# CEPHAMYCIN C PRODUCTION BY *STREPTOMYCES CLAVULIGERUS* MUTANTS IMPAIRED IN REGULATION OF ASPARTOKINASE

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## ABSTRACT

### CEPHAMYCIN C PRODUCTION BY *STREPTOMYCES CLAVULIGERUS* MUTANTS IMPAIRED IN REGULATION OF ASPARTOKINASE

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Aspartokinase is the first enzyme of the aspartate family amino acids biosynthetic pathway. Cephamycin C is a β-lactam antibiotic produced as a secondary metabolite via the enzymatic reactions in the lysine branch of this pathway in *Streptomyces clavuligerus*. The aspartokinase activity of *S. clavuligerus* is under concerted feedback inhibition by two of the end product amino acids, lysine plus threonine. It is also known that carbon flow through the lysine branch of the aspartate pathway is rate limiting step in the formation of cephamycin C.

Therefore, genetic alterations in the regulatory regions of the aspartokinase enzyme are expected to lead to an increased cephamycin C production.

The aim of this study was to obtain *S. clavuligerus* mutants that possess aspartokinase enzyme insensitive to feedback inhibition by lysine and threonine, identification of the mutation(s) accounting for the resistance being the ultimate goal. For this aim, chemical mutagenesis was employed to increase random mutation rate and a population of lysine anti-metabolite resistant *S. clavuligerus* mutants that can grow in the presence of *S*-(2-aminoethyl)-L-cysteine was obtained. The mutants were screened for their aspartokinase insensitivity via enzyme assays and one mutant exhibiting the highest level of deregulation was assessed for its cephamycin C production. The results revealed a 2-fold increase in specific production of the antibiotic.

As a member of β-lactam class antibiotics, cephamycin C has an importance in medicine. Therefore, the mutant strain obtained might be a candidate for industrial production of the compound.

**Keywords:** *Streptomyces clavuligerus*, aspartokinase, cephamycin C biosynthesis, chemical mutagenesis, feedback inhibition, deregulated mutant, antibiotic biosynthesis.

# ÖZ

## ASPARTOKİNAZ DENETİMİ ORTADAN KALDIRILMIŞ *STREPTOMYCES CLAVULİGERUS* MUTANTLARINDA SEFAMİSİN C ÜRETİMİNİN ARAŞTIRILMASI

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Aspartokinaz enzimi, aspartat ailesi amino asitleri biyosentetik yolunun ilk enzimidir. Sefamisin C, *Streptomyces clavuligerus* tarafından ikincil ürün olarak bu yolun lizin dalından itibaren üretilen bir antibiyotiktir. *S. clavuligerus*'a ait aspartokinaz enzimi üzerindeki çalışmalar, bu enzimin aspartat metabolik yolunun son ürünleri olan treonin ve lizin ile geri bildirimli olarak inhibe edildiğini göstermiştir. Sefamisin C üretiminde karbon akışının hız sınırlayıcı aşamasını aspartat yolunun lizin çatalının oluşturduğu bilinmektedir. Bunlara dayanarak, enzimin regulasyon bölgelerine karşılık gelen gen bölgelerinde yapılacak mutasyonların sefamisin C üretiminde artışa neden olacağı beklenmektedir.

Bu çalışmanın amacı, aspartokinaz denetimi ortadan kaldırılmış *S. clavuligerus* mutantları elde ederek aspartokinaz geninde geri bildirim denetiminden sorumlu nokta mutasyonları belirlemektir. Bu amaçla, mikroorganizmada spontan mutasyon hızını artırmak için kimyasal mutajenez tekniği uygulanarak, *S*-(2-aminoetil)-L-sistein anti-metabolit maddesine karşı dirençli bir *S. clavuligerus* populasyonu elde edilmiştir. Elde edilen mutantların aspartokinaz aktvitiveleri belirlenerek enzim geri bildirim duyarsızlığı için taranmış ve en yüksek deregülasyona sahip mutant sefamisin C üretimi bakımından incelenmiştir. Sonuçlar, bu mutantta sefamisin C spesifik üretiminde 2 misli artış olduğunu göstermiştir.

β-laktam antibiyok sınıfının bir üyesi olarak sefamisin C tıbbi alanda öneme sahiptir. Elde edilen mutant sefamisin C'nin endüstriyel üretimi için uygun aday olabilir.

**Anahtar kelimeler:** *Streptomyces clavuligerus*, aspartokinaz, sefamisin C biyosentezi, kimyasal mutajenez, geri bildirim inhibisyonu, deregüle mutant, antibiyotik biyosentezi.

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| | |
|---|---|
| α-AAA | α-Aminoadipic acid |
| ACV | α-Aminoadipyl-cysteinyl-valine |
| AEC | S-(2-aminoethyl)-L-cysteine |
| AK | Aspartokinase; aspartate kinase |
| Asd | Aspartate semialdehyde dehydrogenase |
| *asd* | Aspartate semialdehyde dehydrogenase gene |
| *ask* | Aspartokinase gene |
| bp | Base pairs |
| ca. | circa, approximately |
| DAP | Diaminopimelate |
| *hom* | Homoserine dehydrogenase gene |
| MNNG | *N*'-methyl-*N*'-nitro-*N*'-nitrosoguanidine |

# CHAPTER 1

# INTRODUCTION

## 1.1. The genus *Streptomyces*

The Gram-positive bacteria include two major branches: the low G/C organisms, containing the genera such as *Bacillus*, *Clostridium*, *Staphylococcus* and *Streptococcus*; and the high G/C organisms referred to as the actinomycetes (Kieser *et al.,* 2000). The actinomycetes are large group of filamentous bacteria that form branching filaments. As a result of successful growth and branching, a ramifying network of filaments is formed, called a mycelium (Madigan *et al.,* 1997) (Figure 1.1). Actinomycetes are well-known for their capacity to synthesize a vast repertoire of secondary metabolites, including many useful antibiotics and other products such as the antitumor drug adriamycin, ivermectin; an agent used to combat African river blindness, the immuno-suppressants cyclosporin, FK506 and rapamycin, and the herbicide bialophos (phosphinothricin) (Champness and Chater, 1994). As seen in Table 1.1, actinomycetes do indeed make some two-thirds of the antibiotics that are produced by microorganisms, and amongst them nearly 80% are made by members of the genus *Streptomyces* (Kieser *et al.,* 2000).

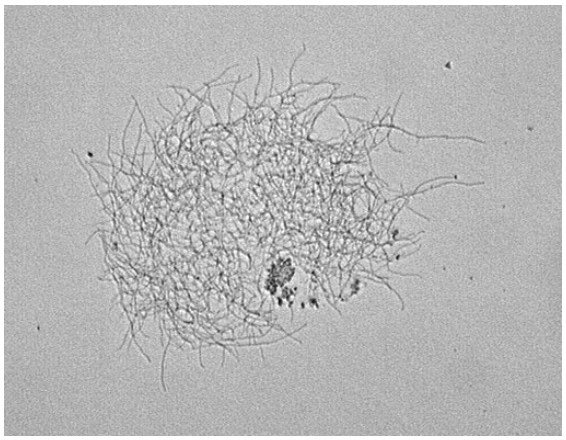

**Figure 1.1:** *S. clavuligerus* under light microscope.

*Streptomyces* is a genus represented by a large number of species and varieties. Over 500 species of *Streptomyces* are recognized by "*Bergey's Manual of Determinative Bacteriology",* although GC base ratios cluster tightly between 69 and 73 mol %. Large (~8 Mb) linear chromosomes and large (10-600 kb) linear plasmids are also striking features of streptomycetes. *Streptomyces* filaments are usually 0.5–1.0 µm in diameter, are of indefinite length, and often lack cross-walls in the vegetative phase. Growth occurs at the tips of the filaments and is often accompanied by branching so that the vegetative phase consists of a complex, tightly woven matrix, resulting in a compact, convoluted colony (Madigan *et al.,* 1997; Keiser *et al*., 2000). The members of the genus *Streptomyces* constitute the major group in actinomycetes. Of 12,000 secondary metabolites with antibiotic activity, 55% are produced by streptomycetes and additional 11% by other actinomycetes (Paradkar *et al*., 2003; Weber *et al.,* 2003).

**Table 1.1.** Approximate numbers of secondary metabolites produced by different organisms, as of 1994 (Kieser *et al.,* 2000).

| Source | Bioactive metabolites | | | "Inactive" metabolites |
|---|---|---|---|---|
| | Antibiotics | Other | Total | |
| Non-actinomycete bacteria | 1400 (12%) | 240 (9%) | 1640 (11%) | 2000-5000 |
| Actinomycetes | 7900 (66%) | 1220 (40%) | 9120 (61%) | 8000-10,000 |
| Fungi | 2600 (22%) | 1540 (51%) | 4140 (28%) | 15,000-25,000 |
| Total microorganisms | 11,900 (100%) | 3000 (100%) | 14,900 (100%) | 25,000-40,000 |
| Lichens | 150 | 200-500 | | ~1000 |
| Algae | 700 | 800-900 | | 1000-2000 |
| Higher plants | 5000 | 25,000-35,000 | | 500,000-800,000 |
| Terrestrial animals | 500 | 10,000-15,000 | | 200,000-300,000 |
| Marina animals | 1200 | 1500-2000 | | 2000-3000 |
| Total higher organisms | 7500 | 35,000-50,000 | | >1,000,000 |

Other noteworthy characteristic of *Streptomyces* is their remarkably complex developmental features in which they differ from most other bacteria (Kalakoutskii and Agre, 1976). Indeed, in terms of their complex life cycle, streptomycetes are superficially more reminiscent of filamentous fungi. They grow by tip extension to form a mycelium of branched hyphae (Flärdh, 2003). In a typical growth cycle, the spores bud into long filamentary hyphae which grow in and on the nutrient surface. The hyphae undergo branching and a dense mycelium (substrate mycelium) is gradually formed. This phase is usually referred as the vegetative growth phase. The vegetative phase is usually followed by a second, aerial growth phase which can be accompanied by the generation of antibiotics. In this phase, the hyphae grow out of the mycelium, sometimes deforming into helical structures,

and ultimately break up into spores which then start the vegetative growth cycle again (Goriely and Tabor, 2003) (Figure 1.2).

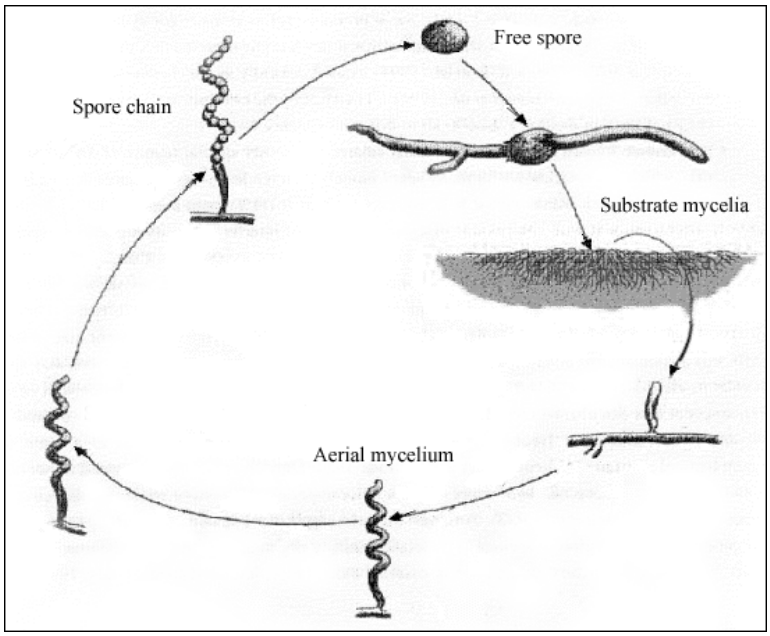


**Figure 1.2.** The life cycle of *Streptomyces coelicolor.* Modified from Kieser *et al.*, 2000.

*Streptomyces clavuligerus* is an actinomycete well known for its ability to produce a variety of β-lactam antibiotics. Of these, isopenicillin N, desacetoxycephalosporin C, and cephamycin C are all derived from the cephamycin C pathway. The same organism produces another group of structurally related β-lactam compounds, the clavams (Figure 1.3). The best known of these compounds, clavulanic acid, possesses weak antibiotic activity but is a very potent β-lactamase inhibitor. Because of its β-lactamase inhibitory properties, clavulanic acid is used clinically in combination with conventional β-lactam antibiotics to treat infections caused by bacteria that would otherwise be resistant to these antibiotics (Paradkar *et al*., 2001). The organism also produces a β-lactamase that is sensitive to clavulanic acid, a β-lactamase inhibitory protein (BLIP), and a BLIP-homologous protein (BLP) (Santamarta *et al*., 2002).

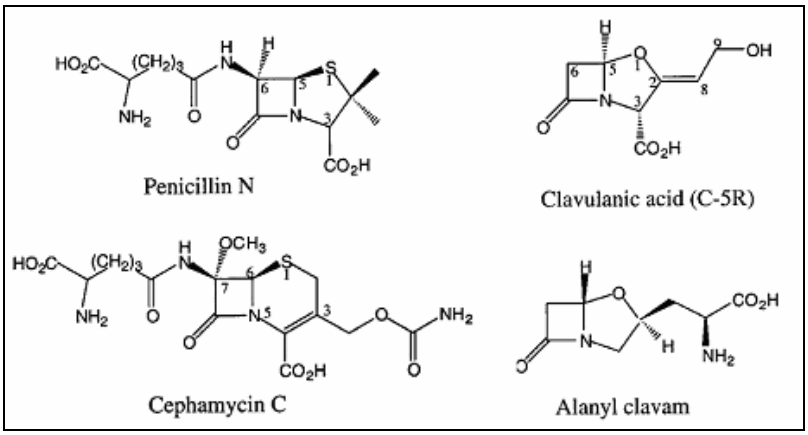


**Figure 1.3**. Some β-lactam products of *S. clavuligerus* (Thai *et al*., 2001).

## 1.2. Cephamycin C as a β-lactam antibiotic and its biosynthesis

The discovery and development of β-lactam antibiotics are among the most powerful and successful achievements in modern science and technology (Demain and Elander, 1999). There are several reasons for this. For one thing, penicillin, the first antibiotic isolated and characterized, is a β-lactam. Second, the inherent toxicity of β-lactam against higher animals is extremely low, yet they are often very effective at killing infecting bacterial pathogen. This is because they inhibit peptidoglycan (polymer unique to true bacteria) synthesis, a reaction that does no exist in the eukaryotic world. Consequently, they are used widely amounting to ca. 60% of the 30 billion dollar world-wide market for antibiotics used for the treatment of human diseases (Glazer and Nikaido, 1998; Demain, 2000). The β-lactam antibiotics include penicillins, cephalosporins, and cephamycins (Madigan *et al.,* 1997).

While β-lactam compounds were first discovered in filamentous fungi, actinomycetes and gram-negative bacteria are also known to produce different types of β-lactams (Liras and Martin, 2006). Bacteria synthesize a variety of β-lactam structures including cephalosporins (mainly as intermediates of biosynthetic pathways), cephamycins, cephabacins, crabaepenemsa and monobactams (Liras, 1999). Taken together, the main producers of penicillins,

cephalosporins and cephamycins are *Penicillium chrysogenum, Acremonium chrysogenum, Nocardia lactamdurans* and *S. clavuligerus.*

All β-lactam compounds contain a four-membered β-lactam ring. The structure of their second ring allows these compounds to be classified into penicillins, cephalosporins, clavams, carbapenems or monobactams. Most β-lactams inhibits bacterial cell wall biosynthesis, while others behave as β-lactamase inhibitors (e.g., clavulanic acid) and even as antifungal agents (e.g. some clavams) (Liras and Martin, 2006).

As mentioned before, the β-lactam antibiotics are potent inhibitors of cell wall synthesis. Peptidoglycan which is primarily responsible for the strength of the wall, is a thin sheet composed of two sugar derivatives, *N*-acetylglucosamine and *N*-acetylmuramic acid, and a small group of amino acids consisting of L-alanine, D-alanine, D-glutamic acid, and either lysine or diaminopimelic acid (DAP). The final stage of cell wall synthesis is the formation of the peptide cross-links between adjacent glycan chains. The formation of peptide cross-links involves an unusual type of peptide bond formation, in a reaction called transpeptidation. The enzymes that accomplish this task, the transpeptidases, are also capable of binding to penicillin or other antibiotics with β-lactam ring. Thus, these transpeptidases are also known as PBP (penicillin binding proteins) (Madigan *et al.,* 1997). Two of the PBP activities include those of the DD-transpeptidase and DD-carboxypeptidase. These enzyme activities utilize an active-site serine strategy in their mechanisms. β-lactam antibiotics also acylate the active site serine of the enzymes. However, in contrast to the case of the peptidoglycan that acylates the active site and allows the terminal D-Ala to serve as the leaving group, acylation of the active site by β-lactam leaves the "leaving group" covalently tethered to the "acyl-enzyme" species. Hence the enzyme is essentially irreversible inactivated, which forms the basis for how β-lactam antibiotics kill bacteria (Lee *et al.,* 2003).

Cephamycins are 7-methoxy-cephalosporins and called second-generation cephalosporins. Methoxylation of cephalosporins increases their inhibitory effect on transpeptidases involved in cell wall synthesis, reduces inactivation by β-

lactamases and increases their activity against gram-negative and anaerobic pathogens, thus making cephamycin C an important clinical antibiotic. Also promising is the fact that they have an extremely low toxicity compared to other antibiotics. As a representative of cephamycins, cephamycin C exhibits activity against many penicillin- and cephalosporin-resistant bacteria (Stapley *et al*., 1979; Glazer and Nikaido, 1998).

In the first steps of cephamycin C biosynthesis, L-lysine is converted to α-aminoadipic acid (α-AAA) through a two step conversion (Kern *et al*., 1980). The deamination of lysine by lysine-6-aminotransferase (LAT) to form 1-piperidiene-6-carboxylate marks the first committed step of cephamycin biosynthesis in *S. clavuligerus*. The second step is mediated by piperideine-6-carboxylate dehydrogenase (PCD) which converts 1-piperideine-6-carboxylate to α-aminoadipic acid (Khetan *et al.*, 1999). Cephamycins are formed in a biosynthetic pathway that includes two steps also involved in penicillin formation (early steps), three steps common with cephalosporin C biosynthesis (intermediate steps) and specific steps for cephamycin C biosynthesis (late steps) (Liras, 1999).

The basic structure of the β-lactam nucleus is formed by condensation of the three precursor amino acids L-α-aminoadipic acid (α-AAA), L-cysteine and L-valine by a mechanism designated as "non-ribosomal peptide synthesis", which involves activation and condensation of the three component amino acids and epimerization of the L- to D-valine to form the tri-peptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). α-AAA is a rare amino acid, hence it can not be incorporated into proteins by the standard ribosomal peptide synthesis (Martin, 1998).

### 1.3. Aspartate pathway

In most bacteria, amino acids of the aspartate family, such as lysine, methionine and threonine, are produced from aspartate by a series of enzymatic steps. Collectively, these pathways constitute the so-called aspartate pathway, a branched pathway whose common steps are regulated to allow the balanced synthesis of the various end products (Zhang *et al.,* 1999) (Figure 1.4).

The primary metabolic pathways which provide substrates for the antibiotic-specific synthetases are principal targets for regulatory mechanisms in controlling antibiotic biosynthesis. In the case of *S. clavuligerus*, the aspartic acid pathway of amino acids might serve as such a target. This pathway (i) provides the α-aminoadipate side chain of cephamycin C, (ii) provides the methyl group at the C-7 position of the antibiotic, (iii) involves cysteine as an intermediate, and (iv) shares common enzymes with the valine biosynthetic pathway; both cysteine and valine moieties are part of the cephamycin molecule. When such a link exists between primary metabolism and antibiotic metabolism, it has been suggested that mechanisms controlling the supply of the required substrates for antibiotic biosynthesis may affect the ability of the cell to produce antibiotics (Aharonowitz *et al*., 1984).

Mendelovitz and Aharonowitz (1982) studied the effect of the cephalosporin precursors and amino acids as well as the regulation of the activity and biosynthesis of three key enzymes in the aspartate pathway on antibiotic production by *S. clavuligerus*. The results suggested that the carbon flow through the lysine-specific branch of the aspartate pathway is a rate-limiting step in the formation of cephamycin C.

### 1.4. Regulation of aspartokinase enzyme

Bacterial biosynthesis of the lysine, methionine and threonine differs from most other pathways of amino acid biosynthesis in that one of the intermediates, diaminopimelate (DAP), has an important metabolic function in its own right as a constituent of the bacterial cell wall peptidoglycan. To ensure that the amino acids produced do not interfere with cell wall biosynthesis, a complex regulatory system is installed in this pathway. Several distinct genomic organizations and a diversity of regulatory mechanisms controlling the metabolic flux through this multi-branched pathway have been identified in bacteria (Zhang *et al.,* 1999).

Since several branch points exist in the aspartate pathway, the flow of carbon from aspartate through lysine to the α-AAA side chain of the cephalosporin molecules

might be controlled by regulatory mechanisms operating at the initial and branching steps of the pathway (Mendelovitz and Aharonowitz, 1982). The first two enzymes in this pathway are aspartokinase (AK, EC.2.7.2.4), which catalyzes the phosphorylation of aspartic acid to produce L-aspartyl-β-phosphate, and aspartate semialdehyde dehydrogenase (Asd, EC.1.2.1.11), which reduces L-aspartyl-β-phosphate into L-aspartic-β-semialdehyde and catalyzes the intervening branch point reaction between the AK and the homoserine dehydrogenase reactions. One of these branches leads to the production of lysine and cell wall, another leads to methionine, threonine, and isoleucine. These two key enzymes in the aspartate pathway are subject to complex regulation by the end-product amino acids in order to ensure that the amino acids produced do not interfere with cell wall biosynthesis. *Ask* and *asd* genes have been found in one operon in most gram-positive microorganisms, such as mycobacteria, *Bacillus*, and corynebacteria (Zhang *et al.*, 2000) and in *S. clavuligerus* as shown by our laboratory (Tunca *et al.*, 2004).

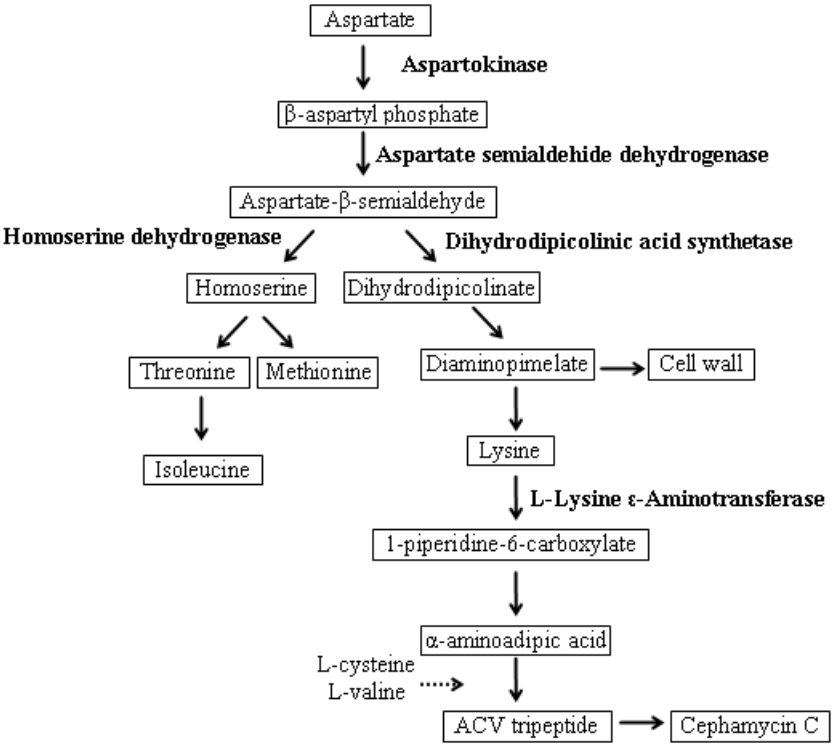


**Figure 1.4.** Biosynthetic pathway of the aspartate family amino acids and precursor flow to cephamycin C.

Because of the complexity of this pathway, different bacterial species have evolved diverse patterns of regulation of AK and aspartate semialdehyde dehydrogenase. For example, *Escherichia coli* has three separate AK isozymes, each controlled by one of the end products of the aspartate pathways, lysine, threonine, and methionine (Zhang *et al.*, 2000). *E. coli* has the following three AK isozymes: AKI-HDI (aspartokinase I-homoserine dehydrogenase I) is a bi-functional protein encoded by the *thrA* gene. It has both AK and homoserine dehydrogenase activities. It is inhibited allosterically by L-threonine and its synthesis is repressed by L-threonine and L-isoleucine. AKII-HDII is encoded by the *metL(M)* gene. It is also bi-functional and its synthesis is repressed by L-methionine. AKIII is encoded by the *lysC* gene, and has aspartokinase activity only. Its synthesis is repressed by L-lysine and its activity is inhibited by L-lysine. AKIII consists of two domains and, in view of its sequence similarity with other AKs, its N-terminal domain is considered to have catalytic activity and its C-terminal domain acts in the inhibitory effect L-lysine exerts on the enzyme (Ogawa-Miyata *et al.*, 2001). *Bacillus subtilis* also has three AKs: aspartokinase I is subject to feedback inhibition by *meso*-diaminopimelate, aspartokinase II is inhibited by L-lysine, while aspartokinase III is inhibited synergistically by L-lysine and L-threonine. The primary role of aspartokinase II and III is to provide precursors for protein synthesis, and aspartokinase I is the primary source of precursors for diaminopimelate and dipicolinate synthesis during sporulation. Only one AK is present in *Pseudomonas*, *Brevibacterium*, and *Corynebacterium* (Zhang *et al.*, 2000).

AK of *S. clavuligerus* and *A. lactamdurans* are feedback regulated by the concerted action of lysine and threonine (Mendelovitz and Aharonowitz, 1982; Hernándo-Rico *et al.*, 2001) (Table 1.2). Aharonowitz *et al.* (1984) demonstrated that aspartokinase deregulated mutants obtained by classical strain development methods (AEC-resistant strains), produced five times higher amount of cephamycin C than the wild type *S. clavuligerus*, accompanied with an increase in DAP accumulation (Mendelovitz and Aharonowitz, 1983; Aharonowitz *et. al.*, 1984). It is also known that the AK of L-lysine overproducing strains, for example

L-lysine analogue resistant *Corynebacterium,* is partially feedback resistant to L-lysine and L-threonine (Ogawa-Miyata *et al.*, 2001).

Tunca *et al*. (2004) cloned and characterized the *ask* gene coding for aspartokinase together with *asd* gene which encodes aspartate semialdehyde dehydrogenase in *S. clavuligerus* NRRL 3585. Also, in our laboratory, *ask* gene was introduced to *S. clavuligerus* in a multi-copy plasmid (Taşkın, 2005) and it was recently demonstrated that multi-copies of *ask* gene resulted in two- to three fold increase in cephamycin C specific production in chemically defined medium (unpublished).

**Table 1.2.** Effect of amino acids and AEC antimetabolite on AK activity. (Modified from Mendelovitz and Aharonowitz, 1983).

| **Additions (5 mM)** | **Relative enzyme activity %** |
|---|---|
| None | 100 |
| L-Threonine | 106 |
| L-Lysine | 140 |
| *S*-(2-aminoethyl)-L-cysteine (AEC) | 118 |
| L-Lysine + L-threonine (1 mM each) | 13 |
| L-Lysine + L-threonine (5 mM each) | 6 |
| AEC + L-threonine | 18 |

## 1.5. Deregulation as a strategy of overproduction in industrial microbiology

Although microorganisms are extremely good in presenting us with an amazing array of valuable products, they usually produce them only in amounts that they need for their own benefit; thus, they tend not to overproduce their metabolites. In strain improvement programs, a strain producing a high titer is usually the desired goal (Adrio and Demain, 2006).

Basic studies on regulation have shown that it is possible to select regulatory mutants which overproduce the end products of primary pathways, using toxic metabolite analogues. Such antimetabolite-resistant mutants often possess

enzymes that are insensitive to feedback inhibition, or enzyme-forming systems resistant to feedback repression. The selection of mutants resistant to toxic analogues of primary metabolites has been widely employed by industrial microbiologists (Adrio and Demain, 2006).

Table 1.3 shows many examples of overproducing microbial metabolites by making use of the antimetabolites. Deregulation of purine synthesis in mutants selected for resistance to analogs of purine was reported for *C. famata* and *B. subtilis* (Stahmann *et al.*, 2000). In addition, strains of *Arthrobacter, Bacillus, Aspergillus,* and *Corynebacterium* that overproduce amino acids, nucleotides and vitamins have been isolated in this manner. In some cases, the rationalized selective agent is biological rather than chemical. Resistance to actinophage has been used to isolate superior vancomycin-producing strains of *Amycolatopsis orientalis* (Parekh *et al.*, 2000).

### 1.6. This study

The purpose of the study was to obtain deregulated aspartokinase mutants of *S. clavuligerus* and to identify the point mutation(s) accounting for the resistance to feedback inhibition. For this aim, chemical mutagenesis was employed to increase spontaneous mutation rate in this organism. Subsequently, the bacterial population was screened for *S*-(2-aminoethyl)-L-cysteine resistance, which is a toxic lysine analogue, so-called antimetabolite, to find out deregulated mutants. One deregulated mutant was selected for the analysis of cephamycin C production. Attempts were also made to amplify the mutant *ask* gene from this organism in order to characterize the mutation accounting for resistance to concerted feedback inhibition.

**Table 1.3** Mutations leading to increased product formation. Modified from Adrio and Demain, 2006.

| **Organism** | **Mutant characteristics** | **Overproduced compound** | **Reference** |
|---|---|---|---|
| *Candida boidinii* | Resistance to ethionine | Methionine | Tani *et al*. (1988) |
| *Streptomyces clavuligerus* | Resistance to aminoehtyl cysteine | Cephamycins | Mendelovitz & Aharonowitz (1983) |
| *Streptomyces pilosus* | Resistance to thialysine | Desferrioxamine | Smith (1987) |
| *Streptomyces cinnamonensis* | Resistance to 2-ketobutyrate inpresence of valine or isoleucine | Monensins A & B | Pospisil *et al*. (1999) |
| *Actinoplanes teichomyceticus* | Resistance to valine hydroxamate | Teichoplanins | Wang *et al*. (1996); Jin *et al*. (2002a) |
| *Candida flareri* | Resistance to iron, to tubercidin,to 2-DOG | Riboflavin | Stahmann *et al*. (2000) |
| *Ashbya gossypii* | Resistance to itaconic acid andaminomethylphosphinic acid | Riboflavin | Stahmann *et al*. (2000) |
| *Penicillium chrysogenum* | Resistance to phenylacetic acid(precursor) | Penicillin G | Barrios-Gonzalez *et al*. (1993) |
| *Amycolatopsis mediterranei* | Sequential resistance to tryptophan(feedback inhibitor), phydroxybenzoate,and propionate(precursor) | Rifamycin B | Jin *et al*. (2002) |

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Bacterial strains and plasmid vectors

The characteristics and sources of bacterial strains used in this study are given in Table 2.1. Spore suspensions of *S. clavuligerus* strains were prepared in 20% glycerol according to the protocol described by Hopwood *et al.* (1985) and stored at -80 °C. Other microorganisms were streaked onto Nutrient Agar (Appendix A), grown at 37 °C and sub-cultured monthly. These cultures were also stored at -80 °C in Nutrient Broth (Appendix A) covered with 15% glycerol. The *E.coli* plasmids were pGEM-T (Figure 2.1) vector and the recombinant plasmid pNST102 (Figure 2.2) (Table 2.1).

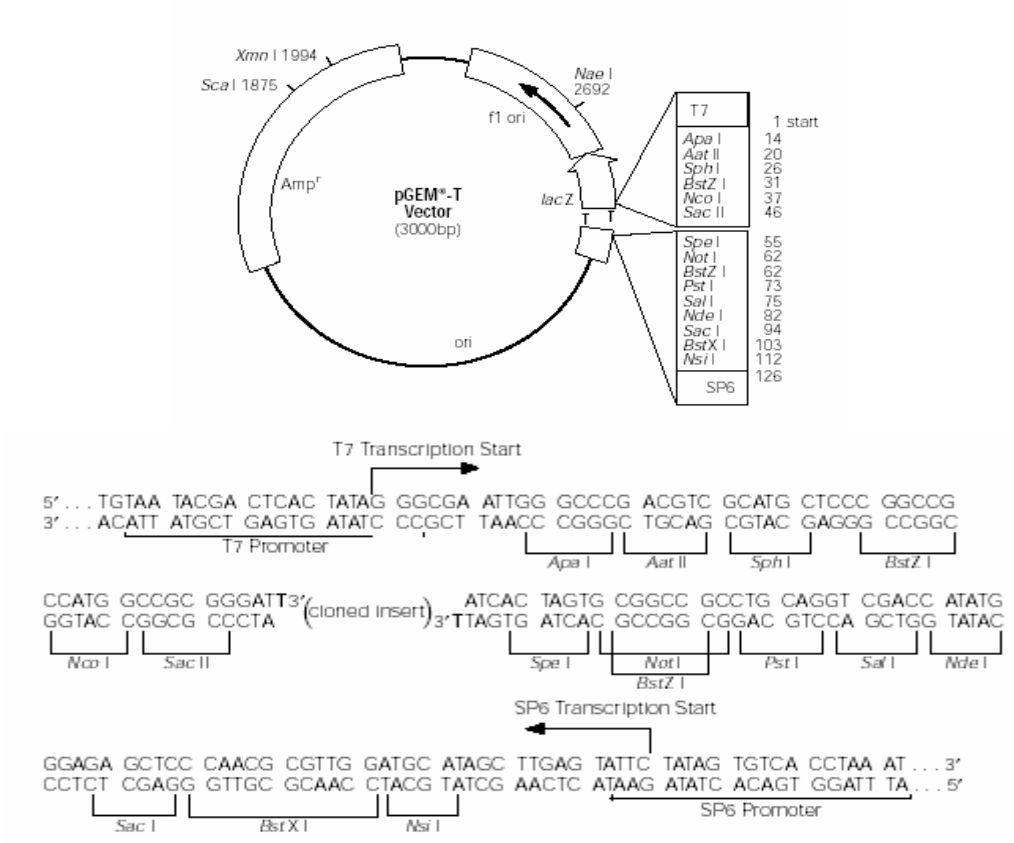


**Figure 2.1.** Map and MCS sequence of the pGEM-T vector

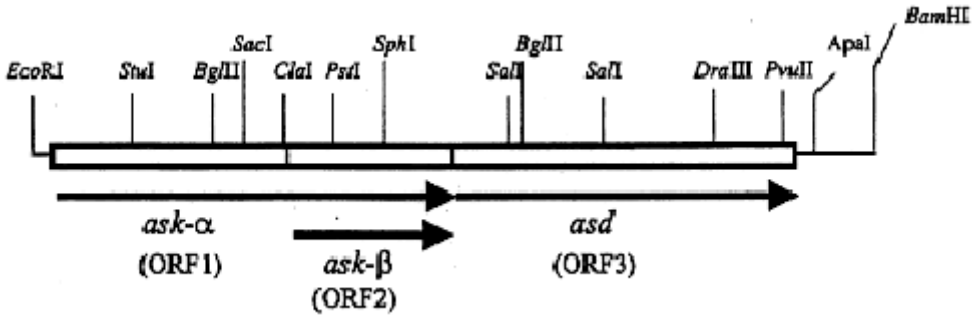


**Figure 2.2.** Map of selected restriction sites in the *S. clavuligerus ask-asd* fragment cloned in pNST102. The relative position and orientation of ORFs are indicated by bold arrows. Modified from Tunca *et al*., 2004

**Table 2.1** List of bacterial strains and plasmids

| **Strains** | **Genotype / Phenotype** | **Source / Reference** |
|---|---|---|
| *S. clavuligerus* NRRL 3585 | Prototroph | Prof. J. Piret, Northeastern University, USA (Higgins and Kastner, 1971) |
| *E. coli ESS* | β-lactam supersensitive *E. coli* strain | Prof. J. Piret, Northeastern University, USA |
| *E. coli* DH5α | F` ϕ80d*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 *sup*E44λ- *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17 | *E. coli* Genetic Stock Center |
| **Plasmids** | | |
| pNST102 | pBluescript II KS + 3.5 kb *Bam*HI-*Bam*HI *S. clavuligerus ask-asd* cluster | Tunca *et al*., 2004 |
| pGEM-T | Amp$^r$ *lacZ*' | Promega |

## 2.2. Culture media

The composition and preparation of culture media are listed in Appendix A.

## 2.3. Buffers and solutions

Buffers, solutions, their composition and preparation are listed in Appendix B.

## 2.4. Chemicals and enzymes

The chemicals and enzymes used as well as their suppliers are listed in Appendix C.

## 2.5. Chemical mutagenesis and screening

Chemical mutagenesis of *S. clavuligerus* NRRL 3585 was accomplished by using the protocol in Mendelovitz and Aharonowitz (1983). *S. clavuligerus* spores stored at -80 °C were suspended in 1 mL 50 mM Tris/HCl buffer (Appendix B) containing 0.01% (v/v) Triton X-100. MNNG was added to a final concentration of 0.5 mg/mL. The mixture was agitated momentarily and incubated in a gyrotory shaker at 30 °C, 300 rpm for one hour. At the end of the incubation period, samples were washed by centrifugation and spread on agar plates containing minimal medium (Appendix A) and AEC at a concentration of 1 mg/mL. Colonies developed within one week were isolated, re-streaked on minimal medium and tested for resistance to AEC.

## 2.6. Preparation of *E.coli* competent cells

*E.coli* competent cells were prepared according to the protocol described by Sambrook *et al.* (1989) with slight modifications. 50 mL of LB broth (Appendix A) was inoculated with *E.coli* from fresh LB agar plate in a 250 mL flask and incubated overnight with shaking at 37 °C to obtain stationary phase culture. 300 µL from this seed culture was inoculated into another flask containing 50 mL LB broth. The culture was incubated for 2-2.5 hours at 37 °C with vigorous shaking around 300 cycles/min in an orbital shaker to obtain an exponentially growing culture. Then the culture was aseptically split into two sterile pre-chilled 40 mL screw-cap centrifuge tubes and stored on ice for 10 min. Next, cells were spun at 4000 rpm for 10 minutes at 4 °C in a Sorvall SS-34 rotor. After centrifugation, supernatants were decanted and each pellet was re-suspended in 5 mL ice-cold 10 mM $CaCl_2$ solution by vortexing. The cells were spun down at 3000 rpm for 10 minutes at 4 °C. Lastly, fluid from cell pellets was decanted and each pellet was re-suspended very gently in 1 mL ice-cold 75 mM $CaCl_2$ solution. The obtained competent cells were stored at -80 °C.

## 2.7. Transformation of *E. coli* competent cells

Competent *E. coli* cells were transformed as described by Sambrook *et al*. (1989) with slight modifications. 100 µL of *E. coli* competent cells were transferred to a pre-chilled microfuge tube. Appropriate amount of DNA (1-50 ng) in a maximum volume of 10 µL was added and mixed gently by pipetting. The tubes were left on ice for 30 minutes. Then, the tubes were placed in a 42 ˚C water bath and heat-pulsed for 90 seconds. Next, the tubes were immediately put on ice for 2 minutes. 900 µL of LB broth was added to each tube and the cultures were incubated for 1.5 hours at 37 ˚C with gentle shaking (180-200 rpm). Finally, 100 µL of transformed competent cells were spread onto selective LB agar plate and incubated overnight at 37 ˚C.

## 2.8. Plasmid isolation from *E. coli*

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were used for isolation of *E. coli* plasmid DNA as specified by the manufacturer.

*E. coli* plasmid DNA was also prepared by using the plasmid mini-preps method described by Hopwood *et al.* (1985). Each strain was grown as patches on selective medium, LB agar containing 100 µg/mL ampicillin. About 1 square cm of cell mass was scraped with a sterile toothpick and put into microfuge tube containing 100 µL cold TSE solution containing 2 mg/mL lysozyme (Appendix B). Each tube was mixed by vortexing to disperse the cells and the toothpick was discarded. Then, the tubes were incubated on ice for 20 minutes. 3/5 volume of lysis solution (Appendix B) was added to each tube and vortexed immediately. The mixture was incubated at room temperature for 10 minutes to lyse the cells and then at 70 ˚C for 10 minutes to denature DNA. Subsequently the tubes were cooled rapidly in ice. An equal amount of phenol-chloroform solution was added (water-saturated, Appendix B), vortexed harshly until a homogeneous and milky white mixture was obtained. Finally, the samples were spun for 5 minutes at

13,000 rpm to separate phases. 10 µL of supernatant was run on agarose gel to assess the yield.

### 2.9. Restriction endonuclease digestions

Restriction enzyme digestions were performed under the conditions specified by the manufacturers.

### 2.10. Agarose gel electrophoresis

Agarose gels were used for DNA analysis at a concentration of 0.9 %. The final concentration of Ethidium Bromide in the gels was 0.5 µg/mL. Gels were run for 1-1.5 hours at 90 Volts. The electrophoresis buffer used was 1X TAE (Appendix B). The DNA bands were visualized on a UV transilluminator (UVP) and photographed by using Vilber Lourmat Gel Imaging System. *Pst*I digested λ DNA and GeneRuler™ 100 bp DNA ladder plus DNA size markers (Appendix C) were used to determine the molecular weights of DNA bands.

### 2.11. Extraction of DNA fragments from gels

The desired fragments were extracted from the gel by using a Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA). The gel slice containing the DNA band was excised from the gel and weighed. Gel extraction was performed according to the manufacturer's instructions. After recovery, an aliquot was run on agarose gel to assess the yield.

### 2.12. Ligations

Ligation reaction of PCR products with pGEM-T vector was performed as follows: 1µL 10X ligase buffer, 1µL (55ng/µL) pGEM-T vector, 500 ng insert DNA, 1µL T4 DNA ligase (3 unit/µL) were mixed and volume was completed to 10 µL with distilled water. The reaction was carried out at 4 $^{o}$C for overnight.

## 2.13. Primer design

PCR primers given in Table 2.2 were designed according to nucleotide sequence of *S. clavuligerus ask-asd* operon (Tunca *et al.* 2004), with the Primer3 online primer design tool (Rozen and Skaletsky, 2000). The primers were synthesized by the Iontek company (Istanbul, Turkey).

**Table 2.2.** PCR primers

| **Name of the primer** | **Sequence of the primer** |
|---|---|
| ask-fwd-00 | 5'- TTCTAGAGTTCGTCCGGCTGCCGGT - 3' |
| ask-rev-00 | 5'- GGATATCCTACCGCCCACTTCCCGA - 3' |
| ask-internal-01 | 5'- GACCGGATCGATCGAGGAGATC - 3' |
| ask-ir-01 | 5'- CCGGGGTGCGAGCGCATGCCG - 3' |
| ask-ir-02 | 5'- ACGTTCTGCAGCACCATGTCG - 3' |
| ask-fwd-01 | 5'- CCTAGTCGAGCAGGCCATTCG - 3' |
| ask-rev-01 | 5'- ACCGACCAGAGCCAGAACAGG -3' |

## 2.14. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in a total volume of 50 and 25 µL. Reaction mixture components were as follows: 1X PCR buffer with $(NH_4)_2SO_4$ and without $Mg^{2+}$ (MBI Fermentas); 3-5 mM $MgCl_2$ solution (MBI Fermentas); primers (1 µM of each); 0.2 mM dNTP mix (MBI Fermentas); 4% DMSO; 1 ng of plasmid DNA or 200-400 ng of chromosomal DNA and 1 Unit of Taq Polymerase (MBI Fermentas). PCR mixture was completed to the appropriate volume with ultra pure water. The PCR amplification condition was as following: 95 $^{o}$C (initial denaturation, 5 min) and 35 cycles of 95 $^{o}$C (denaturation, 1 min), 55-65 $^{o}$C (annealing, 1 min) and 72 $^{o}$C (elongation, 2 min) with 5 min final elongation at the same temperature.

## 2.15. Sample collection for growth determination, cephamycin C bioassay and AK activity measurements

3 mL of *S. clavuligerus* mycelium stock was inoculated into 250 mL triple-baffled flasks containing 50 mL of chemically defined medium (Appendix A). After 24 hours of incubation at 28 ˚C with continuous shaking (220 rpm), optical density ($OD_{595}$) of the suspension of broken mycelia reached 0.2-0.3. 15 mL of this seed culture was inoculated into 250 mL baffled flasks containing 50 mL of chemically defined medium. Six such cultures were started with an initial $OD_{595}$ of 0.030-0.035. Incubation was performed in an orbital incubator at 220 rpm for 120 hours. Samples were taken at the 24, 48, 60, 72, 96 and 120 hours of fermentation. At each given specific hour of fermentation, one of the flasks was taken and samples were collected for optical density, dry cell weight, cephamycin C bioassay and AK activity measurements.

## 2.16. Determination of cultural growth

Cultural growth was monitored by measurement of both optical density (OD) at 595 nm and dry cell weight (DCW). OD measurements were done as indicated by Malmberg *et. al*. (1993). A total of 0.5 mL of the culture was added to a tube containing 0.5 mL of 2.5 M HCl and 3 mL of distilled water. The mixture was homogenized by ultrasonification (Cole Parmer) at 11 Volts for 3 X 10 seconds. The $OD_{595}$ of the resulting suspension was then measured. The cultures were diluted appropriately if the $OD_{595}$ was higher than 0.8. For DCW determinations 5 mL of culture was passed through Whatman cellulose nitrate membrane filters (0.2 μm pore size) by vacuum application. Filters were dried at 80 $^{o}$C for 24 hours and weighed. Three independent measurements were done for both OD and DCW determinations and their average was used in further calculations.

## 2.17. Cephamycin C bioassay

Cephamycin C involved the use of the agar-diffusion method with *E. coli* ESS (a β-lactam supersensitive mutant) as the indicator organism (Aharonowitz and Demain, 1978). For *E. coli* ESS glycerol stock preparation, a 2 mL of *E. coli* ESS overnight culture was centrifuged at 7,000 g for 7-8 minutes and supernatant was discarded. Pellet was re-suspended in 500 µL 50% glycerol and stored at -80 $^{o}$C. 10 mL of soft SNA agar (NB + 0.5% (w/v) agar) (Appendix A) containing 100 µL *E. coli* ESS glycerol stock was poured into a bioassay plate containing 15 mL NB agar (NB + 1.5% (w/v) agar) (Appendix A). Bioassay disks (Oxoid, England) were placed on the surface of a sterile, empty petri plate cover. 20 µL of culture supernatant was impregnated into the disks, 10 µL at a time. 5 µL of acetone was next put on to the disks. After the soft agar was solidified, the disks were placed on the agar surface and plates were incubated overnight at 37 ˚C. Zones of inhibition of growth were measured following the overnight incubation. Cephamycin C production of both the parental and mutant *S. clavuligerus* cells was determined by using the standard curve constructed with cephalosporin C (CPC) as standard (Figure 2.3). One unit of β-lactam produced an inhibition zone equivalent to that formed by 1 g cephalosporin C.

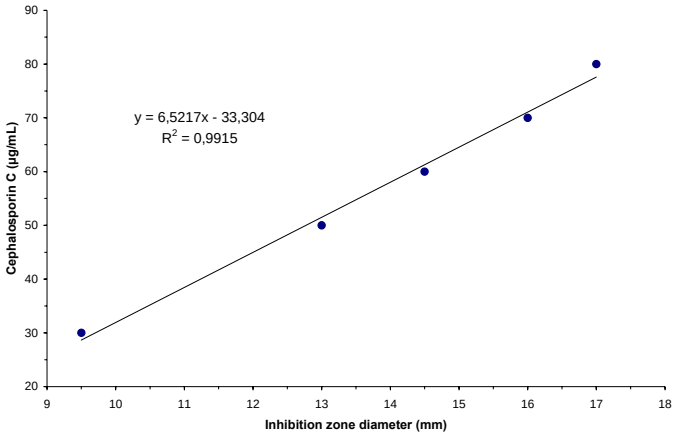


**Figure 2.3.** Cephamycin C bioassay calibration curve

## 2.18. Measurement of aspartokinase activity

Measurements of aspartokinase (AK) activity were performed according to the procedure of Follettie *et al.* (1993) with minor changes. Both the parental and mutant *Streptomyces clavuligerus* cells were grown in 50 mL Trypticase Soy Broth (TSB) medium (Appendix A) at 28 °C for 30 and 48 hours and harvested by centrifugation (8,000 rpm, 15 min, 4 °C) in a Sigma centrifuge. After the cells were washed with 25 mL of 50 mM cold phosphate buffer (Appendix B), crude extracts were prepared by resuspending the cells in 5 mL sonication buffer (Appendix B). For disruption, cells were sonicated for 3 min with 1 min intervals (10 W with 50% pulsing). The cell debris was subsequently removed by centrifugation. AK activity was measured by determining the rate of the formation of the stable aspartate-hydroxymate. Proteins were precipitated from the crude extract by the addition of 5 volumes of saturated ammonium sulfate, and precipitate was collected by centrifugation and resuspended in 1 mL of phosphate buffer (Appendix B). The assay mixture contained 100 mM HEPES (pH 7.8), 400 mM KCl, 12 mM $MgCl_2$, 500 mM hydroxylamine, 10 mM ATP, and 15 mM L-aspartate (pH adjusted to 7.6) in a total volume of 0.5 mL. Partially purified AK was added to the assay mixture and incubated for 60 min at 30 °C. The reaction was terminated by the addition of 0.75 mL stop solution (Appendix B). After centrifugation for 5 min to remove protein debris, $OD_{540}$ of the supernatant was measured. The OD values were translated to millimolars of aspartate-hydroxymate by using the $OD_{540}$ versus aspartate-hydroxymate (mM) standard curve given in Figure 2.4. Background activity measured in the absence of added substrate was subtracted and AK specific activity was reported as nanomoles of aspartylhydroxymate formed per milligram protein per minute.

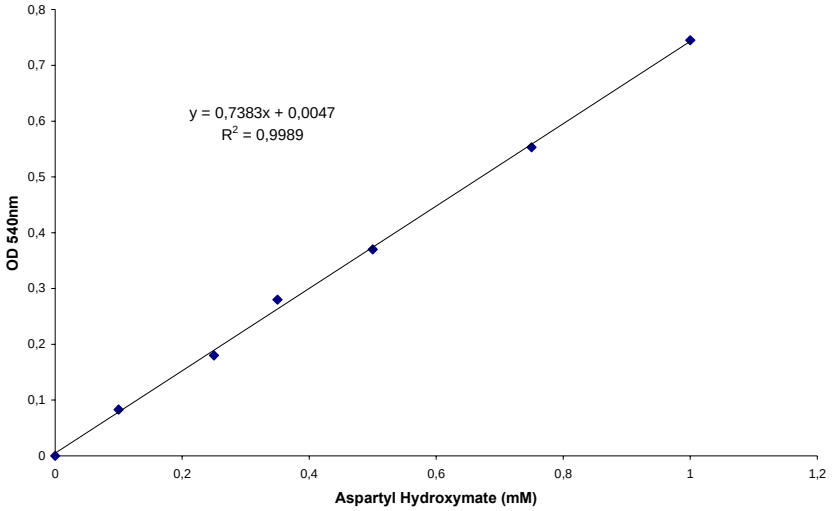


**Figure 2.4.** Absorbance as $OD_{540}$ versus aspartyl hydroxymate concentration (mM) calibration curve.

## 2.19. Determination of protein concentration

Protein concentrations were measured by the Bradford quantification method (Bradford, 1976) with bovine serum albumin (BSA) as the standard. Assay reagent was made by dissolving 100 mg of Coomassie Blue G250 in 50 mL of 95% ethanol. The solution was then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Watman No. 1 filter paper. Volumes of 40, 60, 80 and 100 µl of 1 mg/mL BSA were added to tubes and volumes were adjusted to 500 µL with distilled water. 500 µl of distilled water was added into a tube as reagent blank. 4.5 mL of the assay reagent was added to each tube and mixed gently, but thoroughly. A standard curve as OD at 595 nm versus protein concentration was prepared and the amounts of proteins were determined from the slope of the curve (Figure 2.5).

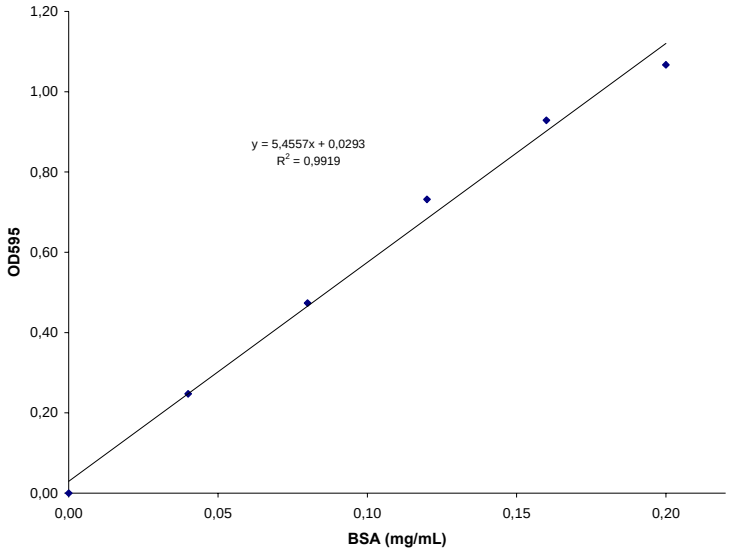


**Figure 2.5.** The calibration curve for protein content determination.

# CHAPTER 3

# RESULT AND DISCUSSION

## 3.1. Chemical mutagenesis in *S. clavuligerus* and screening for AEC-resistant mutants

In order to achieve a high mutation rate and thus increase the probability of finding bacteria possessing AEC-resistant phenotype amongst the whole population of screened cells, chemical mutagenesis was employed. For this purpose, a potent mutagenic agent, namely *N'*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (MNNG, NTG) was used. *S. clavuligerus* NRRL3585 spore suspension was exposed to MNNG aqueous solution for 30 minutes with continuous shaking. Subsequently, the cells were collected and resuspended in distilled water. Equal volumes were spread on petri dishes containing minimal medium with and without AEC (1 mg/mL). In parallel, the same procedure was applied on another sample of spore suspension, this time without the mutagenic agent as a control group.

After about one week of incubation at 30 °C, the first colonies were observed in AEC containing plates. 100 colonies were designated as potential AEC resistant strains and numbered. The selected colonies were re-streaked on AEC containing solid minimal media by using sterile toothpicks. A single plate was divided into 10-20 squares and each colony was streaked into a different region. The colonies that grew best were chosen as they were expected to have better AEC resistance. Consequently, 17 mutant strains were obtained and cultivated in liquid medium for preservation and further analysis. Figure 3.1 compares the growth of the parental strain on solid medium with a mutant strain (no. 84). Growth of the parental strain

on minimal medium containing AEC was considerably inhibited, whereas the mutant strain grew much better.

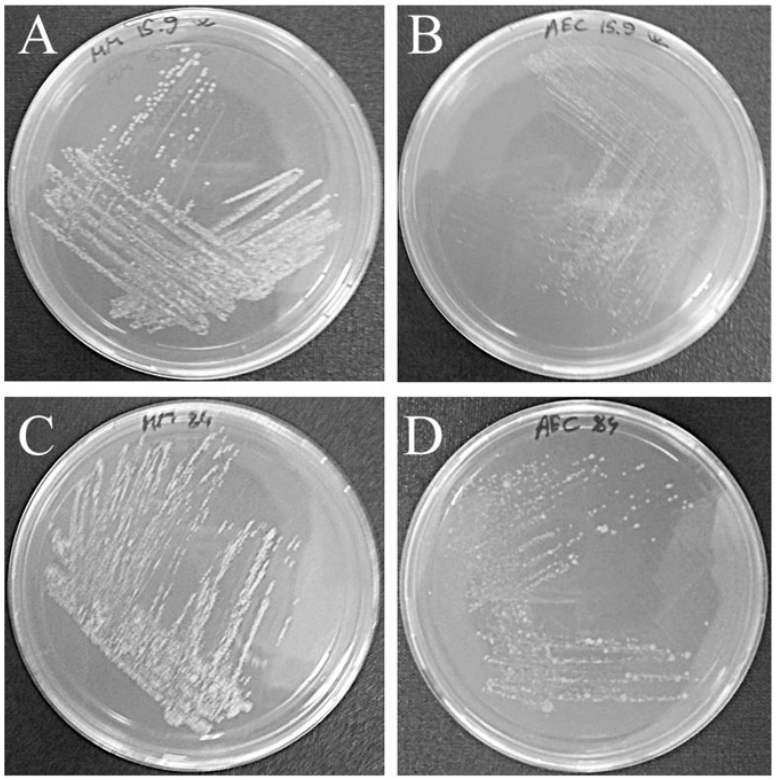


**Figure 3.1** Comparison of the growth of the parental strain and one mutant strain (no. 84) on minimal media. A: Parental strain *S. clavuligerus* on minimal medium, B: Parental strain *S. clavuligerus* on minimal medium containing AEC, C: Mutant strain on minimal medium, D: Mutant strain on minimal medium containing AEC.

### 3.2. Detection of aspartokinase deregulation via enzyme assay

Several genotypes could explain the AEC-resistant phenotype. In addition to the mutation at the regulatory regions of *ask* gene, resistance to the anti-metabolite might also be due to chemical modification of AEC or modification of the structure of the protein originally responsible for AEC transport into the cells (Mendelovitz and Aharonowitz, 1983). Since we were interested in mutations corresponding to the allosteric site of AK, the mutants were screened for their aspartokinase activity and the inhibition pattern in the presence of feedback inhibitors which are lysine plus threonine.

Table 3.1 shows specific and relative aspartokinase activities of the mutants as well as the parental type in the absence and presence of lysine and threonine. The mutant no. 84 had the highest insensitivity to the concerted feedback inhibition.

### 3.3. The effect of aspartokinase deregulation on cephamycin C production

In view of the results of aspartokinase activity determinations in the presence and absence of allosteric inhibitors, AEC-resistant mutant no. 84 was further analyzed for cephamycin C production. Growth and cephamycin C production curves were constructed both for the parental and mutant strains. Their growth was compared in terms of their dry cell weight (DCW) as a function of time. Cephamycin C specific production was reported as μU cephamycin C formed per mg of dry cell weight. Comparisons of the maximum cephamycin C specific production by the parental and mutant strain revealed that the mutant no. 84 produced 2 fold more antibiotic (Figure 3.2).

Using the same strategy, Mendelovitz and Aharonowitz (1983) had found that about 50% of the AEC-resistant strains overproduce cephamycin C in 2- to 7-fold increased levels. In this respect, our result that AEC-resistant mutant no. 84 overproduces the antibiotic by 2-fold is consistent with their finding.

**Table 3.1.** Specific and relative activity of the aspartokinase enzymes of the parental and mutant strains in the absence and presence of the feedback inhibitors. Specific activities are expressed in terms of nanomoles of L-aspartyl-β-hydroxamate formed per minute per mg protein and the corresponding relative activities are given in parentheses, expressed as activity in percentage in relation to the activity detected when no amino acids were added. ND: not determined.

| Strain | Aspartokinase activity in the presence of: | | | |
|---|---|---|---|---|
| | No addition | L-Lysine (8 mM) | L-Lysine and L-Threonine (each 0.4 mM) | L-Lysine and L-Threonine (each 8 mM) |
| Parental strain | 1.78 (100) | 2.65(149) | 1.13 (63) | 0.21(12) |
| Mutant no. 1 | 0.93 (100) | 1.33 (143) | 0.34 (37) | ND |
| Mutant no. 3 | 1.06 (100) | 1.14 (108) | 0.22 (21) | ND |
| Mutant no. 4 | 0.69 (100) | 0.92 (134) | 0.66 (96) | ND |
| Mutant no. 7 | 0.76 (100) | 0.85 (112) | 0.46 (61) | ND |
| Mutant no. 8 | 0.69 (100) | 0.86 (124) | 0.64 (93) | ND |
| Mutant no. 9 | 0.71 (100) | 0.89 (126) | 0.63 (89) | ND |
| Mutant no. 15 | 0.57 (100) | 0.76 (132) | 0.51 (90) | ND |
| Mutant no. 16 | 0.58 (100) | 0.70 (122) | 0.50 (87) | ND |
| Mutant no. 26 | 0.48 (100) | 0.58 (120) | 0.44 (92) | ND |
| Mutant no. 47 | 3.51 (100) | 4.83 (137) | 1.82 (52) | 0.73 (21) |
| Mutant no. 62 | 0.53 (100) | 0.71 (134) | 0.48 (90) | 0.18 (34) |
| Mutant no. 63 | 1.60 (100) | 2.44 (152) | 1.15 (72) | 0.34 (21) |
| Mutant no. 66 | 1.95 (100) | 2.72 (139) | 1.19 (61) | 0.24 (12) |
| Mutant no. 67 | 1.55 (100) | 2.35 (151) | 0.99 (61) | 0.28 (18) |
| Mutant no. 73 | 1.69 (100) | 2.39 (141) | 0.97 (57) | 0.25 (15) |
| Mutant no. 74 | 1.86 (100) | 2.48 (133) | 1.13 (61) | 0.39 (21) |
| Mutant no. 84 | 0.95 (100) | 1.22 (129) | 1.10 (116) | 0.78 (83) |

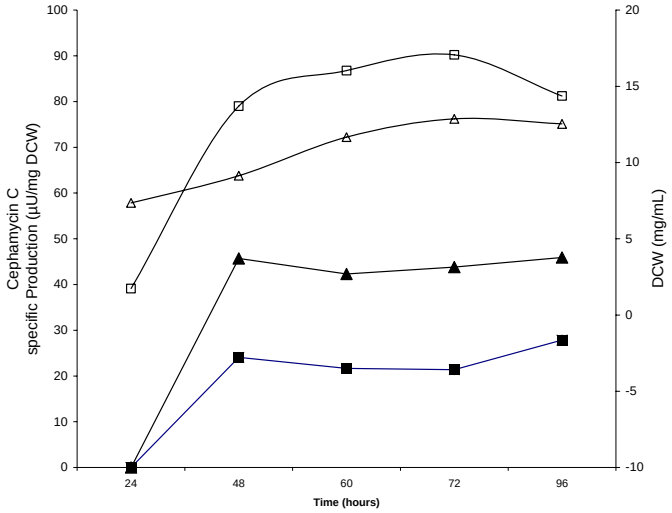


**Figure 3.2.** Growth and specific cephamycin C production by the parental and the mutant strain no 84. Open symbols represent dry cell weight (DCW) and closed ones represent specific cephamycin C production by the parental strain (squares) and the mutant no. 84 (triangles).

### 3.4. Amplification of *ask* gene from chromosomal DNA of the mutant strain

The ultimate aim of the study was to determine the mutation(s) in the *ask* gene that are responsible for the insensitivity of aspartokinase enzyme to the allosteric inhibitors lysine plus threonine. Accordingly, *ask* gene was tried to be amplified using polymerase chain reaction followed by cloning and DNA sequencing.

First attempt was the amplification of the *ask* gene with the ask-fwd-00 and ask-rev-00 primers (Figure 3.3) which were originally designed by Taşkın (2005). The primers possessed restriction sites at their 5' ends that are not complementary to the *ask* gene sequence. Since primers were long (25 bp), the presence of these restriction site sequences at the ends of the primers was not expected to decrease the specificity in hybridization.

Initial attempts to amplify the aspartokinase gene using chromosomal DNA of *S. clavuligerus* under the conditions routinely used in our laboratory for the amplification of the gene from the recombinant plasmid pNST102 yielded only a

little amount of the product which was not sufficient for sequencing. Optimization of PCR conditions to get sufficient amounts of product that is the band corresponding to 1609 bp, included testing of various $Mg^{2+}$, DMSO and template concentrations as well as temperature and duration of annealing and denaturation steps. In Figure 3.4, 3.5 and 3.6 some examples of the optimization results are presented.

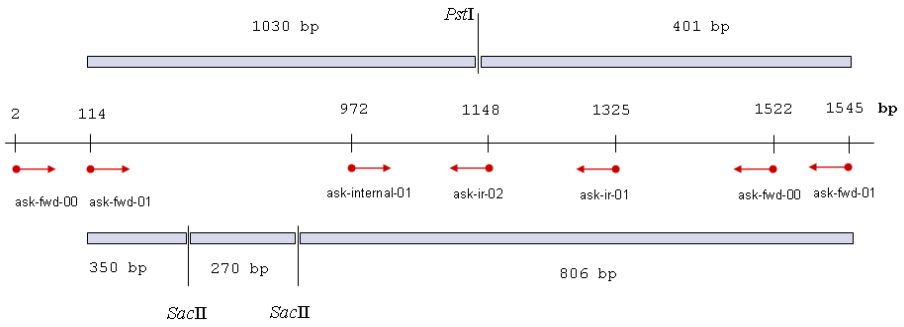


**Figure 3.3.** Positions of the primers and restriction enzyme cutting sites on *ask* gene.

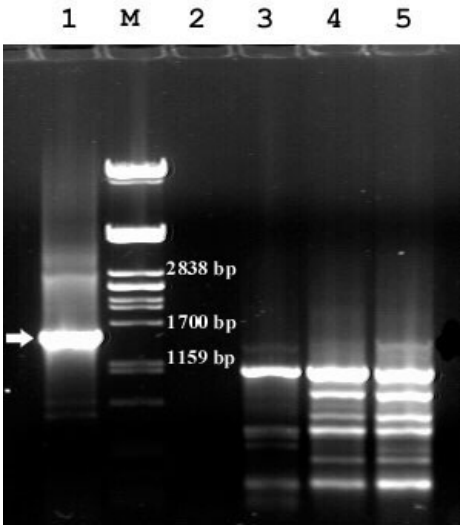


**Figure 3.4.** Effect of $Mg^{2+}$ concentration on PCR amplification of the *ask* gene using chromosomal DNA of the mutant no. 84 as the template. The primer pair employed was ask-fwd-00/ask-rev-00. The enzyme was Taq Polymerase (MBI Fermentas) and the annealing temperature was 60 °C. Lane 1: pNST102 as the template as a positive control; Lanes 2-5: $Mg^{2+}$ concentrations of 0, 1.5, 3.0 and 5.0 mM, respectively; M: *Pst*I digested/λ DNA size marker. 1609 bp band corresponding to the *ask* gene amplified from pNST102 is indicated with the white arrow.

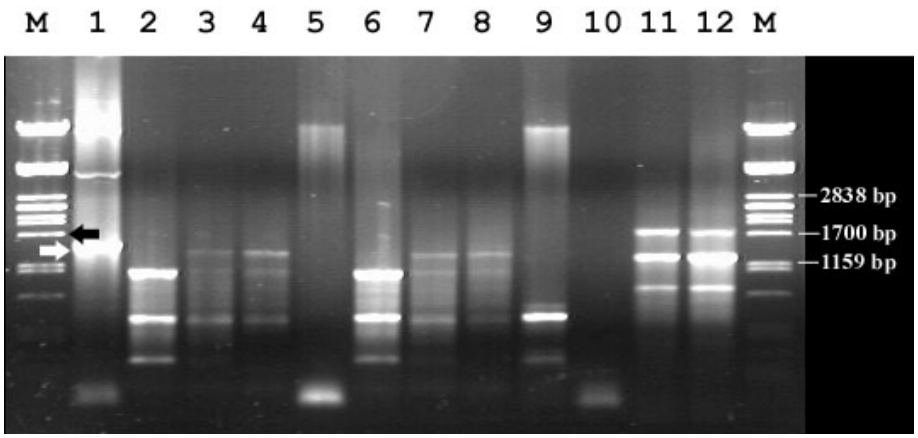


**Figure 3.5.** Effect of DMSO concentration on PCR amplification of the *ask* gene. The primer pair used was ask-fwd-00/ask-rev-00. The enzyme was Taq Polymerase (MBI Fermentas) and the annealing temperature was 63 °C. Lane 1: pNST102 as the template as a positive control; Lane 2: Parental chromosomal DNA as the template with 4% DMSO; Lane 3: Parental chromosomal DNA as the template with 10% DMSO; Lane 4: Parental chromosomal DNA as the template with 4% DMSO plus Q solution (Qiagen Kit); Lane 5: Parental chromosomal DNA as the template without DMSO; Lane 6-9: Mutant (no. 84) chromosomal DNA as the template in the same order; Lane 10: Negative control (PCR without template); Lane 11 and 12: PCR product using homoserine dehydrogenase gene (1.3 kb) primers in order to test the quality of the chromosomal DNA; M: *Pst*I digested/λ DNA size marker. 1700 bp DNA size marker is indicated with the black arrow and 1609 bp *ask* gene amplified from pNST102 with the white arrow.

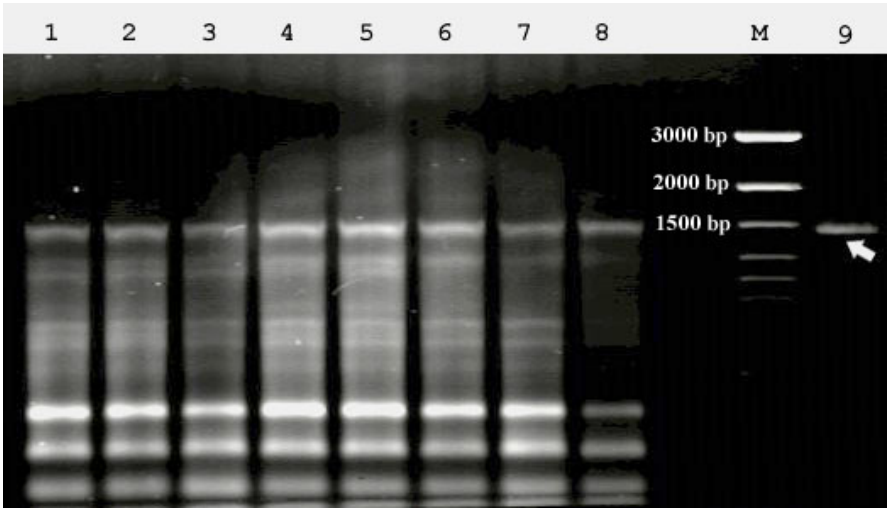


**Figure 3.6.** Effect of template DNA amount on PCR amplification of the *ask* gene. The primer pair used was ask-fwd-01/ask-rev-01. The enzyme was Taq Polymerase (MBI Fermentas) and the annealing temperature was 64 °C. Lane 1-8: Mutant no. 84 chromosomal DNA as the template in amounts of 210 (Lanes 1, 2), 150 (Lanes 3, 4), 120 (Lanes 5, 6) and 90 (Lanes 7, 8) ng, respectively. M: Gene-Ruler™ 100 bp DNA ladder

plus DNA size marker. Lane 9: pNST102 as the template as a positive control. The *ask* gene (1431 bp) amplified from pNST102 is indicated with white arrow.

In order to assess the identity of putative *ask* band and use it in DNA sequencing reactions, about 250 ng of DNA was needed. However, optimization of PCR conditions did not provide the fragment in a sufficient yield. The solution for this problem was the cloning of the fragment eluted from the gel into the pGEM-T vector. The PCR bands obtained by using the chromosomal DNA of the parental and mutant strains (no. 16 and no. 84) which possessed the same electrophoretic mobility with the *ask* amplicon obtained by using pNST102 as the template were cut and eluted from the agarose gel (Figure 3.6). After ligating the eluted DNA bands to the pGEM-T vector, *E. coli* competent cells were transformed with the resulting recombinant plasmids. The Figure 3.7 shows the result of plasmid isolation from the some *E. coli* recombinants carrying the putative *ask* amplicon obtained from chromosomal DNA of the mutant no. 84. Likewise, the putative *ask* amplicon obtained from mutant no. 16 was also cloned in pGEM-T vector.

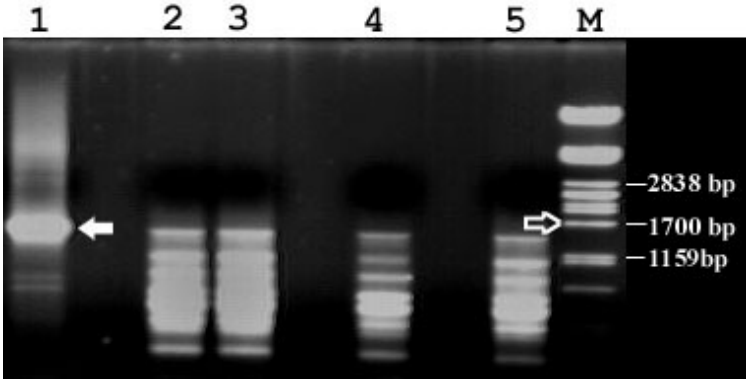


**Figure 3.7.** PCR amplification using the ask-fwd-00 and ask-rev-00 primer pair when the parental and mutant chromosomal DNAs were used as the templates. Lane 1: pNST102 as the template as a positive control; Lane 2 and 3: Chromosomal DNA of the mutant no. 84 as the template; Lane 4: Chromosomal DNA of the mutant no. 16 as the template; Lane 5: Chromosomal DNA of the parental strain as the template. M: *Pst*I digested/λ DNA size marker. 1700 bp DNA size marker is indicated with a hollow arrow and 1609 bp *ask* gene with a white arrow.

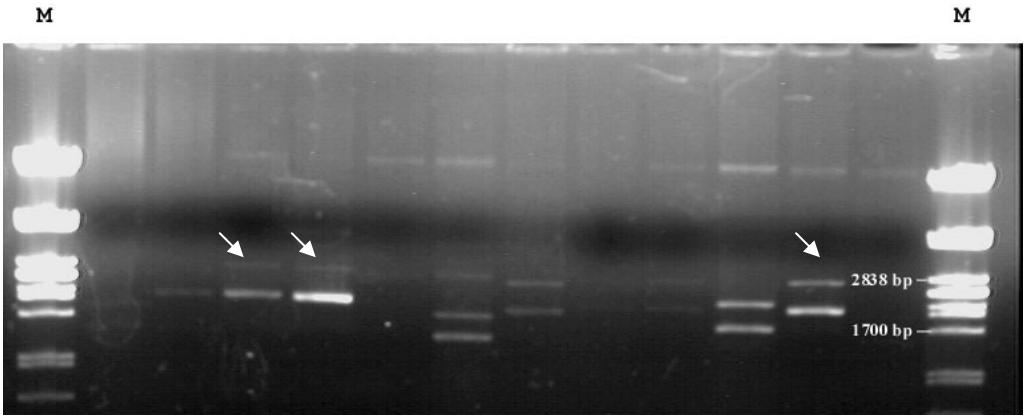


**Figure 3.8.** Analysis of plasmids of *E. coli* recombinants carrying the *ask* amplicon obtained from chromosomal DNA of the mutant no. 84. M: *Pst*I digested/λ DNA size marker. The plasmids that are relatively larger in size (indicated with arrows) were selected for further analysis.

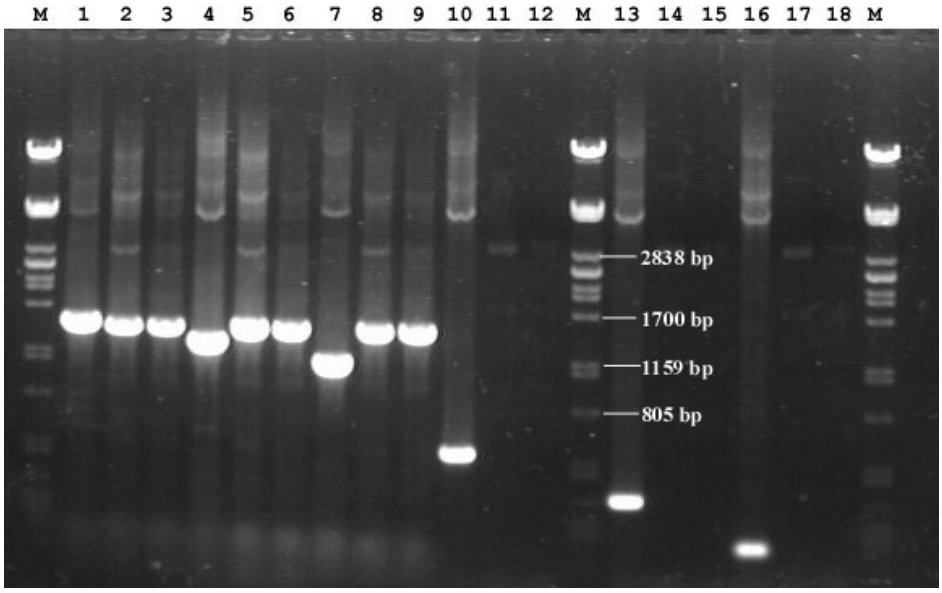


**Figure 3.9.** PCR with various primer pairs to test the identity of the insert cloned into the pGEM-T vector to the *ask* gene. Lanes 1, 4, 7, 10, 13 and 16: pNST102 as the template as a positive control; Lanes 2, 5, 8, 11, 14 and 17: Chromosomal DNA of the mutant no. 16 as the template; Lanes 3, 6, 9, 12, 15 and 18: Chromosomal DNA of the mutant no. 84 as the template. Lanes 1-3, 4-6, 7-9, 10-12, 13-15 and 16-18: PCR by using ask-fwd-00/ask-fwd-00, ask-fwd-00/ask-ir-01, ask-fwd-00/ask-ir-02, ask-internal-01/ask-rev-00, ask-internal-01/ask-ir-01 and ask-internal-01/ask-ir-02 primer pairs, respectively. M: *Pst*I digested/λ DNA size marker.

Figure 3.11 represents the position of the primers and the size of the products they expected. The Figure 3.8 illustrates amplification of the cloned fragments with various combinations of primers in order to verify the identity to the *ask* gene of the fragment cloned into the pGEM-T vector. PCR amplification using the recombinant plasmids as the template was expected to yield the same product with those obtained from pNST102 as the template with the same primer pairs if the cloned fragments were really the *ask* gene. However, the results clearly demonstrated that the fragments amplified from the mutant no. 16 and 84 and cloned into the pGEM-T vectors were not the aspartokinase gene.

Following the failure in amplification of aspartokinase gene with ask-fwd-00 and ask-rev-00 primer pair, a new primer pair namely, ask-fwd-01 and ask-rev-01 complementary to the regions shown in Figure 3.11 was designed. The primers did not contain any restriction endonuclease sites at their ends, to increase the specificity of hybridization. Figure 3.10 illustrates the amplification results using these primers.

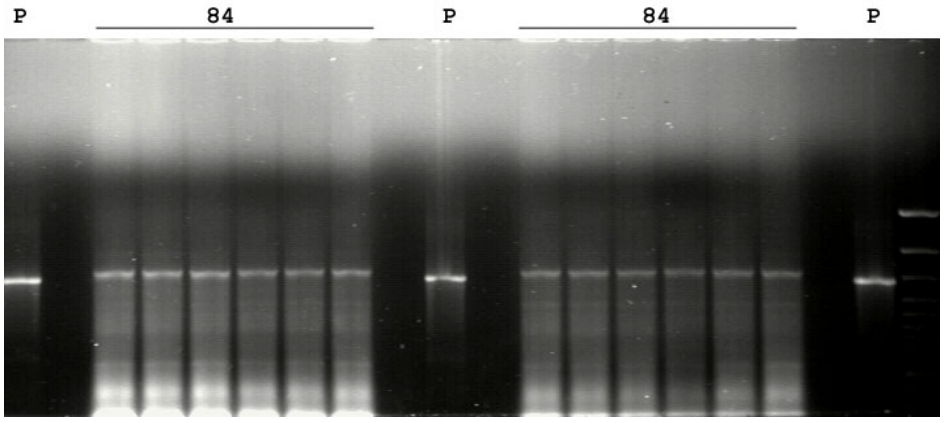


**Figure 3.10**. PCR amplification with the ask-fwd-01 and ask-rev-01 primer pair using Taq polymerase (MBI Fermentas). P: pNST102 as the template as a positive control; 84: Chromosomal DNA of the mutant no. 84 as the template.

Subsequent verification via cutting the eluted DNA of the putative *ask* amplicon from the mutant no. 84 with restriction enzymes (Figure 3.11) revealed that although fragments amplified appear to have the same size with the *ask* gene amplified from pNST102, the fragments were not corresponding to the *ask* gene.

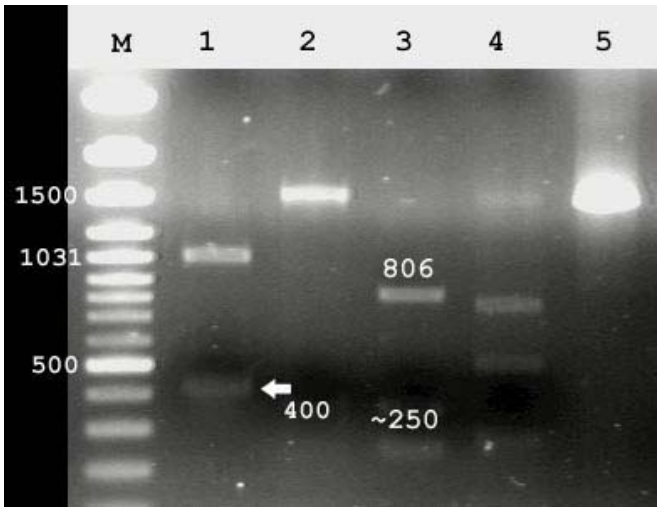


**Figure 3.11.** Restriction enzyme digestion of PCR product of *ask* gene. Lane 1 and 2: *Pst*I digestion of *ask* gene amplified from pNST102 plasmid and chromosomal DNA of mutant no.84 respectively; Lane 3 and 4: *Sac*II digestion of *ask* gene amplified from pNST102 plasmid and chromosomal DNA of mutant no. 84 respectively; Lane 5: Uncut *ask* amplified from pNST102; M: Gene-Ruler™ 100 bp DNA ladder plus DNA size marker.

One of the possible reasons of the consistent difficulty of amplifying *ask* gene from the chromosomal DNA of *S. clavuligerus* is the high G/C content of the template. It is known that DNA regions highly rich in guanine and cytosine nucleotides tend to form stable secondary structures (Loewen and Switala, 1995; Henke *et al.*, 1997). In genome sequencing projects, DNA regions that often contain stable secondary structure with high G/C content are encountered. These regions are to not only difficult to amplify by PCR for template preparations, but also determine the DNA sequences using standard cycle sequencing method (Izawa *et al.*, 2006). Since G/C content of *Streptomyces* chromosomal DNA ranges between 69 and 73 % (Keiser *et al*., 2000), it is probable that secondary structures formed at and around the *ask* gene leads to the partial or complete inhibition of polymerase chain reaction. A combination of multiple displacement amplification and transcriptional sequencing methods is proposed by Izawa *et al.* (2006) for the analysis of sequences with G/C content ranging from 65% to 85%.

*E. coli* has three AKs, and two of them (AKI and II) are conjugated with homoserine dehydrogenase. The activity of AKI-HDI is inhibited by threonine, and its synthesis is repressed by threonine plus isoleucine, while AKII-HDII

activity is repressed by methionine (Kikuchi *et al.*, 1999). In both enzymes, AK activity is localized to the N-terminal region and homoserine dehydrogenase activity is localized to the C-terminal regions (Theze *et al.*, 1974). Aspartokinase III (AKIII), encoded by lysC at 91.1 min, is a single function enzyme that is inhibited and repressed by lysine (Cassan *et al.* 1986). By *in vitro* chemical mutagenesis of the cloned *lys*C gene, Kikuchi *et al.* (1999) isolated lysine-insensitive mutants which were then demonstrated to have missense mutations in amino acid residues 323-352, and at position 250 of AKIII. Overall, 5 different mutants were obtained and shown to contain *lys*C1 AC<u>G</u>→A<u>T</u>G 344 Thr→Met, *lys*C2 T<u>C</u>A→T<u>T</u>A 345 Ser→Leu, *lys*C6 <u>G</u>AG→<u>A</u>AG 250 Glu→Lys, *lys*C12 <u>G</u>AA→<u>A</u>AA 323 Gly→Asp and *lys*C47 A<u>C</u>C→A<u>T</u>C 352 Thr→Ile, respectively.

In corynebacteria, the concerted inhibition by threonine and lysine is exerted at a regulatory site that involves $Ser^{301}$ of AK, as shown for *Corynebacterium glutamicum* (Kalinowski *et al.*, 1991). A mutant resistant to the lysine analogue AEC showed a $Ser^{301}$ to $Tyr^{301}$ change. Similarly, in *Corynebacterium flavum* N13, $Gly^{345}$ of AK has been reported to be involved in the concerted inhibition of AK by lysine and threonine (Follettie *et al.*, 1993). Hernándo-Rico *et al* (2001) studied the structure of the *ask-asd* operon and formation of AK subunits in the cephamycin producer *Amycolatopsis lactamdurans*. Both $Ser^{301}$ and $Gly^{345}$ were present in the amino acid sequence of *A. lactamdurans* AK and they confirmed their involvement in concerted feedback regulation of AK in *A. lactamdurans* by *in vitro* mutagenesis of these two amino acid residues. *S. clavuligerus ask* gene which was first cloned and characterized in our laboratory (Tunca *et al*., 2004) also contains both $Ser^{301}$ and $Gly^{345}$ in the regulatory region in a sequence motif of DMVLQNV <u>S</u>$^{301}$NTSSGRTDI TFTLSKANGP KAVASLEKİK EELGFSSVLY DDHV<u>G</u>$^{345}$KVSLV as compared to DMVLQNV <u>S</u>$^{301}$VEDGTTDI TFTCPRSDGR RAMEİLKKLQ VQGNWTNVLY DDQV<u>G</u>$^{345}$KVSLV in *C. glutamicum*. The present study was originally designed with the aim of finding out amino acid substitutions, if any, other than $Ser^{301}$ and $Gly^{345}$ in the regulatory region accounting for concerted feedback deregulation in *S. clavuligerus*. However, the

consistent failure in amplification of the mutant templates did not allow identification of the sites of other possible mutations.

As recently demonstrated in our laboratory, a drastic increase in aspartokinase activity in *S. clavuligerus* provided by introducing *ask* gene on a multi-copy plasmid was not accompanied by an ample increase in cephamycin C yields (Taşkın, 2005). Antibiotic production increased by only 2 to 3 fold in the recombinant organism. One possible reason for this should be feedback inhibition of the aspartokinase enzyme by its end product amino acids. Therefore, it seems quite rationale to express *ask* multi-copies in an AK deregulated background, the mutant no. 84 obtained in the present study being a promising candidate. Since both this mutant and the recombinant strain already overproduce cephamycin C (each at least 2 fold as compared to the parental strain), their recombination is expected to provide a cumulative increase in antibiotic titres. However, care should be taken to keep the mutation stable, by controlling the strain on AEC-containing agar plates at intervals, since the mutation contains the risk of reverting back.

# CHAPTER 4

# CONCLUSION

- By using chemical mutagenesis technique about 100 AEC-resistant mutants were obtained. 17 of them were tested for their aspartokinase enzyme insensitivity to concerted feedback inhibition. The aspartokinase from one selected mutant, namely the mutant no. 84, showed the highest degree of insensitivity to feedback inhibition which resulted in two fold increase in specific production of cephamycin C.

- PCR conditions for the amplification of the putative *ask* gene in sufficient yields from the chromosomal DNA of this mutant were tried to be optimized.

- In PCR reactions, the bands amplified from the chromosomal DNA of the mutant strain corresponding to the size of the *ask* gene were extracted from the agarose gel and subsequently eluted. These amplicons were taken into verification tests either directly and/or after further amplification via cloning into pGEM-T vector. The findings of the verification tests which involved further amplification using the internal primers and/or restriction enzyme digestion revealed that none of the amplified fragments corresponded to the *ask* gene.

- Yet, the strain holds promise for being used as a host for the expression of *ask* gene on a multi-copy plasmid recently constructed in our laboratory.

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# APPENDIX A

## COMPOSITION AND PREPARATION OF CULTURE MEDIA

**Liquid Media**

| **Luria Broth (LB)** | **g/L** |
|---|---|
| Luria Broth | 25 |

Sterilized at 121 °C for 15 minutes

| **Nutrient Broth (NB)** | **g/L** |
|---|---|
| Nutrient Broth | 8 |

Sterilized at 121 °C for 15 minutes

| **Tryptone Soy Broth with Sucrose (TSB)** | **g/L** |
|---|---|
| Tryptic Soy Broth | 30 |

Sterilized at 121 °C for 15 minutes

| **Chemically Defined Medium** | **g/L** |
|---|---|
| $K_2HPO_4$ | 3.5 |

| | |
|---|---|
| MOPS | 20.9 |

pH is adjusted to 6.9 and volume completed to 780 mL with distilled water.

Sterilized at 121 °C for 15 minutes.

After sterilization following components were added to the medium:

| | **mL/L** |
|---|---|
| L-Asparagine (10 g/L) | 200 |
| $MgSO_4 \cdot 7H_2O$ (0.615 g/mL) | 2 |
| Glycerol (50%) | 20 |
| *Trace salt solution | 1 |

| **Trace salt solution** | **g/100 mL** |
|---|---|
| $MnCl_2 \cdot 4H_2O$ | 0.1 |
| $FeSO_4 \cdot 7H_2O$ | 0.1 |
| $ZnSO_4 \cdot 7H_2O$ | 0.1 |
| $CaCl_2$ | 0.1 |

**Solid Media**

| **LB Agar** | **g/L** |
|---|---|
| Luria Broth | 25 |
| Agar | 15 |

Sterilized at 121 °C for 15 minutes

| **NB Agar** | **g/L** |
|---|---|
| Nutrient Broth | 8 |
| Agar | 15 |

Sterilized at 121 °C for 15 minutes

| **Soft NB Agar** | **g/L** |
|---|---|
| Nutrient Broth | 8 |
| Agar | 5 |

Sterilized at 121 °C for 15 min.

| **Minimal Medium Agar** (Kieser *et al.*, 2000; modified) | **g/L** |
|---|---|
| $(NH_4)_2SO_4$ | 2.1 |
| $K_2HPO_4$ | 0.5 |
| $MgSO_4 \cdot 7H_2O$ | 0.2 |
| $FeSO_4 \cdot 7H_2O$ | 0.01 |
| Glycerol (50%) | 20 ml |
| Agar | 20 |

Sterilized at 121 °C for 15 minutes. Glycerol solution was sterilized separately.

## APPENDIX B

## BUFFERS AND SOLUTIONS

| **Phosphate Buffer** (pH 7.5, 50 mM) | **g/L** |
|---|---|
| $KH_2PO_4$ | 6.8 |
| KOH | 1.9 |

pH adjusted with KOH

**Sonication Buffer**

150 mM KCl

5% Glycerol (v/v)

1 mM EDTA (added at the time of use)

1 mM PMSF (dissolved in Isopropanol, added at the time of use)

**Stop solution**

10% (w/v) $FeCl_3 \cdot 6H_2O$

3.3% (w/v) TCA

0.7 N HCl

Dissolved in 100 mL distilled water and protected from light.

| **Tris/HCl buffer** (pH 7.5, 50 mM) | **g/L** |
|---|---|
| Tris-HCl | 6 |

pH adjusted with KOH, sterilized at 121 °C for 15 minutes

**TSE Buffer** (Hintermann, 1981)

| | |
|---|---|
| Sucrose | 10.3% |
| Tris-HCl (pH 8.0) | 25 mM |
| EDTA (pH 8.0) | 25 mM |
| Lysozyme | 2 mg/mL |

**Lysis Solution**

0.3 M NaOH

2% SDS

**Phenol-Chloroform Solution** (water-saturated, Hintermann, 1981)

| | |
|---|---|
| Phenol | 500 g |
| Chloroform | 500 mL |
| Distilled water | 400 mL |

The solution was stored at room temperature and protected from light.

**X-Gal** (5-bromo-4-chloro-3-indolyl-B-D-galactoside)

| | |
|---|---|
| X-Gal | 20 mg |
| Dimethylformamide | 1 mL |

The solution was stored at –20 ˚ C and protected from light.

**IPTG** (Isopropyl-β-D-thiogalactoside)

| | |
|---|---|
| IPTG | 100 mg |
| Distilled water | 1 mL |

The solution was filter sterilized and stored at –20 ˚ C.

**AK Assay Mixture**

100 mM HEPES (pH 7.8)

400 mM KCl

12 mM $MgCl_2$

500 mM Hydroxylamine

10 mM ATP

15 mM L-Aspartate

**Tris-Acetate-EDTA Buffer** (TAE, 50X)

| | |
|---|---|
| Tris base | 242 g |
| Glacial acetic acid | 57.1 mL |
| EDTA (0.4 M, pH 8.0) | 125 mL |

Dissolved in 1 L distilled water. When used, diluted to 1X with distilled water.

## APPENDIX C

## CHEMICALS AND THEIR SUPPLIERS

### Chemicals

| | |
|---|---|
| AEC | Sigma |
| Agar | Merck |
| Agarose | Prona |
| Ampicillin | Sigma |
| L-Aspartate | Sigma |
| BSA | Sigma |
| $CaCl_2 \cdot 2H_2O$ | Merck |
| Cephalosporin C (CPC) | Sigma |
| Chloroform | Merck |
| Coomassie Brilliant Blue G-250 | Merck |
| Dimethyl formamide | Merck |
| DMSO | Sigma |
| EDTA | AppliChem |
| Ethanol | Botafarma |

| | |
|---|---|
| Ethidium bromide | Sigma |
| $FeCl_3 \cdot 6H_2O$ | Sigma |
| Glacial Acetic Acid | Merck |
| Glycerol | Merck |
| HCl | Fluka |
| IPTG | MBI Fermentas |
| Isopropanol | Merck |
| $K_2SO_4$ | Merck |
| KCl | Merck |
| $K_2HPO_4$ | Merck |
| $KH_2PO_4$ | Merck |
| KOH | Merck |
| Luria Broth | Q-Biogene |
| L-Lysine | Sigma |
| Malt Extract | LabM |
| MNNG | Sigma |
| MOPS | Sigma |
| $MgCl_2.6H_2O$ | Merck |
| $MnCl_2.4H_2O$ | Merck |
| NaCl | Merck |

| | |
|---|---|
| $NaH_2PO_4$ | Merck |
| $(NH_4)_2SO_4$ | Merck |
| NaOH | Merck |
| Nutrient Broth | Merck |
| PEG-1000 | Aplichem |
| Phenol | Merck |
| Phenylmethylsulfonylfluoride (PMSF) | Sigma |
| SDS | Merck |
| Sucrose | Merck |
| L-Threonine | Sigma |
| Trichloroacetic acid (TCA) | Sigma |
| Tris Base | Merck |
| Tris-HCl | Merck |
| Triton X-100 | Sigma |
| Tryptic Soy Broth | Oxoid |
| X-Gal | MBI Fermentas |
| Yeast Extract | Difco |
| $ZnSO_4$ | Sigma |

**Enzymes**

| | |
|---|---|
| Lysozyme | Q-Biogene |
| RNAse | MBI Fermentas |
| T4 DNA Ligase | MBI Fermantas |
| *Sac*II | New England |
| *Pst*I | New England |
| Yellow/Tango Buffer | MBI Fermentas |
| NEB2 Buffer | New England |
| *Taq* DNA polymerase | MBI Fermentas |

**Size Markers**

| | |
|---|---|
| *Pst*I digested/λ DNA | MBI Fermentas |
| GeneRuler™ 100 bp DNA ladder plus | MBI Fermentas |

**Kits**

| | |
|---|---|
| DNA Extraction Kit | Qiagen |
| Plasmid isolation kit | Qiagen |