β -Lactam Antibiotic Production by *Streptomyces clavuligerus* Mutants Impaired in Regulation of Aspartokinase

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This work describes isolation and characterization of *Streptomyces clavuligerus* mutants resistant to the lysine analogue *S*-(2-aminoethyl)-L-cysteine (AEC). The mutation to AEC resistance was shown to affect the feedback regulation of aspartokinase; 70% of the mutants isolated had aspartokinase activity insensitive to concerted feedback inhibition by lysine plus threonine. Among these mutants, 70% (about 50% of the total AEC-resistant strains isolated) showed significant overproduction of β -lactam antibiotics.

INTRODUCTION

The cephalosporins, cephamycin, and the penicillin N types of β -lactam antibiotics, which are produced by fungi and streptomycetes are derived mainly from three amino acids: α -aminoadipic acid (AAA), cysteine and valine. Extensive physiological, biochemical and genetic studies have revealed the direct involvement of primary metabolites in the regulation of β -lactam antibiotic production (Drew & Demain, 1977). The metabolic origin of the AAA moiety of β -lactam antibiotic produced by fungi differs from that of the streptomycetes; in fungi AAA is an intermediate of the lysine biosynthetic pathway (Umbarger, 1978), whereas in streptomycetes AAA originates as a catabolic product of lysine produced from the diaminopimelic acid (DAP)-lysine branch of the aspartate pathway (Kirkpatrick *et al.*, 1973; Kern *et al.*, 1980). Therefore, the involvement of lysine metabolism in controlling β -lactam antibiotic synthesis in fungi and streptomycetes becomes of special interest, from both a comparative biochemical as well as the practical point of view.

Suppression of penicillin formation by addition of L-lysine to cultures of *Penicillium chrysogenum* was first observed by Demain (1957). It has been attributed to feedback regulatory effects of lysine on homocitrate synthetase, the first step in the fungal lysine biosynthetic pathway (Masurekar & Demain, 1972; Luengo *et al.*, 1980). In contrast, addition of L-lysine and a mixture of DL- and meso-DAP to a culture of *Streptomyces clavuligerus* in chemically defined medium significantly stimulated β -lactam antibiotic production (Mendelovitz & Aharonowitz, 1982). It therefore became interesting to see whether alteration of the intracellular lysine pool by genetic means could affect β -lactam antibiotic production in a similar fashion to that obtained with external lysine addition. The use of lysine antimetabolites for selection of lysine-regulatory mutants was chosen to achieve this goal. Masurekar & Demain (1974) were able to obtain lysine-regulatory mutants of *P. chrysogenum* which overproduced and excreted lysine and were deficient in production of penicillin. Further, a revertant of such a mutant was found to recover the ability to produce penicillin. Bacterial mutants resistant to lysine analogues have already been used commercially for lysine production. Among these, mutants of *Brevibacterium flavum* resistant to *S*-(2-aminoethyl)-L-cysteine (AEC) produced lysine most effectively (Sano & Shiio, 1970).

Abbreviations: AAA, α -aminoadipic acid; AEC, *S*-(2-aminoethyl)-L-cysteine; AK, aspartokinase; DAP, diaminopimelic acid; DDPS, dihydrodipicolinate synthetase.

The objective of this work was to select for AEC-resistant mutants of *S. clavuligerus*, to investigate possible alteration in regulation of their lysine biosynthetic pathway, and to determine whether such a mutation might affect cephamycin formation. As will be shown, 70% of AEC-resistant mutants of *S. clavuligerus*, in which feedback regulation of aspartokinase activity was impaired, produced elevated levels of antibiotics.

METHODS

Bacterial cultivation. *Streptomyces clavuligerus* NRRL 3585 (Higgins & Kastner, 1971) was used throughout this study. The chemically defined medium and growth conditions employed were reported previously (Aharonowitz & Demain, 1977). Selection of analogue-resistant mutants was carried out on agar containing all minimal medium components except asparagine. The latter was replaced by 40 mM-NH₄Cl as nitrogen source. Spores were prepared on solid medium which contained (l⁻¹): 10 g malt extract (Difco), 4 g yeast extract, 4 g glycerol, 100 µg CoCl₂, and 20 g agar, adjusted to pH 7.0.

Isolation of AEC-resistant mutants. Spores suspended in 1 ml 50 mM-Tris/HCl buffer, pH 7.5, containing 0.01% (v/v) Triton X-100 were transferred to an Erlenmeyer flask (125 ml) containing 7.5 ml 5.0 mM-Tris/HCl buffer, pH 7.5. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was added to a final concentration of 0.5 mg ml⁻¹. The mixture was agitated momentarily and incubated in a gyrotory shaker at 30 °C. Samples were taken, at time intervals, washed by centrifugation and spread on agar plates containing defined medium (see above) and AEC at a concentration of 1 mg ml⁻¹. Colonies which developed within one week were isolated, restreaked on sporulation medium and tested for resistance to AEC.

Aspartokinase assay. Aspartokinase activity was measured by the formation of aspartylhydroxamate (Stadtman *et al.*, 1961) with the 30–65% saturated (NH₄)₂SO₄ fraction of crude sonic extracts. The reaction mixture contained (in 1 ml): 0.1 M-Tris/HCl buffer pH 7.6, 0.02 M-ATP, 0.02 M-aspartate, 0.01 M-MgSO₄, 1.4 mM-β-mercaptoethanol, 0.8 M-NH₂OH. HCl neutralized with KOH, and enzyme as indicated. Incubation was at 30 °C. Specific activity was expressed as nmol aspartylhydroxamate formed min⁻¹ (mg protein)⁻¹.

Fermentations. All cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of the chemically defined medium described by Aharonowitz & Demain (1977). Incubation was at 30 °C for 120 h on a gyrotory shaker with a 2 in (5 cm) stroke at 250 r.p.m.

Antibiotic assay. Samples were assayed for the production of cephalosporins by the standard disc agar plate technique, using as an assay organism an *Escherichia coli* strain Ess that is supersensitive to β-lactam antibiotics (Kohsaka & Demain, 1976). In this assay we detected only the cephamycin C, which was the dominant product (as was confirmed by HPLC analysis). Clavulanic acid had only 5–10% of the activity of cephalosporin C in the assay (Aharonowitz & Demain, 1977).

RESULTS

Effect of AEC on growth and aspartokinase activity

When the wild-type strain was grown on a solid chemically defined medium containing increasing concentrations (>1 mg ml⁻¹) of the lysine analogue AEC, growth was completely inhibited. However, in liquid medium of the same composition, the concentration required to achieve 50% inhibition of growth was 1.5 mg ml⁻¹. Addition of L-lysine, at concentrations four times lower than that of the inhibitor, reversed the inhibitory effect of AEC and allowed normal growth to occur.

Since aspartokinase (AK) activity of *S. clavuligerus* is under concerted feedback inhibition by lysine plus threonine (Mendelovitz & Aharonowitz, 1982), the possible substitution of lysine by its analogue AEC and hydroxylysine in the AK assay was investigated. The results in Table 1 demonstrate feedback inhibition of AK activity by the concerted effect of threonine plus AEC, similar to that achieved by threonine plus lysine. The combination of threonine plus hydroxylysine inhibited AK activity to a lesser extent. It was assumed that addition of threonine to the AEC-containing growth medium would increase the growth inhibitory effect mediated by AEC. However, threonine actually increased the inhibitory effect of AEC only over a very narrow range of concentrations (20–200 µg ml⁻¹; Fig. 1); upon further increase of threonine concentration, the inhibitory effect of AEC was reversed.

Regulation of AK activity in the AEC-resistant mutants

Each of the AEC-resistant colonies was tested for growth, AK activity and antibiotic production. Although most colonies tested appeared morphologically similar, differences in

Table 1. Effect of threonine, lysine and lysine analogues on aspartokinase activity

The standard hydroxamate assay was used. The enzyme preparation was the 30 to 65% ammonium sulphate fraction prepared from a 48 h culture. Protein content in each assay mixture was 0.94 mg. In a control reaction, with no added amino acid, 21.6 nmol aspartohydroxamate min⁻¹ were formed, representing 100% relative activity.

Addition (5 mM)	Relative enzyme activity (%)
None	100
L-Threonine	106
L-Lysine	140
Hydroxylysine	95
S-(2-aminoethyl)-L-Cysteine (AEC)	118
L-Lysine + L-threonine	6
AEC + L-threonine	18
Hydroxylysine + L-threonine	64

Fig. 1. Effect of threonine on growth of *S. clavuligerus* in presence of AEC. Cells were grown in the chemically defined medium containing 1 mg AEC ml⁻¹ and increasing concentrations of threonine. Maximum growth at 66 h observed in the control culture was 1250 Klett units.

their growth patterns were observed in the liquid medium. Some of the mutants were poor growers and some formed dense clumps. Of the several different genotypes that could account for the AEC resistance phenotype, those which possessed a genetic alteration in the AK regulatory site were of special interest. It was expected that in such mutants the concerted inhibitory effect exerted by threonine plus lysine (or its analogues) on AK activity would be absent. Accordingly, a cell extract from each AEC-resistant mutant obtained was tested for AK activity in the presence and absence of the putative feedback inhibitors. In 32 out of the 44 mutants tested, AK activity differed from that of the wild-type in that it was unaffected by concentrations of lysine plus threonine that caused about 50% inhibition of AK activity in the wild-type strain. Table 2 presents data on 12 such mutants resistant to AEC. The AK activity of another three mutants (strains 1, 20 and 38) was inhibited in the presence of these two amino acids (Table 2). AK activity, in presence of 8 mM-lysine, was always higher than in the control (no addition), the degree of stimulation being between 20–100%. Significant differences in AK activity were usually also obtained in the presence of 8 mM-threonine. These changes in AK activity provoked by threonine might be explained by differences in enzyme concentration in different reaction mixtures (Shabtai *et al.*, 1975).

Two mutants (strains 11 and 52) were assayed for repression and inhibition of dihydrodipicolinate synthetase (DDPS) by lysine, DAP, isoleucine, homoserine, methionine, AAA and lysine. No change with respect to feedback regulation of DDPS in the mutant compared to the wild-type was observed; DAP repressed DDPS synthesis by 30%, whereas DAP and AAA each inhibited DDPS activity by 30%.

Table 2. *Effect of L-lysine and L-threonine on aspartokinase activity in AEC-resistant mutants*

Organisms were grown in chemically defined medium and harvested in their late growth phase (48–72 h). Aspartokinase activity was assayed in the ammonium sulphate fraction as described in the legend to Table 1.

Mutant no.	Relative aspartokinase activity in the presence of:				Aspartokinase deregulation
	No addition	L-Lysine (8 mM)	L-Threonine (8 mM)	L-Lys (0.4 µM) + L-Thr (0.4 mM)	
Wild-type	100	173	115	35	—
<i>aec(R)1</i>	100	141	71	35	—
<i>aec(R)3</i>	100	142	93	100	+
<i>aec(R)5</i>	100	127	110	100	+
<i>aec(R)10</i>	100	129	109	98	+
<i>aec(R)11</i>	100	139	104	100	+
<i>aec(R)15</i>	100	124	109	90	+
<i>aec(R)20</i>	100	151	87	29	—
<i>aec(R)22</i>	100	159	100	93	+
<i>aec(R)27</i>	100	178	119	95	+
<i>aec(R)28</i>	100	148	203	98	+
<i>aec(R)35</i>	100	219	122	142	+
<i>aec(R)38</i>	100	161	104	61	—
<i>aec(R)50</i>	100	193	192	116	+
<i>aec(R)52</i>	100	128	117	107	+
<i>aec(R)54</i>	100	146	143	114	+

Table 3. *Specific antibiotic production by AEC-resistant and wild-type strains of S. clavuligerus*

Cultures were grown in chemically defined medium without any added amino acids except asparagine, which served as the nitrogen source. At various times, samples were taken for pH, biomass, and antibiotic analysis.

Mutant no.	Aspartokinase deregulation*	Dry cell wt† (mg ml ⁻¹)	Maximum antibiotic concn (µg ml ⁻¹)	Specific production of antibiotic‡ [µg (mg dry cell wt) ⁻¹]	Relative specific production value
Wild-type	—	1.8	84	47	1.0
<i>aec(R)3</i>	+	0.8	95	122	2.6
<i>aec(R)5</i>	+	1.2	260	217	4.6
<i>aec(R)10</i>	+	1.3	122	94	2.0
<i>aec(R)11</i>	+	0.7	127	181	3.9
<i>aec(R)15</i>	+	1.3	420	323	6.9
<i>aec(R)22</i>	+	1.3	285	219	4.9
<i>aec(R)27</i>	+	1.5	210	140	3.0
<i>aec(R)28</i>	+	1.1	345	313	6.7
<i>aec(R)35</i>	+	1.4	150	107	2.3
<i>aec(R)38</i>	—	1.8	49	27	0.6
<i>aec(R)50</i>	+	1.5	405	270	5.7
<i>aec(R)52</i>	+	1.2	285	237	5.0
<i>aec(R)54</i>	+	1.1	230	209	4.4

* +, Aspartokinase activity which is not inhibited when assayed in the presence of 0.4 mM-L-lysine plus 0.4 mM-L-threonine.

† The dry cell weight shown was the maximum achieved during fermentation of 120 h.

‡ Maximum cephalosporin titre divided by the maximum dry cell weight achieved during fermentation.

Antibiotic production by the AEC-resistant mutants

All AEC-resistant mutants were grown in the chemically defined medium (four independent growth cycles) for 120 h and broth samples were assayed for antibiotic. Among those mutants in which AK activity was deregulated, 23 out of 32 (about 70%) produced antibiotic at higher titres and at higher specific production values [µg antibiotic (mg dry cell weight)⁻¹] than the wild-type.

In fermentations of the other nine cultures, production values did not significantly differ from those of the wild-type. Among the AEC-resistant mutants possessing a non-deregulated AK activity, 3 out of 12 tested (25%) still yielded improved titres (between 120 and 280 $\mu\text{g ml}^{-1}$) corresponding to 2–5 times more antibiotic produced based on specific production values. The results shown in Table 3 represent fermentation data for 12 AEC-resistant mutants deregulated in their AK activity and one which was not deregulated. The antibiotic titres achieved in these fermentations ranged from 1.1 to 5.9 times higher than the wild-type titre. Furthermore, due to the decrease in the maximum biomass obtained with the AEC-resistant mutants, the calculated relative specific production values of those strains were even higher. Mutant *aec*(R)38, the one which retained a normal AK regulation pattern, could grow to the same extent as the wild-type. However, its capacity to produce antibiotics was significantly lower.

DISCUSSION

Our results show that superior β -lactam antibiotic-producing strains of *S. clavuligerus* were prevalent among mutants selected for resistance to the lysine analogue, AEC. Although several genotypes can explain the AEC-resistant phenotype, genetic alteration which results in an impaired regulation of AK activity was of particular interest to us.

The AK activity of *S. clavuligerus* was previously shown to be feedback-inhibited by the concerted effect of threonine plus lysine (Mendelovitz & Aharonowitz, 1982). Lysine could be replaced by AEC for concerted feedback inhibition of AK activity (Table 1). Since AEC also inhibited growth of *S. clavuligerus*, an effect that could be reversed by lysine addition, it seemed reasonable to assume that AEC acted as a lysine antimetabolite. Presumably then, the combination of AEC and threonine in the growth medium should have enhanced growth inhibition. However, such an effect was only observed at low threonine concentrations; when threonine concentration was increased, restoration of normal growth levels was observed. The reason for this phenomenon is unclear. However, observations reported by Gross & Burkhardt (1973) suggest possible involvement of amino acid transport mechanisms. Two uptake systems for lysine, arginine and their analogues exist in *Streptomyces hydrogenans* (Gross & Burkhardt, 1973). One system operates when low (<0.1 mM) extracellular lysine (or lysine analogue) concentrations are available, whereas when higher lysine and arginine concentrations are available, lysine is taken up by a second system which generally mediates the uptake of neutral amino acids. This latter system has a lower affinity for lysine and arginine, but its transport capacity is higher than the first, lysine-specific, system. Since we have used 5 mM-AEC in order to inhibit *S. clavuligerus* growth, the second transport system was probably operating. Thus, upon increasing threonine concentration, AEC uptake could be competitively inhibited.

It was the *in vivo* effect of AEC that enabled selection of AEC-resistant mutants of the sort from which AK-deregulated strains could be screened. Indeed, such mutants occurred at high frequency among the AEC-resistant types (Table 2).

The data presented here suggest a similarity between the AK systems of *S. clavuligerus* and *Brevibacterium flavum*. In *B. flavum*, AK activity appears to be due to a single enzyme which is concertedly feedback-inhibited by lysine plus threonine (Shiio & Miyajima, 1969); AEC-resistant mutants of the *B. flavum* strain were found to possess a desensitized AK free from feedback inhibition by lysine plus threonine, and also produced lysine in excess (Sano & Shiio, 1970).

Since L-lysine and DL-meso-DAP each stimulated specific β -lactam antibiotic production by *S. clavuligerus* (Mendelovitz & Aharonowitz, 1982), and since feedback control was exerted by lysine plus threonine on AK, it was assumed that carbon flow from aspartate to lysine and AAA would be rate-limiting for antibiotic synthesis. Therefore, increased antibiotic synthesis was expected upon release of enzymes involved in lysine biosynthesis from such feedback effects. Consistent with this idea was the finding that 70% of the mutants impaired in the feedback regulation of their AK activity (about 50% of the total AEC-resistant population) produced significantly higher amounts of antibiotics, in terms of both volumetric titre and specific production. However, with respect to antibiotic production capacity, other phenotypes were

also found among the AEC-resistant strains, specifically: (a) those which were deregulated in their AK activity but did not produce higher amounts of antibiotics (20% of the total); (b) those which were unaffected in AK activity, but did produce high amounts of antibiotics (6% of the total); and (c) those which possessed an unaffected AK activity and produced normal amounts of antibiotics (20% of the total). Types (b) and (c) could be the result of genetic alterations in genes responsible for AEC transport or AEC modification. The reason why type (b) mutants are antibiotic producers is, however, still unclear.

Although the majority of the AEC-resistant AK-deregulated mutants produce higher antibiotic titres as well as higher specific production values, the degree of increase did not correlate exactly with the degree of impairment in AK regulation. The possible involvement of more than a single mutation could not be ruled out. Further analysis of biochemical defects in the AK protein extracted from different mutants is still required for a better understanding of its role in regulating the carbon flow towards the lysine branch of the aspartate pathway. Two other enzymic activities in the lysine branch might be suitable targets for regulation. One, DAP-decarboxylase, was suggested to be under regulation in *Streptomyces lipmanii* (Kirkpatrick *et al.*, 1973) and is now under study in *S. clavuligerus* in our laboratory. As was suggested by Kern *et al.* (1980), a second possible target for regulation in the pathway leading to α -aminoadipate is L-lysine ϵ -aminotransferase.

The rationale behind the isolation of lysine analogue-resistant mutants was to obtain lysine overproducers. Such mutants of *P. chrysogenum* overproduced lysine and underproduced penicillin (Masurekar & Demain, 1974). In sharp contrast, corresponding mutants of *S. clavuligerus* were found to overproduce cephalosporins.

The link between primary and secondary metabolism shown in this work demonstrates the feasibility of obtaining strains that produce large amounts of antibiotics through selection for mutants deregulated in the biosynthesis of a primary metabolite directly involved as a precursor in the synthesis of the antibiotic in question.

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