then they would be expected to have a lower mutation frequency. Postreplicative repair requires DNA synthesis. Germinated spores would be undergoing DNA synthesis at a much higher rate than ungerminated spores and so would be expected to have postreplicative repair. This may explain the higher rate of mutagenesis with germinated spores than with ungerminated spores.

Mutagenesis with NTG was used to compare mutant production with UV irradiation. Mutagenic lesions induced by NTG are produced at a higher rate at the replication fork than at other areas of the chromosome (Godfrey, 1974). This appears to be the case with *S. clavuligerus* as germinated spores produced higher mutation frequencies than did ungerminated spores. Godfrey (1974) had found that cells undergoing DNA replication mutated at a rate 6 times that of cells which were not replicating. This does not appear to be the case in this experiment. The difference in the rate of mutagenesis between the two types of spores is low. The rate of mutagenesis is only 1.4 times higher with germinated spores than with ungerminated spores. There is a greater difference in the mutation frequencies at higher survival rates than at the lower rates. Perhaps the long incubation times used with NTG allowed DNA synthesis to begin in the ungerminated spores even though the spores were suspended in a non-growth medium which does not support growth, and this led to a lessening of the effects of germination. Stonesifer and

Baltz (Stonesifer and Baltz, 1985; Baltz and Stonesifer, 1985a) have suggested that NTG mutagenesis is not totally dependent on error prone DNA repair. The error prone system is only responsible for 90% of NTG mutagenesis, unlike UV mutagenesis which requires error prone repair. This also may explain why there is not as big a difference with the two types of spores as there was with UV treatment. Again the incubation times may have allowed some postreplicative repair in the ungerminated spores.

From the above results of mutagenesis it was decided to use UV light treatment of germinated spores as the mutagenic treatment of choice. The overall frequency of mutation was greater and the inherent dangers of working with NTG were eliminated. AEC resistant mutants were then isolated using this technique.

During the course of this study transposon mutagenesis became available as a method that could be used in some *Streptomyces* species (Chung, 1987) had isolated Tn4556 from *S. fradiae* and shown that it would transpose in various *Streptomyces* species. Since the transposon is carried on the plasmid pMT660, and this plasmid is a derivative of pIJ702 which is known to function in *S. clavuligerus*, it seemed likely that the transposon system would function in *S. clavuligerus*. The transposon was received from Dr. Chung as a gift in the form of an *S. lividans* culture containing Tn4560 in the pMT660 plasmid. The transposon was introduced into *S. clavuligerus* and AEC resistant mutants were isolated. Problems were encountered with the selection of transposon containing mutants. The cells were initially grown on selective media containing thiostrepton to isolate transformants which contained the plasmid. The cells were then grown in media which alternately contained viomycin or lacked viomycin. The viomycin

concentrations which were used with *S. lividans* proved to be much too low for selection of viomycin resistant *S. clavuligerus*. Concentrations of 300 µg/ml had to be used with *S. clavuligerus*. Even at this concentration, growth of the wildtype was not completely inhibited. However at this concentration growth was retarded to a degree where wildtype could be differentiated from mutants containing the transposon. Chung (personal communication) has recently changed the selection marker in Tn4556 and this will greatly aid use of this transposon in the future.

When AEC resistant mutants were obtained using transposon containing cells it was necessary to prove that the mutants contained the transposon. Hybridization studies were used to detect the presence of the transposon. Chromosomal DNA was isolated from four mutants and used for hybridization studies. The plasmid which carried the transposon was a derivative of pIJ702 (Birch and Cullum, 1985). Labelled pIJ702 was then used to check for the presence of contaminating plasmid DNA. If the viomycin resistance had been acquired by recombination of the plasmid or by the presence of the plasmid itself, the labelled pIJ702 would have hybridized to the DNA. No hybridization was seen indicating that there were no homologous regions in the chromosomal DNA to the plasmid vector. Labelled pUC1169 was then used as a hybridization probe. Any specific hybridization to this probe should be due to the transposon component. Two bands appeared in the mutants and not the wildtype. The hybridization was presumably due to transposon as no hybridization was seen with the vector. The transposon had transposed from the plasmid to the chromosomal DNA. This is not proof that the AEC resistance mutations are due to insertion of the transposon, only that the transposon transposed. Although the bands appear on the autoradiograph at

the same position in all the mutants, this does not necessarily mean that the transposon is in the same position in all the mutants. However, if the AEC resistance is due to insertion of the transposon, then similar digests would be expected, as the transposon maybe inserted in the same region. This would give fragments that are of approximately the same size regardless of the insertion point of the transposon. To determine if the AEC resistance was due to the insertion of the transposon DNA, the transposon and the flanking DNA would have to be cloned from these mutants and analyzed.

A range of mutants was now available to study AEC resistance and the overproduction of antibiotics. Initial studies showed highly variable antibiotic production. Mutants which were classed as overproducers in one trial seemed to underproduce in the next trial. To try and standardize the antibiotic production measurements, production was followed as a function of time in different media. Wildtype and mutant strains were grown in both TCSS and MM to determine the effects of complex media compared to defined media. Mutants were also grown in MM+AEC to determine the effects of AEC on antibiotic production.

Antibiotic production in TCSS seemed to reach a peak in all the cultures at approximately 56 - 80 h. The growth of the cultures, measured as optical density, reached a peak at about 48 h. All the mutants seemed to overproduce antibiotic to some extent compared to the wildtype cultures. Mutants 5 and UV1 produced the highest levels of antibiotic of all the mutants. Growth of the cultures was good in all cases with the mutants having as good or better growth characteristics than the wildtype.

Growth and antibiotic production of wildtype and mutant strains in MM

was less than that in TCSS. Some variation was seen in antibiotic production. Both overproduction and underproduction was seen with the mutants. Peak production of antibiotic was delayed in the MM compared to TCSS. Highest levels of production occurred at approximately 38 h whereas peak growth occurred at 40 h. Both of the transposon mutants grew at a lower rate than the other cultures and produced lower levels of antibiotic. The spontaneous AEC resistant mutants seemed to produce the highest levels of antibiotic in this medium. UV1, which was the highest producer in TCSS, still overproduces compared to the wildtype but produces less antibiotic than the spontaneous mutants in MM.

AEC resistant mutants were also grown in MM containing AEC. The addition of AEC seemed to inhibit the growth of the organisms to some extent. The peak level of growth obtained was substantially less than in MM alone. Antibiotic production was not affected by the addition of AEC in mutant 5 but did seem to be decreased slightly in the other mutants. Mutants 8 and UV1 appeared to be affected by AEC to the greatest extent of all the mutants. Production was lowered by over 50% in both mutants when compared to levels in TCSS. However, in several cases this difference was seen at a single point during the growth curve and may be due to differences in the assay at that time. Although samples were stored at -75°C and assayed at one time, there is variation inherent in bioassays which may range from 10 -20%. Triplicate samples were used to minimize variations. Each bioassay plate has a limited number of samples and some variation between plates is possible. The transposon mutants showed very little antibiotic production in both the MM and MM+ AEC medium.

Overproduction of antibiotic has been correlated with deregulation of

aspartokinase activity (Mendelovitz and Aharonowitz, 1983). Aspartokinase deregulation could affect antibiotic production in two ways. Mutants could be deregulated in the production of aspartokinase and overproduce the enzyme. This would lead to overproduction of antibiotic by leading to an overproduction of α-aminoadipic acid as this has been shown to be the limiting factor in antibiotic production. Mutants could also be deregulated in that they produce the same amount of aspartokinase as the wildtype but the enzyme has different characteristics with respect to inhibition by amino acids. Again this could lead to an overproduction of α-aminoadipic acid. To determine if this was the case with the mutants isolated here, aspartokinase activity was measured. A comparison of aspartokinase in wildtype and the highest antibiotic producer (mutant 5) of the AEC resistant mutants was undertaken. Aspartokinase activity was measured as a function of time to determine if production of the enzyme occurred at different stages in the growth of the wildtype and mutant. The optimum production of aspartokinase appeared at 48 h for both cultures when grown on TCSS. The mutant also had highest aspartokinase activity at 48 h in MM and MM+AEC. The wildtype seemed to produce highest levels of aspartokinase a little later than the mutant in MM, with activity reaching a peak at 64 h. The optimum time reported for aspartokinase production by Mendelovitz and Aharonowitz (1982) was between 48 and 64 h. Levels of aspartokinase activity were higher in TCSS than in MM. The levels were similar between the two cultures in TCSS but in MM mutant 5 produced higher levels.

The aspartokinase production in each of the mutants was then examined. Two different amino acids of the aspartate family were used to determine the effects

on aspartokinase biosynthesis. All of the mutants overproduced aspartokinase compared to the wildtype. This would suggest that biosynthesis of aspartokinase is deregulated. The effects of lysine and threonine were not consistent between the mutants. The results of this experiment are significantly different than those of Mendelovitz and Aharonowitz (1982). They report that aspartokinase production in wildtype was increased with the addition of lysine, remained approximately the same when threonine was added, and decreased when both lysine and threonine were added. In this study wildtype production was increased when lysine was added, decreased when threonine was added, and increased again when both lysine and threonine were added. Each subsequent mutant tested was affected by amino acid supplementation in a different way. The variable effects of addition of amino acids has been reported to occur with AEC resistant mutants by Mendelovitz and Aharonowitz (1982) when activity was determined but was not reported for biosynthesis of the enzyme. When AEC resistant mutants were grown in MM+AEC the only consistent response was the effect of AEC on aspartokinase production. In each case AEC drastically reduced the amount of the aspartokinase. Lysine increased the production of aspartokinase and as AEC is a lysine analog it would be expected to have the same effect. The effects of the two compounds are opposite with lysine increasing production and AEC decreasing production. Two of the mutants did not produce detectable levels of enzyme when AEC was added to the medium. Lysine is not present in MM and must be produced by the organism for growth to occur. The aspartokinase pathway leads to the production of several essential amino acids. It is unclear how growth could occur with undetectable levels of aspartokinase.

The same amino acids that were used to examine the effects of amino acid supplementation on biosynthesis of aspartokinase were then used to determine the effects on activity of the enzyme. The effects on activity differed from the wildtype in two cases. Lysine generally inhibited the activity of aspartokinase except for mutants 1 and 3. This is not as was reported by Mendelovitz and Aharonowitz (1983). They reported that lysine stimulated activity in all cases, and lysine in combination with threonine inhibited aspartokinase. Threonine by itself inhibited aspartokinase activity slightly to a level intermediate between lysine and the lysine plus threonine combination. Although Mendelovitz and Aharonowitz (1983) had reported a decrease in the activity of aspartokinase in the presence of both amino acids it was not as great as that reported here. The criterion that was used by Mendelovitz and Aharonowitz (1983) to define deregulation of aspartokinase was that the inhibition of aspartokinase activity by lysine plus threonine should not exceed 50%. Using this definition, only one AEC resistant mutant isolated in this study meets this requirement (mutant 1).

In summary, this study did not find evidence for deregulation of aspartokinase activity as a mechanism for AEC resistance in *S. clavuligerus*. Mutants did appear to overproduce aspartokinase in comparison to the wildtype culture, and the aspartokinase showed the same characteristics as the wildtype with respect to inhibition by amino acids. Perhaps AEC resistance leading to overproduction of antibiotics is not due to a deregulation of aspartokinase as has been suggested by Mendelovitz and Aharonowitz (1983) but rather to a significant overproduction of the enzyme. Overproduction of the enzyme which is still sensitive to feedback inhibition by lysine and threonine, may lead to an

overproduction of α-aminoadipic acid. Much higher levels of amino acids would be needed to inhibit the overproduced aspartokinase. This would have the same effect as deregulation of feedback inhibition as more lysine would be available for antibiotic production. The addition of lysine to cultures has been shown to increase cephamycin C production in *S. clavuligerus* (Mendelovitz and Aharonowitz, 1982).

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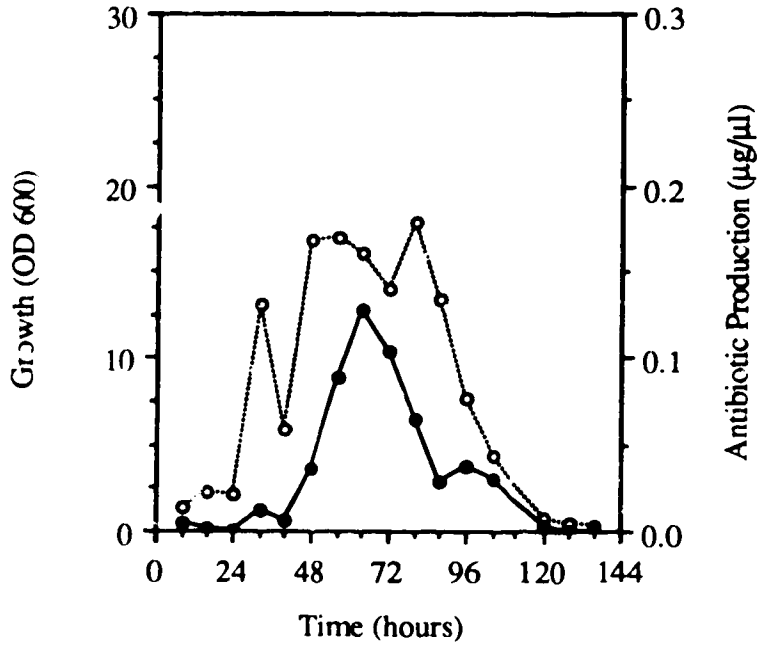
Figure 8. The production of antibiotic byAEC resistant mutants grown in TCSS. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate TCSS. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. T2. b. T4. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a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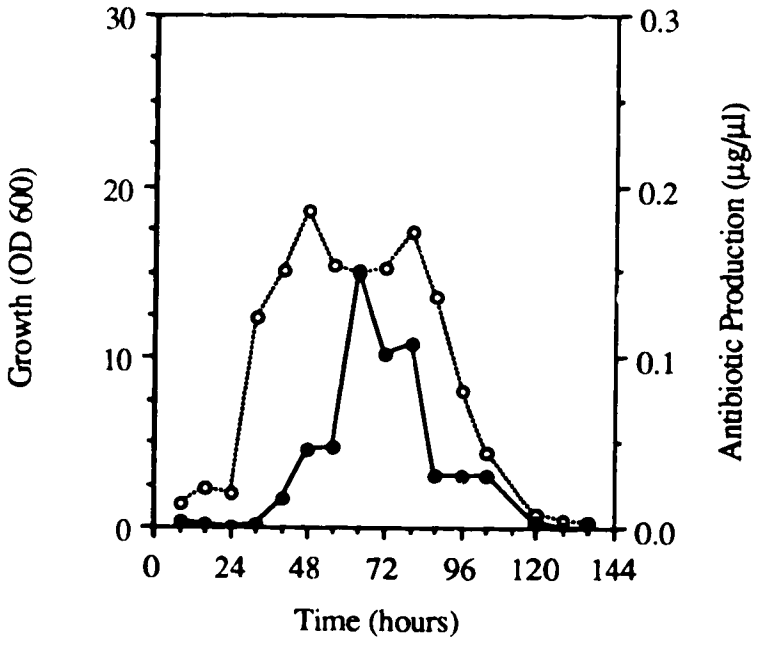
Time (hours)
Antibiotic Production (µg/µl)

b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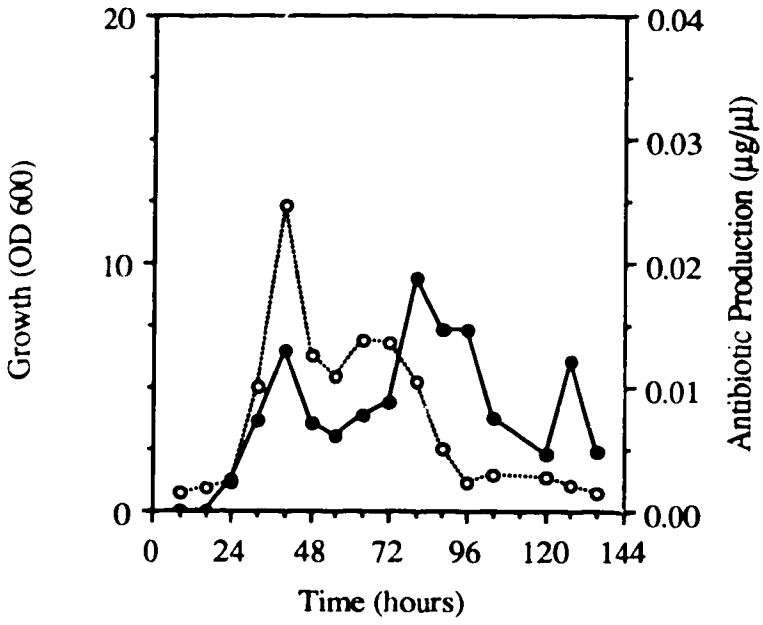
Time (hours)
Growth (OD 600)
Antibiotic Production (µg/µl)

Figure 9. The production of antibiotic by wildtype and AEC resistant mutants grown in MM. Seed cultures of wildtype and AEC resistant mutants were grown for 48 h and used to inoculate MM. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. Wildtype. b. Spontaneous mutant 5. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a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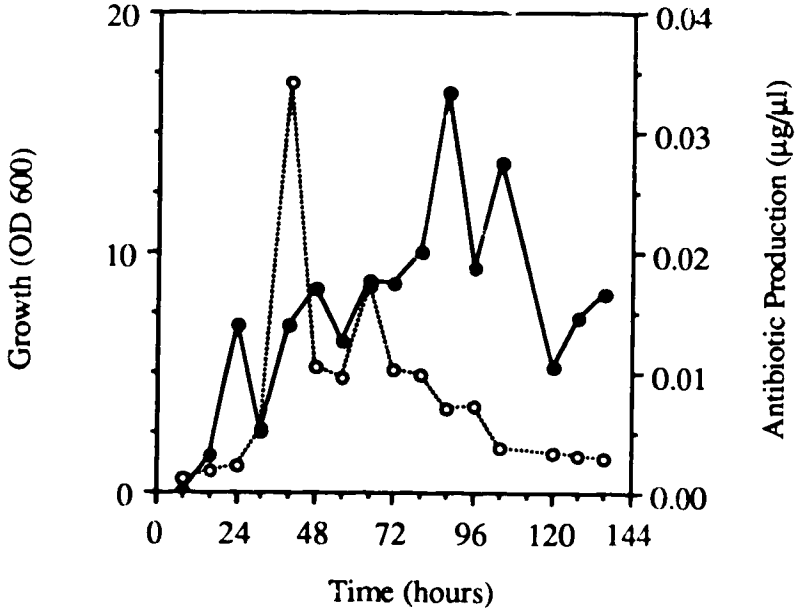
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

b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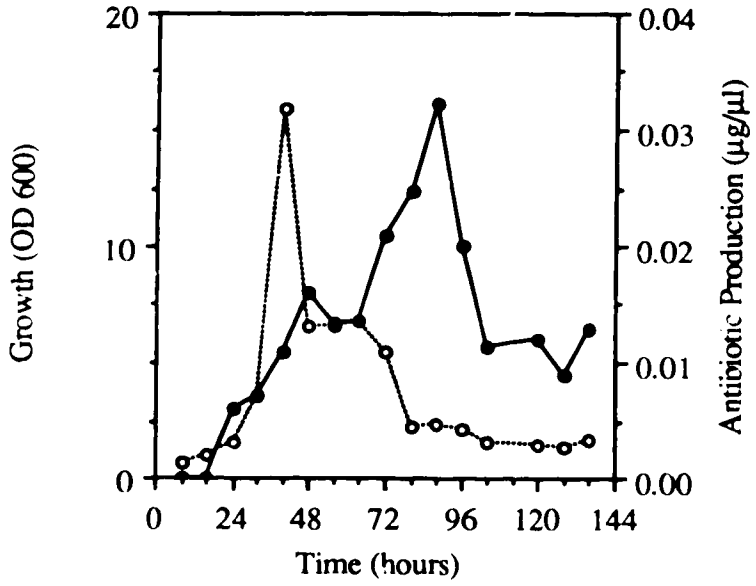
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

Figure 10. The production of antibiotic by AEC resistant mutants grown in MM. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate MM. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. Spontaneous mutant 8 b. UV1. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a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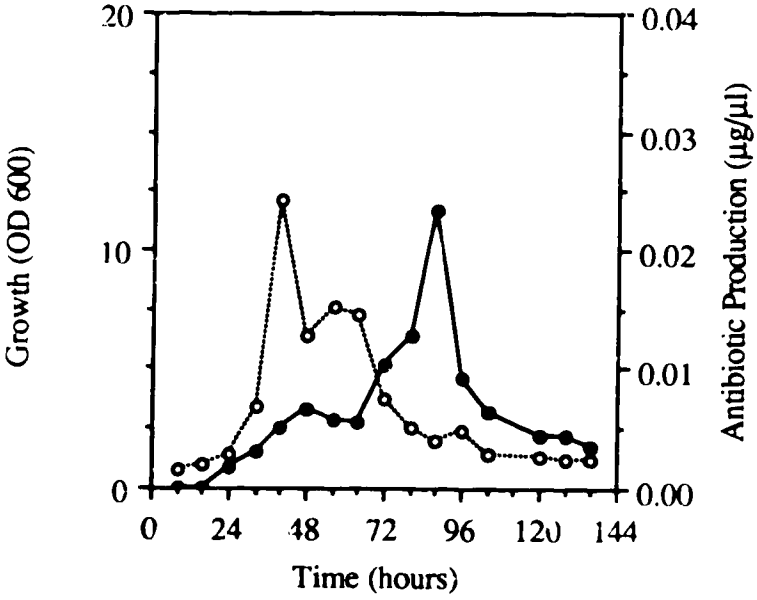
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

b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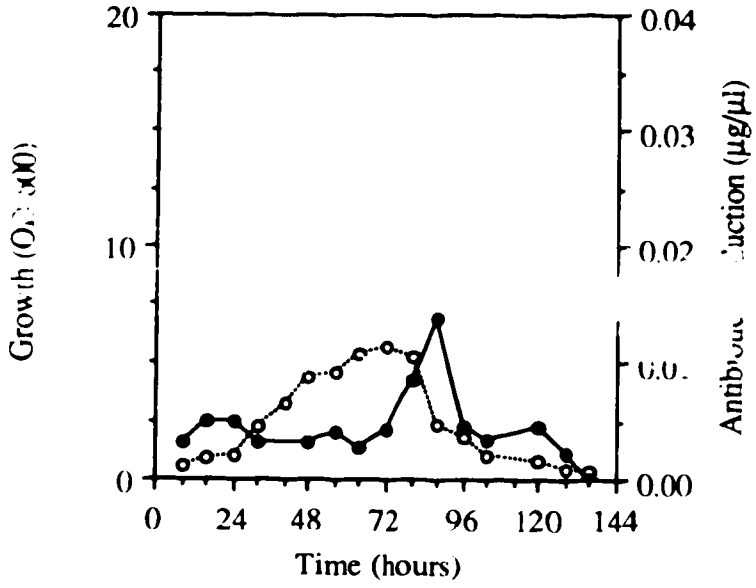
Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)

Figure 11. The production of antibiotic by AEC resistant mutants grown in MM. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate MM. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. T2 b. T4. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a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Time (hours)

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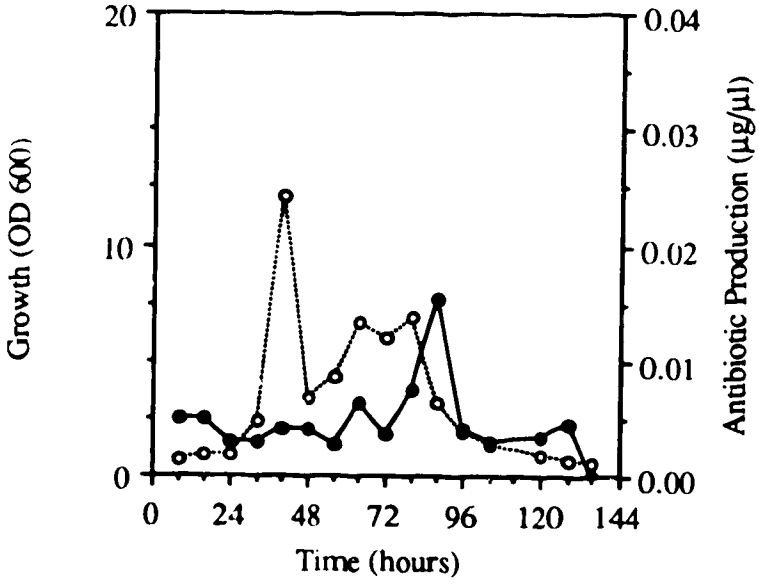
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Antibiotic Production (µg/µl)
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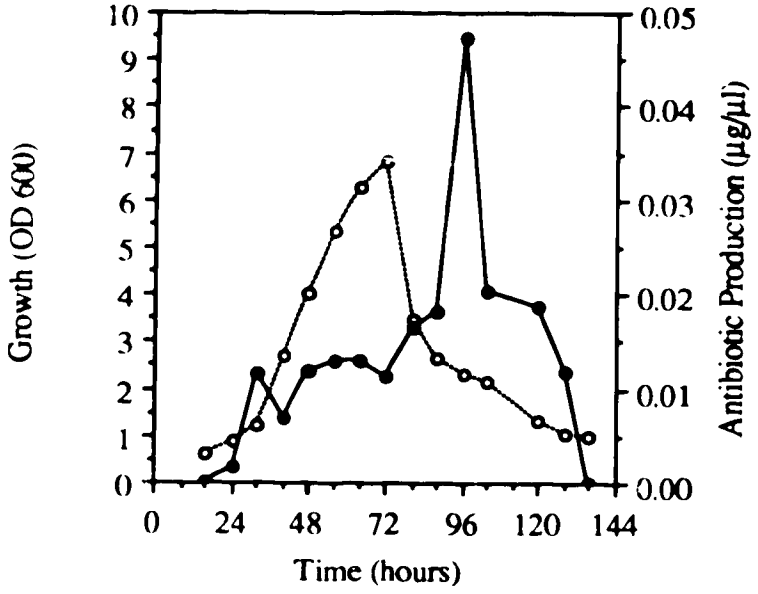
Time (hours)

Figure 12. The production of antibiotic by AEC resistant mutants grown in MM+AEC. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate MM+AEC. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. Spontaneous mutant 5 b.Spontaneous mutant 8.

(○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


Growth (OD 600)
Antibiotic Production (µg/µl)
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b

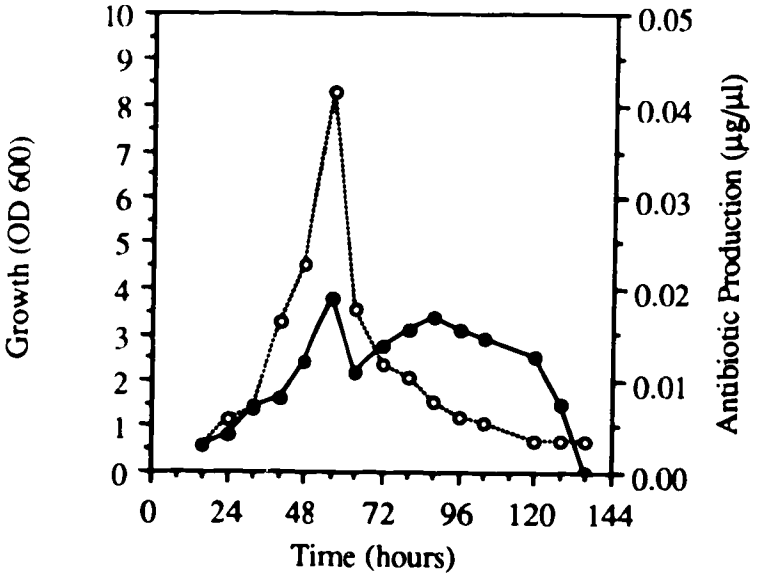
Growth (OD 600)
Antibiotic Production (µg/µl)
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Figure 13. The production of antibiotic by AEC resistant mutant (UV1) grown in MM+AEC. Seed cultures of the AEC resistant mutant were grown for 48 h and used to inoculate MM+AEC. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

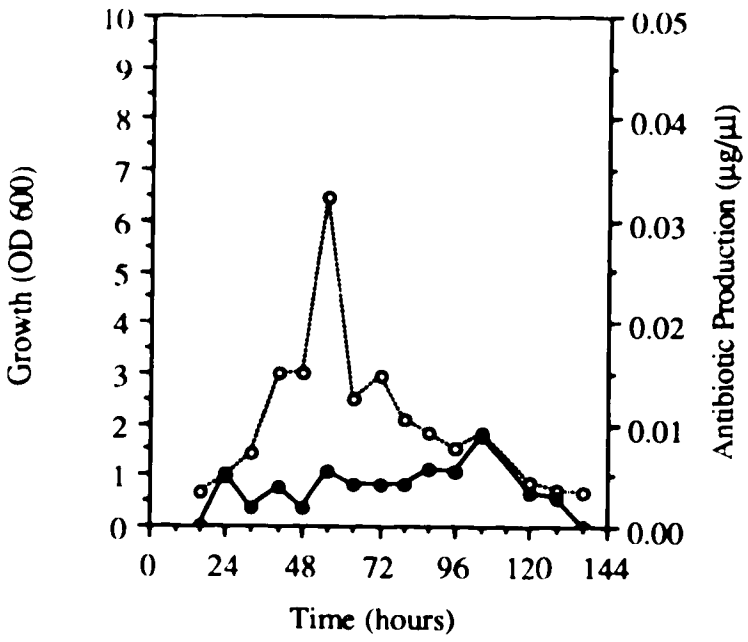
Growth (OD 600)
Antibiotic Production (µg/µl)
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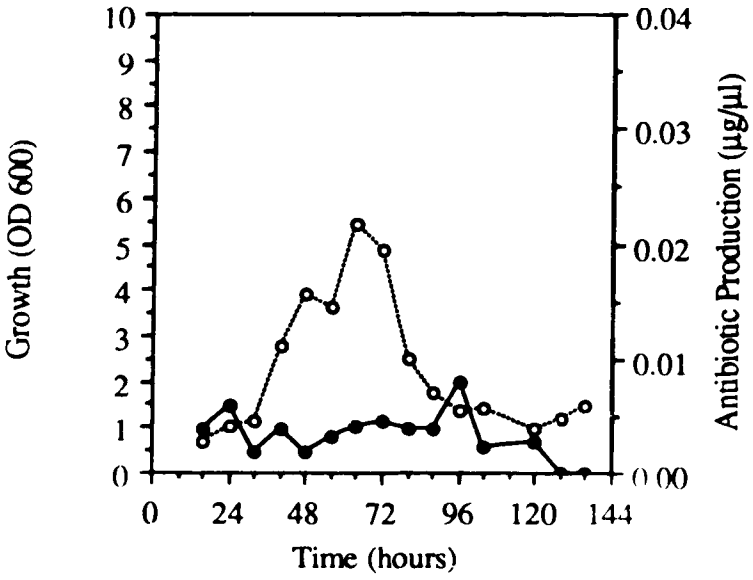
Figure 14. The production of antibiotic by AEC resistant mutants grown in MM+AEC. Seed cultures of AEC resistant mutants were grown for 48 h and used to inoculate MM+AEC. At 8 h intervals the optical density was determined at 600 nm. Samples of the culture supernatant were also removed. These samples were used to determine antibiotic levels by the bioassay procedure outlined in II.6.4. a. T2 b.T4. (○ - Growth (OD_{600}), ● - Antibiotic Production (μg/ml))

a


Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)
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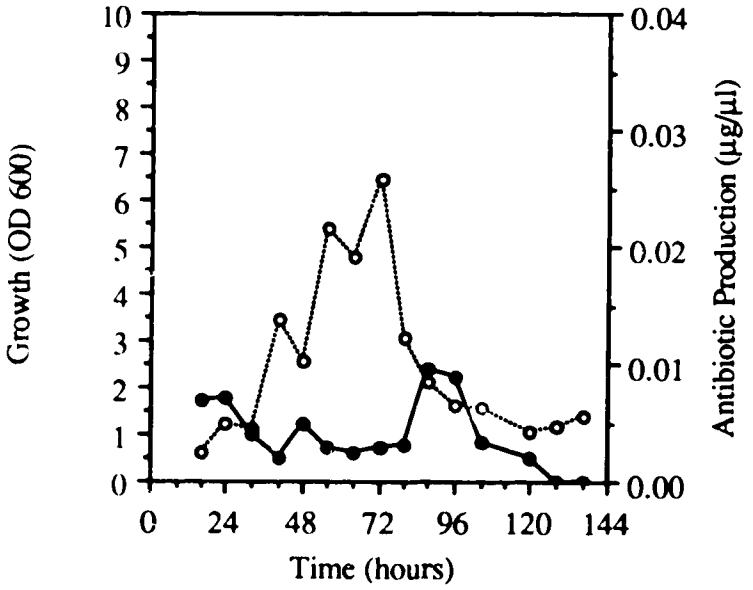
b


Growth (OD 600)
Antibiotic Production (µg/µl)
Time (hours)
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the above experiment and was therefore used to investigate whether the AEC resistant of this isolate was similarly due to effects on aspartokinase regulation. To determine the optimum time for aspartokinase production a time course experiment was carried out (II.2.3.2) in which aspartokinase content of cells was measured throughout the growth period. Seed cultures of wildtype and spontaneous mutant 5 were used to inoculate TCSS, and MM, and spontaneous mutant 5 was also used to inoculate MM+AEC. Cell free extracts were prepared at 8 h intervals for 96 h and these used to determine aspartokinase activity (refer to II.6.6.). The results are shown in Figures 15 and 16. The time of optimum production appears to be at 48 h. Wildtype and mutant 5 produce about the same amount of aspartokinase in TCSS, however in MM mutant 5 produces more aspartokinase than wildtype and the aspartokinase activity is produced in the early stages of the growth cycle. Mutant 5 produces similar amounts of aspartokinase (in terms of specific activity) in both MM and in MM+AEC.

III.5.3. Regulatory Characteristics of Aspartokinase in AEC Resistant Mutants and Wildtype Isolates

III.5.3.1. Effect of Amino Acids on Production of Aspartokinase

To determine what effect amino acids had on the level of production of aspartokinase, lysine, threonine and AEC were added to the medium. Seed cultures of wildtype and AEC resistant mutants were used to inoculate MM containing no additional amino acids; 10 mM lysine; 10 mM threonine; 10 mM lysine + 10 mM threonine; and 1.5 mg/ml (7 mM) AEC. The cell free extracts prepared from wildtype and AEC resistant mutant strains were used to determine aspartokinase activity. Based on the results of the study described above, cells were harvested at

Figure 15. The production of aspartokinase by wildtype and spontaneous AEC resistant mutant 5 during growth in TCSS. Seed cultures of wildtype and mutant 5 were used to inoculate TCSS. At 8 h intervals 100 ml of culture was removed and cell free extracts prepared. The protein content was assayed and also the aspartokinase activity. Enzyme activity is measued as units/mg protein. One enzyme unit equals 1 nmol/min. (● -Wildtype, ○ -Mutant 5)

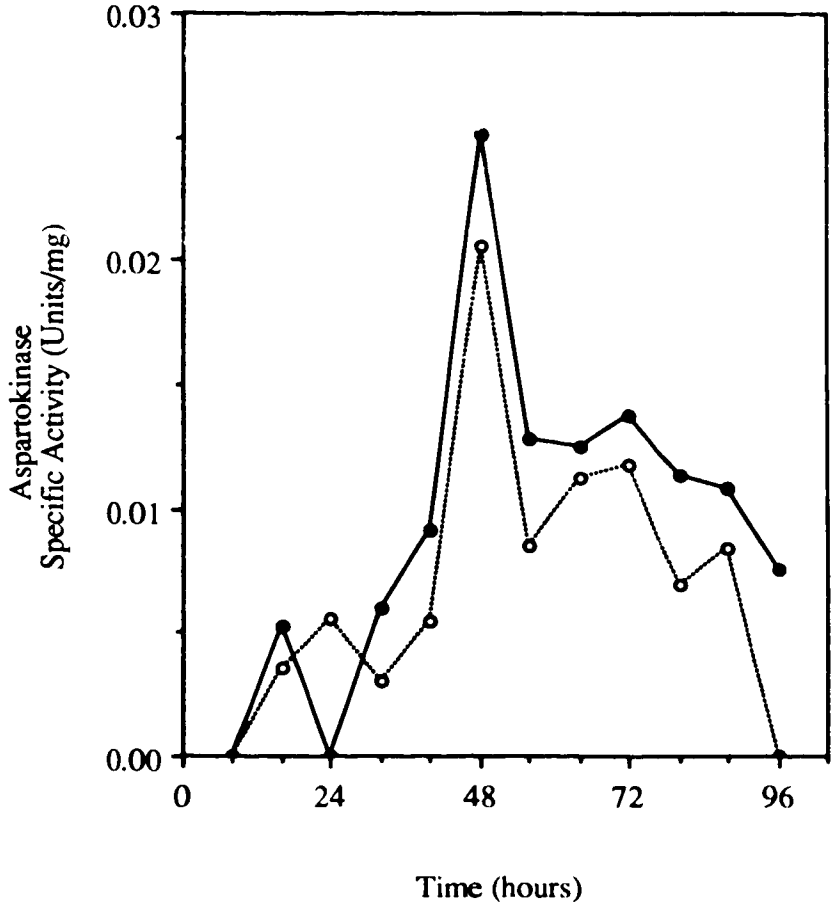
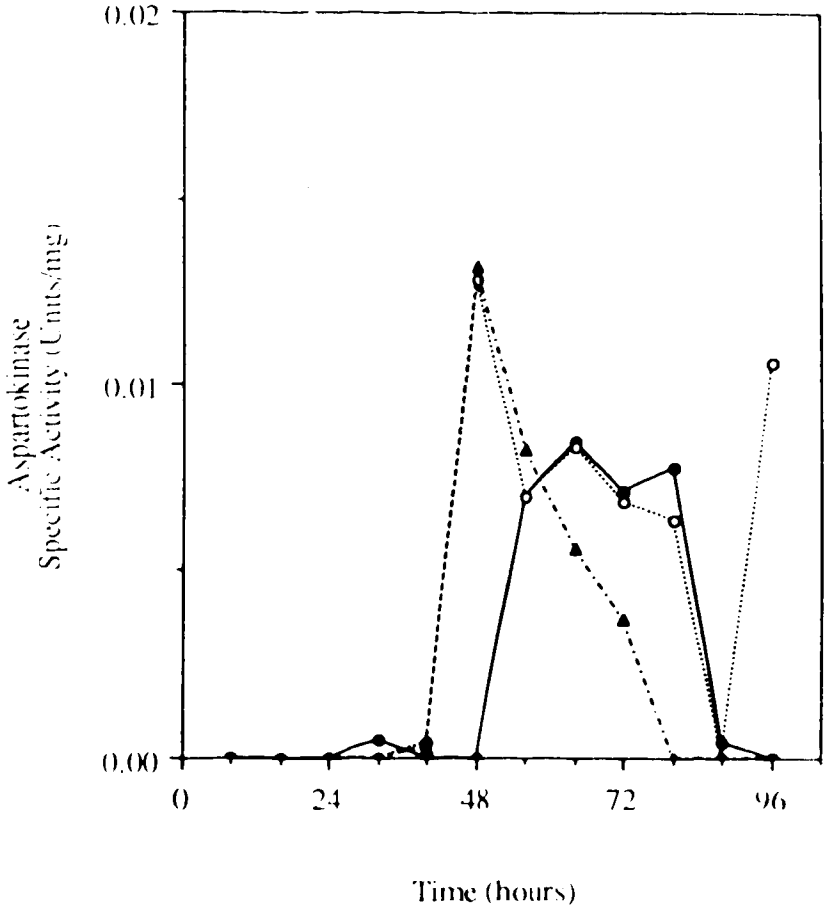

0.03
0.02
0.01
0.00
Aspartokinase
Specific Activity (Units/mg)
0
24
48
72
96
Time (hours)

Figure 16. The production of aspartokinase by wildtype and spontaneous AEC resistant mutant 5 during growth in MM and MM+AEC. Seed cultures of wildtype and mutant 5 were used to inoculate MM, mutant 5 was also used to inoculate MM+AEC. At 8 h intervals 100 ml of culture was removed and cell free extracts prepared. The protein content was assayed and also the aspartokinase activity. Enzyme activity is measued as units/mg protein. One enzyme unit equals 1 nmol/min. (● -Wildtype, ○ -Mutant 5 (MM), ▲ -Mutant 5 (MM+AEC))


0.02
0.01
0.00
Aspartokinase
Specific Activity (Units/mg)
0
24
48
72
96
Time (hours)

56 h for the production of cell extracts for the assay of aspartokinase activity. The results are expressed in Table 4. The aspartokinase activity of each isolate growing in MM was set to 100% and aspartokinase activities of the extracts from the other growth conditions were expressed relative to that. Wildtype *S. clavuligerus* showed increased aspartokinase production when MM was supplemented with L-lysine at 10 mM as well as with L-lysine + L- threonine, each at 10 mM. These results are as expected and compare with those obtained by Mendelovitz and Aharonowitz (1982) as they reported that L-lysine and L-lysine + L-threonine incresed production. Addition of L-threonine by itself inhibited the production of aspartokinase whereas it was reported by Mendelovitz and Aharonowitz (1982) that aspartokinase biosynthesis was not affected by threonine alone. The effect of AEC on biosynthesis of aspartokinase was not reported by Mendelovitz and Aharonowitz (1982) as only wildtype cultures were used in studies on aspartokinase production. Amino acid supplementation of MM had a variable effect on the levels of production of aspartokinase in the different mutant strains. Increasing biosynthesis of aspartokinase was found in mutants 8 and UV2, decreasing biosynthesis in UV1 and T1, and unchanged biosynthesis in mutant T4. Threonine increased production in mutants 8 and T1, decreasing biosynthesis in mutants UV1,UV2 and T4. The combination of lysine and threonine increased production in 8, UV1 and UV2 and lowered it in T1 and T4. Although AEC is a lysine analog it decreased the biosynthesis of aspartokinase to very low levels, in mutants UV2 and T1 to undetectable levels. However all the mutants exhibited higher levels of aspartokinase than did wildtype. The level of aspartokinase biosynthesis in mutant strains compared to wildtype is shown in Table 5. For each growth medium, aspartokinase production of the various mutants is expressed relative to the

Table 1. The effects of amino acid on aspartokinase biosynthesis in wildtype and AEC resistant cultures.

	ASPARTOKINASE ACTIVITY[a]				
	Amino Acid Added to Medium[b]				
Culture	None	Lys	Thr	Lys+Thr	AEC
Wildtype	100	114	85	118	*
8	100	153	146	115	27.9
UV 1	100	93.9	95.8	134	8.9
UV 2	100	123	80.4	161	nd
T1	100	82.2	111	85.3	nd
T4	100	99.8	86.7	87.7	11

a. Activity expressed relative to activity in MM for each culture after addition of amino acids

b. Lys = 10 mM lysine
Thr = 10 mM threonine,
Lys+Thr = 10 mM lysine + 10 mM threonine
AEC = 7 mM AEC

* = not tested

nd = not detectable

Specific activity of the wildtype cultures in MM 6.68 units/mg protein

Table 5 Aspartokinase production of AEC resistant cultures compared to wildtype

	ASPARTOKINASE ACTIVITY			
	Amino Acids Added to Medium			
Culture	None	Lys	Thr	Lys +Thr
8	124	171	213	121
UV 1	154	126	173	175
UV 2	163	176	154	222
T1	157	113	205	114
T4	135	118	128	110

a. Aspartokinase activity is expressed as a percentage of the wildtype activity under the same conditions. Basal medium = minimal medium
b. Lys = 10 mM lysine
Thr = 10 mM threonine,
Lys+Thr = 10 mM lysine + 10 mM threonine
AEC = 7 mM AEC
Specific activity of the wildtype cultures in MM 6.68 units/mg protein

aspartokinase activity of the wildtype culture growing on the same medium. All of the mutants appear to overproduce the enzyme to some extent, compared to the wildtype.

III.5.2. Effects of Amino Acids on Aspartokinase Activity in AEC Resistant Mutants and Wildtype

Mendelovitz and Aharonowitz (1982) have reported that L-lysine increases activity of aspartokinase while L-threonine has no effect on activity. Lysine and threonine in combination decrease activity. Results obtained in Table 5 indicate that both L-lysine and L-threonine individually decrease activity in wildtype and that the combination of L-lysine + L-threonine has an inhibitory effect that is about additive. Mendelovitz and Aharonowitz (1982) suggest that a mutant is deregulated in aspartokinase activity if the addition of L-lysine + L-threonine does not inhibit activity to a level of 50% of wild type. According to this definition only one mutant (spontaneous mutant 1) was deregulated in aspartokinase activity. The levels of aspartokinase activity in the presence of combined L-lysine + L-threonine were unchanged or lowered in all mutants and wild type except for mutant 1 when compared to aspartokinase activity with no amino acids present. The effect of amino acids on activity of the aspartokinase of mutant 1 is similar to that reported for deregulation of aspartokinase activity.

Table 6 The effects of amino acids on the aspartokinase activity of AEC resistant and wildtype cultures

	ASPARTOKINASE ACTIVITY			
	Amino Acid Added to Assay Mixture			
Culture	None	Lys	Thr	Lys+Thr
Wildtype	100	72.1	48.2	28.4
1	100	145	100	85.7
3	100	144	79.2	30.1
5	100	54.2	30.4	15
8	100	54.3	30.3	12.2
UV 1	100	56.3	36.9	13.5
T2	100	56.4	38.6	18.3
T3	100	30.9	22.3	15
T4	100	38.9	21.9	9.6

a. Aspartokinase activity is expressed as a percentage of the activity of which is seen with no amino acids added to the assay mixture
b. Lys = L-lysine at a final concentration of 5 mM
Thr = L-threonine at a final concentration of 5 mM
Lys+Thr = L-lysine and L-threonine both at a final concentration of 5 mM
Specific activity of the wildtype cultures in MM 6.68 units/mg protein

IV. DISCUSSION

Resistance to AEC has been reported to be associated with antibiotic overproduction in *S. clavuligerus* (Mendelovitz and Aharonowitz, 1983). To study this further, and to try and determine the mechanism of AEC resistance in *S. clavuligerus* , spontaneous mutants resistant to AEC were isolated. Initially, the level of sensitivity to this compound had to be determined in *S. clavuligerus*. This proved to have several difficulties associated with it. Inhibition of growth by AEC was found, however there was not total inhibition. Wildtype *S. clavuligerus* grew in the presence of AEC at concentrations of 1 mg/ml. By pregrowing the cultures in MM, growth was substantially limited at AEC concentrations of 1 mg/ml. This concentration was then used to obtain spontaneous mutants for subsequent experiments. When cultures were grown in liquid media wildtype cultures were difficult to distinguish from AEC resistant cultures. Growth of the wildtype cultures was slow to start in comparison to the AEC resistant cultures but seemed to reach the same level of growth. This may have been due to selection for spontaneous mutants in these cultures. To try to overcome this problem the concentration of AEC in liquid cultures was increased to 1.5 mg/ml. At this concentration the wildtype cultures grew poorly and were easily separated from AEC resistant cultures. The AEC resistant cultures did not appear to be effected by this increase in AEC concentration. Mendelovitz and Aharonowitz also reported using higher concentrations of AEC in liquid cultures.

A second problem arose when the AEC resistant colonies were first isolated. After 4 days growth, single resistant colonies appeared on plates of MM+ AEC. When these plates were incubated further, more colonies appeared

surrounding these colonies. The colonies were smaller than the original colonies and were thought to be scavenger colonies. When AEC resistant isolates were streaked out as on MM+AEC and the streaks were crossed by streaks of wildtype culture, no scavenger colonies were found. The wildtype streak grew very slowly on the plate, but grew as well distant from the AEC resistant streak and close to it. It was decided to use just the initially appearing, fast growing colonies when isolating spontaneous mutants and also when isolating induced AEC resistant mutants in subsequent experiments.

As stated earlier, in the introduction, *Streptomyces* are known to have high levels of genetic instability. It was shown by Mevarech (personal communication) that methotrexate resistance in *Halobacterium* was correlated with an amplification of chromosomal DNA. In order to determine if this could also be a mechanism for AEC resistance in *S. clavuligerus* , evidence for DNA amplification was sought. The first experiment carried out to determine if amplification was occurring, was a reversion experiment. If DNA amplification had occurred, this amplified DNA would be expected to be lost rapidly when the selective pressure for it was removed. After several rounds of cultivation in the absence of AEC, none of the spontaneous mutants was found to have reverted to wildtype. Mutant 4 did show a higher level of reversion than the other mutants selected. As mutant 4 had shown the highest reversion, this mutant was used to further investigate amplification. Amplification produces a characteristic pattern of amplified bands when chromosomal DNA is examined by agarose gel electrophoresis after restriction endonuclease digestion. This amplification of bands was not seen with mutant 4. Amplification did not appear to be associated with AEC resistance.

When spontaneous AEC resistant mutants were assayed for antibiotic production, the high levels reported by Mendelovitz and Aharonowitz (1983), of up to 500% higher than wildtype, were not found. This may have been due to the nature of the mutation, as Mendelovitz and Aharonowitz (1983) had used induced mutants. Mutagenesis of *Streptomyces* to produce various mutants has been reported but no consensus exists as to the optimum procedures for generating mutants. The optimum mutagen and the time in the life cycle of the cell were determined for *S. clavuligerus*. *Streptomyces* are filamentous organisms and each mycelial filament will have more than one copy of the chromosome. For mutagenesis it is desirable that there be only one chromosome/cell so that mutations are not complemented. In initial studies, mycelium was fragmented ultrasonically to separate the individual cells. Plate counts done to enumerate the organism after this step were highly variable. This may have been due to only partial breakage of the mycelia and subsequent breakage during plating. To achieve the greatest amount of breakage, survival of the cells had to be sacrificed. Because of this variability, sonicated mycelia were not used in mutagenesis experiments. Comparison of growing and dormant stages in the lifecycle of the cell was achieved by using ungerminated spores and germinated spores. Germinated spores should be equivalent to vegetative cells, however there are relatively few genomes/germinated spore as opposed to mycelium which has many genomes.

Two different mutagens were compared in this study. As stated earlier, these mutagens produce different effects and have both been used in *S. clavuligerus*. Ultraviolet light does not appear to produce significant differences in the survival of ungerminated spores or germinated spores. The frequency of

mutant production is higher in the germinated spores than the ungerminated spores. As there would appear to be the same number of lethal hits produced in both types of spores, the reason for more mutagenic hits in the germinated spores is unclear. Excision repair is error free (Witkin, 1976) while postreplicative repair is error prone. If the ungerminated spores were using only excision repair then they would be expected to have a lower mutation frequency. Postreplicative repair requires DNA synthesis. Germinated spores would be undergoing DNA synthesis at a much higher rate than ungerminated spores and so would be expected to have postreplicative repair. This may explain the higher rate of mutagenesis with germinated spores than with ungerminated spores.

Mutagenesis with NTG was used to compare mutant production with UV irradiation. Mutagenic lesions induced by NTG are produced at a higher rate at the replication fork than at other areas of the chromosome (Godfrey, 1974). This appears to be the case with *S. clavuligerus* as germinated spores produced higher mutation frequencies than did ungerminated spores. Godfrey (1974) had found that cells undergoing DNA replication mutated at a rate 6 times that of cells which were not replicating. This does not appear to be the case in this experiment. The difference in the rate of mutagenesis between the two types of spores is low. The rate of mutagenesis is only 1.4 times higher with germinated spores than with ungerminated spores. There is a greater difference in the mutation frequencies at higher survival rates than at the lower rates. Perhaps the long incubation times used with NTG allowed DNA synthesis to begin in the ungerminated spores even though the spores were suspended in a non-growth medium which does not support growth, and this led to a lessening of the effects of germination. Stonesifer and

Baltz (Stonesifer and Baltz, 1985; Baltz and Stonesifer, 1985a) have suggested that NTG mutagenesis is not totally dependent on error prone DNA repair. The error prone system is only responsible for 90% of NTG mutagenesis, unlike UV mutagenesis which requires error prone repair. This also may explain why there is not as big a difference with the two types of spores as there was with UV treatment. Again the incubation times may have allowed some postreplicative repair in the ungerminated spores.

From the above results of mutagenesis it was decided to use UV light treatment of germinated spores as the mutagenic treatment of choice. The overall frequency of mutation was greater and the inherent dangers of working with NTG were eliminated. AEC resistant mutants were then isolated using this technique.

During the course of this study transposon mutagenesis became available as a method that could be used in some *Streptomyces* species (Chung, 1987) had isolated Tn4556 from *S. fradiae* and shown that it would transpose in various *Streptomyces* species. Since the transposon is carried on the plasmid pMT660, and this plasmid is a derivative of pIJ702 which is known to function in *S. clavuligerus*, it seemed likely that the transposon system would function in *S. clavuligerus*. The transposon was received from Dr. Chung as a gift in the form of an *S. lividans* culture containing Tn4560 in the pMT660 plasmid. The transposon was introduced into *S. clavuligerus* and AEC resistant mutants were isolated. Problems were encountered with the selection of transposon containing mutants. The cells were initially grown on selective media containing thiostrepton to isolate transformants which contained the plasmid. The cells were then grown in media which alternately contained viomycin or lacked viomycin. The viomycin

concentrations which were used with *S. lividans* proved to be much too low for selection of viomycin resistant *S. clavuligerus*. Concentrations of 300 µg/ml had to be used with *S. clavuligerus*. Even at this concentration, growth of the wildtype was not completely inhibited. However at this concentration growth was retarded to a degree where wildtype could be differentiated from mutants containing the transposon. Chung (personal communication) has recently changed the selection marker in Tn4556 and this will greatly aid use of this transposon in the future.

When AEC resistant mutants were obtained using transposon containing cells it was necessary to prove that the mutants contained the transposon. Hybridization studies were used to detect the presence of the transposon. Chromosomal DNA was isolated from four mutants and used for hybridization studies. The plasmid which carried the transposon was a derivative of pIJ702 (Birch and Cullum, 1985). Labelled pIJ702 was then used to check for the presence of contaminating plasmid DNA. If the viomycin resistance had been acquired by recombination of the plasmid or by the presence of the plasmid itself, the labelled pIJ702 would have hybridized to the DNA. No hybridization was seen indicating that there were no homologous regions in the chromosomal DNA to the plasmid vector. Labelled pUC1169 was then used as a hybridization probe. Any specific hybridization to this probe should be due to the transposon component. Two bands appeared in the mutants and not the wildtype. The hybridization was presumably due to transposon as no hybridization was seen with the vector. The transposon had transposed from the plasmid to the chromosomal DNA. This is not proof that the AEC resistance mutations are due to insertion of the transposon, only that the transposon transposed. Although the bands appear on the autoradiograph at

the same position in all the mutants, this does not necessarily mean that the transposon is in the same position in all the mutants. However, if the AEC resistance is due to insertion of the transposon, then similar digests would be expected, as the transposon maybe inserted in the same region. This would give fragments that are of approximately the same size regardless of the insertion point of the transposon. To determine if the AEC resistance was due to the insertion of the transposon DNA, the transposon and the flanking DNA would have to be cloned from these mutants and analyzed.

A range of mutants was now available to study AEC resistance and the overproduction of antibiotics. Initial studies showed highly variable antibiotic production. Mutants which were classed as overproducers in one trial seemed to underproduce in the next trial. To try and standardize the antibiotic production measurements, production was followed as a function of time in different media. Wildtype and mutant strains were grown in both TCSS and MM to determine the effects of complex media compared to defined media. Mutants were also grown in MM+AEC to determine the effects of AEC on antibiotic production.

Antibiotic production in TCSS seemed to reach a peak in all the cultures at approximately 56 - 80 h. The growth of the cultures, measured as optical density, reached a peak at about 48 h. All the mutants seemed to overproduce antibiotic to some extent compared to the wildtype cultures. Mutants 5 and UV1 produced the highest levels of antibiotic of all the mutants. Growth of the cultures was good in all cases with the mutants having as good or better growth characteristics than the wildtype.

Growth and antibiotic production of wildtype and mutant strains in MM

was less than that in TCSS. Some variation was seen in antibiotic production. Both overproduction and underproduction was seen with the mutants. Peak production of antibiotic was delayed in the MM compared to TCSS. Highest levels of production occurred at approximately 38 h whereas peak growth occurred at 40 h. Both of the transposon mutants grew at a lower rate than the other cultures and produced lower levels of antibiotic. The spontaneous AEC resistant mutants seemed to produce the highest levels of antibiotic in this medium. UV1, which was the highest producer in TCSS, still overproduces compared to the wildtype but produces less antibiotic than the spontaneous mutants in MM.

AEC resistant mutants were also grown in MM containing AEC. The addition of AEC seemed to inhibit the growth of the organisms to some extent. The peak level of growth obtained was substantially less than in MM alone. Antibiotic production was not affected by the addition of AEC in mutant 5 but did seem to be decreased slightly in the other mutants. Mutants 8 and UV1 appeared to be affected by AEC to the greatest extent of all the mutants. Production was lowered by over 50% in both mutants when compared to levels in TCSS. However, in several cases this difference was seen at a single point during the growth curve and may be due to differences in the assay at that time. Although samples were stored at -75°C and assayed at one time, there is variation inherent in bioassays which may range from 10 -20%. Triplicate samples were used to minimize variations. Each bioassay plate has a limited number of samples and some variation between plates is possible. The transposon mutants showed very little antibiotic production in both the MM and MM+ AEC medium.

Overproduction of antibiotic has been correlated with deregulation of

aspartokinase activity (Mendelovitz and Aharonowitz, 1983). Aspartokinase deregulation could affect antibiotic production in two ways. Mutants could be deregulated in the production of aspartokinase and overproduce the enzyme. This would lead to overproduction of antibiotic by leading to an overproduction of α-aminoadipic acid as this has been shown to be the limiting factor in antibiotic production. Mutants could also be deregulated in that they produce the same amount of aspartokinase as the wildtype but the enzyme has different characteristics with respect to inhibition by amino acids. Again this could lead to an overproduction of α-aminoadipic acid. To determine if this was the case with the mutants isolated here, aspartokinase activity was measured. A comparison of aspartokinase in wildtype and the highest antibiotic producer (mutant 5) of the AEC resistant mutants was undertaken. Aspartokinase activity was measured as a function of time to determine if production of the enzyme occurred at different stages in the growth of the wildtype and mutant. The optimum production of aspartokinase appeared at 48 h for both cultures when grown on TCSS. The mutant also had highest aspartokinase activity at 48 h in MM and MM+AEC. The wildtype seemed to produce highest levels of aspartokinase a little later than the mutant in MM, with activity reaching a peak at 64 h. The optimum time reported for aspartokinase production by Mendelovitz and Aharonowitz (1982) was between 48 and 64 h. Levels of aspartokinase activity were higher in TCSS than in MM. The levels were similar between the two cultures in TCSS but in MM mutant 5 produced higher levels.

The aspartokinase production in each of the mutants was then examined. Two different amino acids of the aspartate family were used to determine the effects

on aspartokinase biosynthesis. All of the mutants overproduced aspartokinase compared to the wildtype. This would suggest that biosynthesis of aspartokinase is deregulated. The effects of lysine and threonine were not consistent between the mutants. The results of this experiment are significantly different than those of Mendelovitz and Aharonowitz (1982). They report that aspartokinase production in wildtype was increased with the addition of lysine, remained approximately the same when threonine was added, and decreased when both lysine and threonine were added. In this study wildtype production was increased when lysine was added, decreased when threonine was added, and increased again when both lysine and threonine were added. Each subsequent mutant tested was affected by amino acid supplementation in a different way. The variable effects of addition of amino acids has been reported to occur with AEC resistant mutants by Mendelovitz and Aharonowitz (1982) when activity was determined but was not reported for biosynthesis of the enzyme. When AEC resistant mutants were grown in MM+AEC the only consistent response was the effect of AEC on aspartokinase production. In each case AEC drastically reduced the amount of the aspartokinase. Lysine increased the production of aspartokinase and as AEC is a lysine analog it would be expected to have the same effect. The effects of the two compounds are opposite with lysine increasing production and AEC decreasing production. Two of the mutants did not produce detectable levels of enzyme when AEC was added to the medium. Lysine is not present in MM and must be produced by the organism for growth to occur. The aspartokinase pathway leads to the production of several essential amino acids. It is unclear how growth could occur with undetectable levels of aspartokinase.

The same amino acids that were used to examine the effects of amino acid supplementation on biosynthesis of aspartokinase were then used to determine the effects on activity of the enzyme. The effects on activity differed from the wildtype in two cases. Lysine generally inhibited the activity of aspartokinase except for mutants 1 and 3. This is not as was reported by Mendelovitz and Aharonowitz (1983). They reported that lysine stimulated activity in all cases, and lysine in combination with threonine inhibited aspartokinase. Threonine by itself inhibited aspartokinase activity slightly to a level intermediate between lysine and the lysine plus threonine combination. Although Mendelovitz and Aharonowitz (1983) had reported a decrease in the activity of aspartokinase in the presence of both amino acids it was not as great as that reported here. The criterion that was used by Mendelovitz and Aharonowitz (1983) to define deregulation of aspartokinase was that the inhibition of aspartokinase activity by lysine plus threonine should not exceed 50%. Using this definition, only one AEC resistant mutant isolated in this study meets this requirement (mutant 1).

In summary, this study did not find evidence for deregulation of aspartokinase activity as a mechanism for AEC resistance in *S. clavuligerus*. Mutants did appear to overproduce aspartokinase in comparison to the wildtype culture, and the aspartokinase showed the same characteristics as the wildtype with respect to inhibition by amino acids. Perhaps AEC resistance leading to overproduction of antibiotics is not due to a deregulation of aspartokinase as has been suggested by Mendelovitz and Aharonowitz (1983) but rather to a significant overproduction of the enzyme. Overproduction of the enzyme which is still sensitive to feedback inhibition by lysine and threonine, may lead to an

overproduction of α-aminoadipic acid. Much higher levels of amino acids would be needed to inhibit the overproduced aspartokinase. This would have the same effect as deregulation of feedback inhibition as more lysine would be available for antibiotic production. The addition of lysine to cultures has been shown to increase cephamycin C production in *S. clavuligerus* (Mendelovitz and Aharonowitz, 1982).

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