B. TAŞKIN

EFFECT OF HOMOLOGOUS MULTIPLE COPIES OF ASPARTOKINASE GENE ON CEPHAMYCIN C BIOSYNTHESIS IN *STREPTOMYCES CLAVULIGERUS*

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

EFFECT OF HOMOLOGOUS MULTIPLE COPIES OF ASPARTOKINASE GENE ON CEPHAMYCIN C BIOSYNTHESIS IN *STREPTOMYCES CLAVULIGERUS*

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M. Sc., Department of Biotechnology

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Streptomyces clavuligerus is a gram-positive filamentous bacterium well known for its ability to produce an array of β-lactam compounds (secondary metabolites) including cephamycin C, clavulanic acid and other structurally related clavams. Of these, cephamycin C is a second generation cephalosporin antibiotic having great medical significance. Biosynthesis of the β-lactam nucleus begins with the non-ribosomal condensation of L-α-aminoadipic acid (α-AAA), L-cysteine and L-valine to form the tripeptide α-aminoadipiyl-cysteinyl-valine (ACV). In *Streptomyces clavuligerus*, α-aminoadipic acid (α-AAA) is a catabolic product of L-lysine produced from the lysine branch of the aspartate pathway and its biosynthesis represents a key secondary metabolic regulatory step in carbon flow to β-lactam synthesis through this core pathway.

The *ask* (aspartokinase)-*asd* (aspartate semialdehyde dehydrogenase) gene cluster which encodes for the first key enzymes of aspartate pathway has already been cloned from *S. clavuligerus*, characterized and heterologously expressed for the first time in our laboratory. Amplification of *ask-asd* cluster or *ask* gene alone in a multi-copy *Streptomyces* plasmid vector and determination of the effects of multiple copies on cephamycin C biosynthesis were the goals of the present study. For this purpose, three different strategies were employed. Of these, two strategies involving the use of vector pIJ702 did not work because of the instability of resulting recombinant plasmids. In the third and last strategy, we used another multicopy *Streptomyces* vector, pIJ486, which we showed in this study to be very stable for the same purpose. Meanwhile, an efficient protoplast transformation protocol was developed in our laboratory. Ask gene was cloned into this vector and *S. clavuligerus* protoplasts were efficiently transformed with the recombinant plasmid (pTB486) using the newly-developed protocol. After stable recombinants were obtained, the effects of the multiple copies of *ask* gene on cephamycin C biosynthesis were determined. There was a profound reduction in the rate and extent of growth of Ask overproducers, as experienced by testing two independent *ask*-multicopy recombinants. Although one such recombinant strain (designated *S. clavuligerus* TB 3585) had a 5.5 fold increased level of Ask activity as compared to the parental strain, it displayed only a 1.1 fold increase in specific production of cephamycin C.

Keywords: *Streptomyces clavuligerus*; gene cloning; aspartokinase; aspartate semialdehyde dehydrogenase; cephamycin C biosynthesis.

ÖZ

STREPTOMYCES CLAVULIGERUS'DA ASPARTOKİNAZ GENİNİN HOMOLOG ÇOKLU KOPYALARININ SEFAMİSİN C ÜRETİMİNE ETKİSİ

Taşkın, Bilgin

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Streptomyces clavuligerus gram-pozitif, filamentöz bir bakteri olup, sefamisin C, klavulanik asit ve diğer klavamlar gibi β-laktam bileşikleri (sekonder metabolitler) üretme yeteneğine sahip olmasıyla tanınmaktadır. Bu bileşiklerden sefamisin C, ikinci kuşak bir sefalosporin antibiyotiği olup büyük tıbbi öneme sahiptir. β-laktamların biyosentezi L-α-aminoadipik-asit (α-AAA), L-sistein ve L-valin'in ACV üçlü peptidini oluşturmak üzere ribozomal olmayan bağlanması ile başlar. *S. clavuligerus*'da α-aminoadipik asit (α-AAA), aspartat metabolik yolunun lizin-spesifik dalından gelen L-lizinin katabolik bir ürünü olup biyosentezi bu önemli metabolik yolun β-laktamları oluşturan karbon akışında anahtar bir sekonder denetleyicidir.

Laboratuvarımızda daha önce yürütülen çalışmalarda *S. clavuligerus*'un aspartik asit biyosentetik yolunun ilk iki enzimini kodlayan *ask* (aspartokinaz)-*asd* (aspartat

semialdehid dehidrojenaz) operonunun tamamı ilk kez klonlanmış, karakterize edilmiş ve heterolog gen ifadeleri elde edilmiştir. Şimdiki çalışmada, klonlanan *ask-asd* operonunun ya da sadece *ask* geninin çok kopyalı bir *Streptomyces* plazmid vektör üzerinde amplifikasyonu ve çoklu kopyalarının sefamisin C biyosentezine etkilerinin belirlenmesi amaçlanmıştır. Bu amaç doğrultusunda üç ayrı strateji denenmiştir. Bunlardan pIJ702 vektörünü içeren ilk iki deneme rekombinant vektörün instabilitesi nedeniyle olumsuz sonuçlanmıştır. Üçüncü ve de sonuncu denemede, bir diğer *Streptomyces* vektörü olan pIJ486 aynı amaç için kullanılmış ve pIJ702'den çok daha stabil bir vektör olduğu bulunmuştur. Laboratuvarımızda paralel olarak yürütülen bir diğer çalışmada *S. clavuligerus* için oldukça etkin bir protoplast transformasyon protokolu geliştirilmiştir. pIJ486 vektörüne *ask* geni klonlandıktan sonra rekombinant vektör (pTB486) *S. clavuligerus* protoplastlarına bu protokol kullanılarak aktarılmıştır. Stabil rekombinant hücreler elde edildikten sonra bu genin çoklu kopyalarının sefamisin C biyosentezine etkileri araştırılmıştır. Ask genini ifade eden rekombinantların üreme hızları ve miktarları önemli ölçüde düşmüştür. Bu sonuç, iki ayrı ve bağımsız *ask*-çoklu kopya rekombinantı ile yapılan denemelerle kanıtlanmıştır. Rekombinant *S. clavuligerus* hücrelerinde (TB 3585 suşu) Ask aktivitesi parental hücrelere kıyasla 5.5 kat artmış olmasına rağmen sefamisin C üretim miktarı sadece 1.1 kat artış göstermiştir.

Anahtar kelimeler: *Streptomyces clavuligerus*; gen klonlaması; aspartokinaz; aspartat semialdehid dehidrojenaz; sefamisin C biyosentezi

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α-AAA	: α-aminoadipic acid
Asd	: aspartate semialdehyde dehydrogenase
Ask	: aspartokinase
ACV	: α-aminoadipyl-cysteinyl-valine
DAP	: diaminopimelate
CPC	: cephalosporin C
U	: unit
aa(s)	: amino acid(s)
bp	: base pairs
orf(s)	: open reading frame(s)

CHAPTER 1

INTRODUCTION

1.1. Streptomycetes as antibiotic producers

Streptomycetes are gram-positive, chemoheterotrophic soil bacteria belonging to the order *Actinomycetales*. Large (>8 Mb), high G/C, linear chromosomes and large linear plasmids are characteristic of the genus, circular plasmids being also abundant (Paradkar *et al*., 2003). The most striking feature of the genus *Streptomyces* and closely related genera is their ability to produce a wide variety of secondary metabolites. These natural products have been an extraordinary rich source for lead structures in the development of new successful drugs. In addition to the field of antimicrobials, further compounds have been approved as immunosuppressants (FK-506, rapamycin, ascomycin), anticancer compounds (bleomycin, dactinomycin, doxorubicin, staurosporin), antifungal compounds (amphotericin B, nystatin), herbicides (phosphinothricin), drugs used in the treatment of diabetes (acarbose) and anthelmintic agents (avermectin, milbemycin). Of the 12,000 secondary metabolites with antibiotic activity, 55% were produced by streptomycetes and additional 11% by other actinomycetes (Weber *et al*., 2003).

Streptomycetes differ conspicuously from most other bacteria in their growth and developmental biology and are superficially more reminiscent of filamentous fungi. Thus, 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 is gradually formed. This phase is usually referred as the vegetative growth phase. A typical example of vegetative growth is shown in Figure 1.1. 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).

Hyphal differentiation is an important factor influencing the production of secondary metabolites. A general feature of *Streptomyces* is that production of secondary metabolites is associated with low specific growth rates, and it is the lowering of specific growth rate that signals a cascade of regulatory events resulting in chemical differentiation (secondary metabolism) and morphological differentiation (Jonsbu *et al*., 2002). A mechanistic and physiological understanding of tip growth, hyphal branching and mycelium fragmentation leads to improvement of their value as producers of antibiotics (Flärdh, 2003).

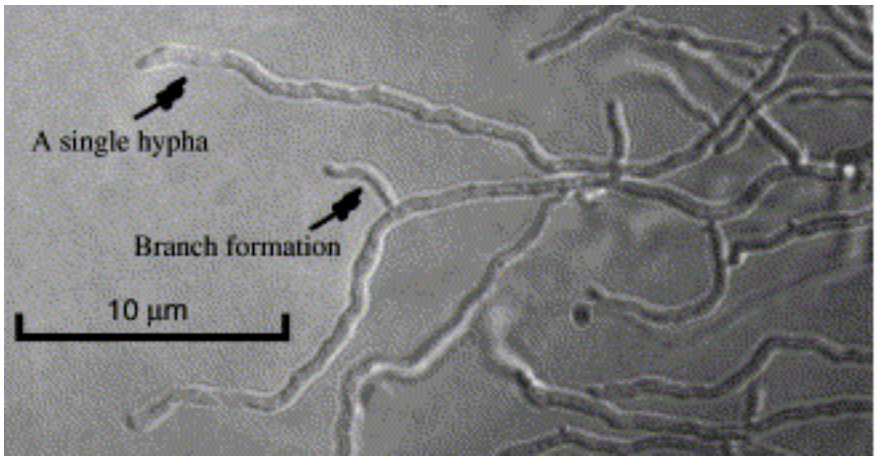
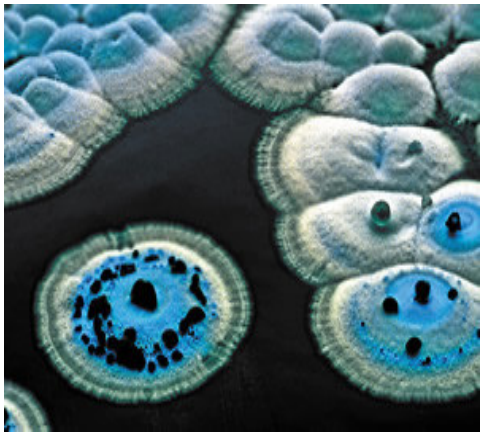


Figure 1.1. Mycelial growth of *S. coelicolor* on solid medium (Goriely and Tabor, 2002).



(a)

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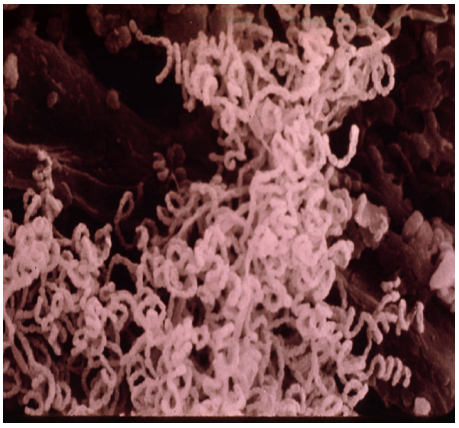

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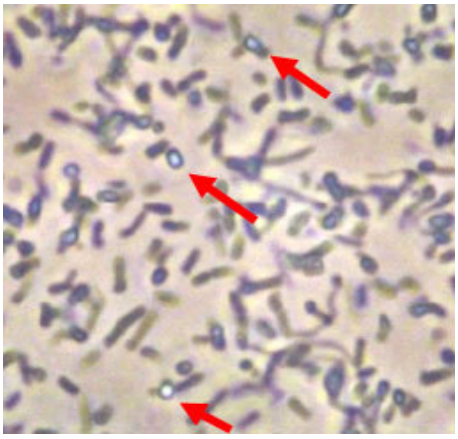


(d)

Figure 1.2. Morphological differentiation of *Streptomyces.* (a) *Streptomyces coelicolor* colonies. (b) An antibiotic droplet secreted from a *Streptomyces* colony. (c) The filamentous mycelium of *Streptomyces* (http://biology.kenyon.edu/Microbial_Biorealm/bacteria/gram-positive/streptomyces/streptomyces.html). d) *Streptomyces* spores (http://www-micro.msb.le.ac.uk/video/Streptomyces.html).

1.2. *Streptomyces clavuligerus* and β-lactam antibiotics

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).

β-lactams are probably the most important class of antibiotics for several reasons. For one thing, penicillin, the first antibiotic isolated and characterized, is a β-lactam.

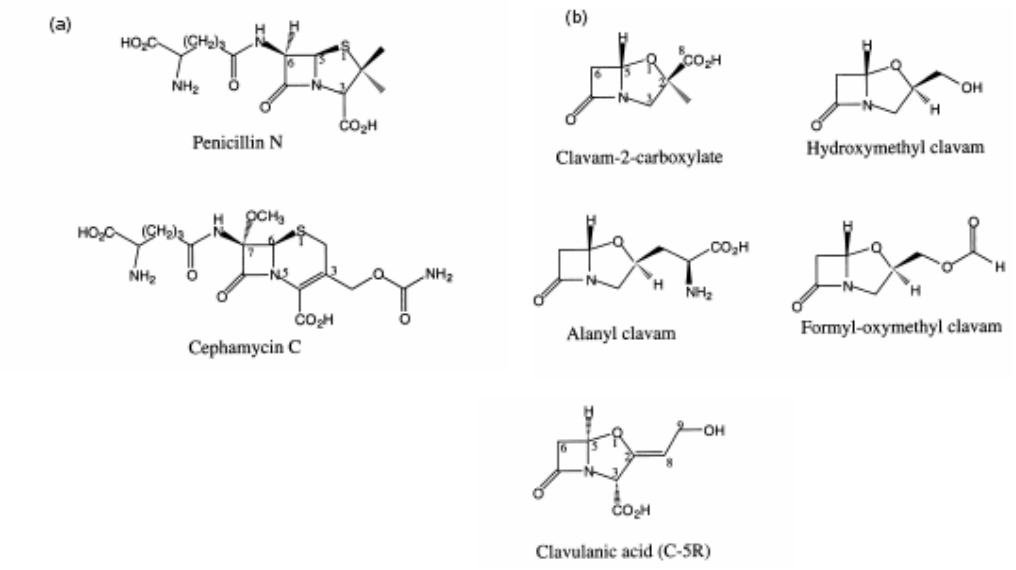


Figure 1.3. β-Lactam products of *S. clavuligerus*. (a) Conventional β-lactam antibiotics. (b) Clavam metabolites (Thai *et al*., 2001).

Second, the inherent toxicity of β-lactams against higher animals is extremely low, yet they are often very effective at killing infecting bacteria. This is because they inhibit peptidoglycan synthesis, a reaction that does not exist in the eukaryotic world (Glazer and Nikaido, 1998). β-lactams have been used extensively for treatment of various bacterial infections for more than half a century and today penicillins are commodity-type products with annual production volumes exceeding 60,000 tons (Thykaer and Nielsen, 2003).

Penicillin-binding proteins (PBPs) are responsible for the final stages of bacterial cell wall assembly. These enzymes are targets of β-lactam antibiotics. Two of the PBP activities include DD-transpeptidase and DD-carboxypeptidase activities. DD-transpeptidases carry out the indispensable cross-linking step of the cell wall synthesis. The cross-linking reaction gives the structural rigidity to the cell wall that is critical for bacterial survival. The DD-transpeptidase activity swaps one amide bond for another, in the process of which the cross-linked cell wall is formed (1→2→4; Figure 1.4A). The DD-carboxypeptidase activity moderates the degree of cross-linking by removing the terminal D-Ala of the peptidoglycan (Figure 1.4B), hence preempting the possibility of cross-linking. Both the DD-carboxypeptidase and DD-transpeptidase activities utilize an active-site serine strategy in their mechanisms. β-lactam antibiotics also acylate the active site serine of DD-carboxypeptidases and DD-transpepdidases. 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-lactams leaves the "leaving group" covalently tethered to the "acyl-enzyme" species. Hence, the enzyme is essentially irreversibly inactivated, which is the basis for how β-lactam antibiotics kill bacteria (Lee *et al*., 2003).

1.3. Cephamycin C biosynthetic pathways in *Streptomycetes*

A wide variety of microorganisms are known to produce β-lactams ranging from filamentous fungi to both gram-positive and gram-negative bacteria (Thykaer and

Nielsen, 2003). Filamentous fungi produce only β-lactam antibiotics with penicillin or cephalosporin structure. Bacteria synthesize a variety of β-lactam structures

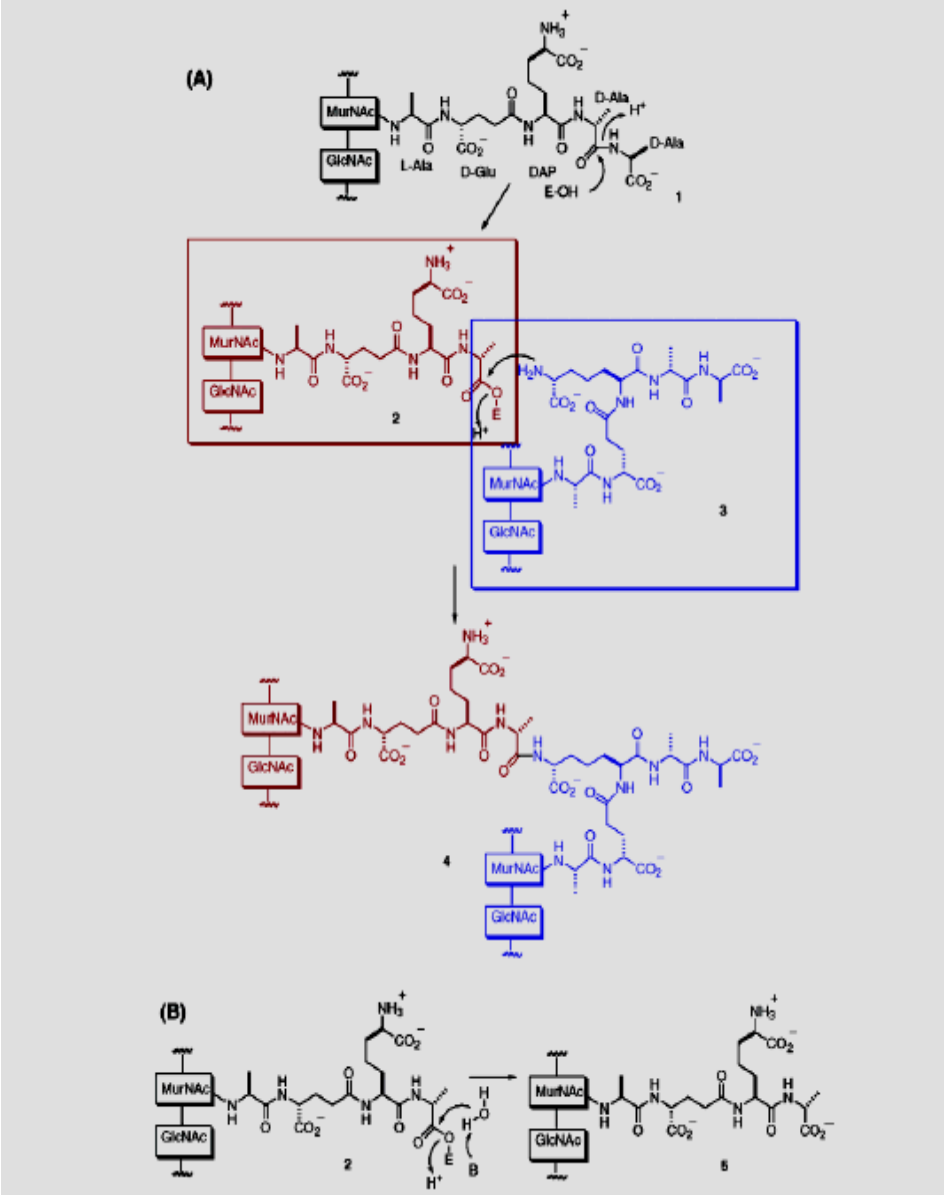


Figure 1.4. (A) Mechanistic steps in cross-linking of the cell wall by DD-transpeptidases. (B) Mechanistic steps in the activity of DD-carboxypeptidases.

including cephalosporins (mainly as intermediates of biosynthetic pathways), cephamycins, cephabacins, clavams, carbapenems and monobactams (Liras, 1999). The production of penicillins, cephalosporins and cephamycins are the best characterized processes. The main producers of these products are *Penicillium chrysogenum*, *Acremonium chrysogenum*, *Nocardia lactamdurans* and *Streptomyces clavuligerus*. An overview of the biosynthetic pathways of the β-lactams produced by these organisms is given in Fig. 5. The biosynthesis of penicillins, cephalosporins and cephamycins have the two first steps in common (Thykaer and Nielsen, 2003).

The first step in the biosynthesis of β-lactam antibiotics is the condensation of the three amino acids L-α-aminoadipic acid, L-cysteine and L-valine with the concomitant epimerization of L-valine to D-valine to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). This reaction is catalysed by a single multifunctional enzyme ACV synthetase (ACVS) (Theilgaard *et al*., 1997).

In bacteria, the α-aminoadipic acid comes from the catabolism of lysine (Demain and Davies, 1999). In the first steps in the cephamycin C biosynthesis, lysine is converted to α-aminoadipic acid (α-AAA) through a two step conversion. The deamination of lysine by lysine-6-aminotransferase (LAT) to form 1-piperidiene-6-carboxylate marks the first committed step of cephamycin biosynthesis in *Streptomyces 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) (Figure 1.6).

The second step in the biosynthetic route to penicillin and cephalosporins is the conversion of ACV to isopenicillin N (IPN). A single enzyme, isopenicillin N synthase (IPNS), catalyzes oxidation (desaturation) of the linear tripeptide to form the bicyclic isopenicillin N, which possesses weak but significant antibiotic properties (Aharonowitz, Cohen and Martin, 1992). Isopenicillin N is the branch point at which the biosynthesis of penicillin is separated from the biosyntheses of cephalosporins and cephamycins (Thykaer and Nielsen, 2003).

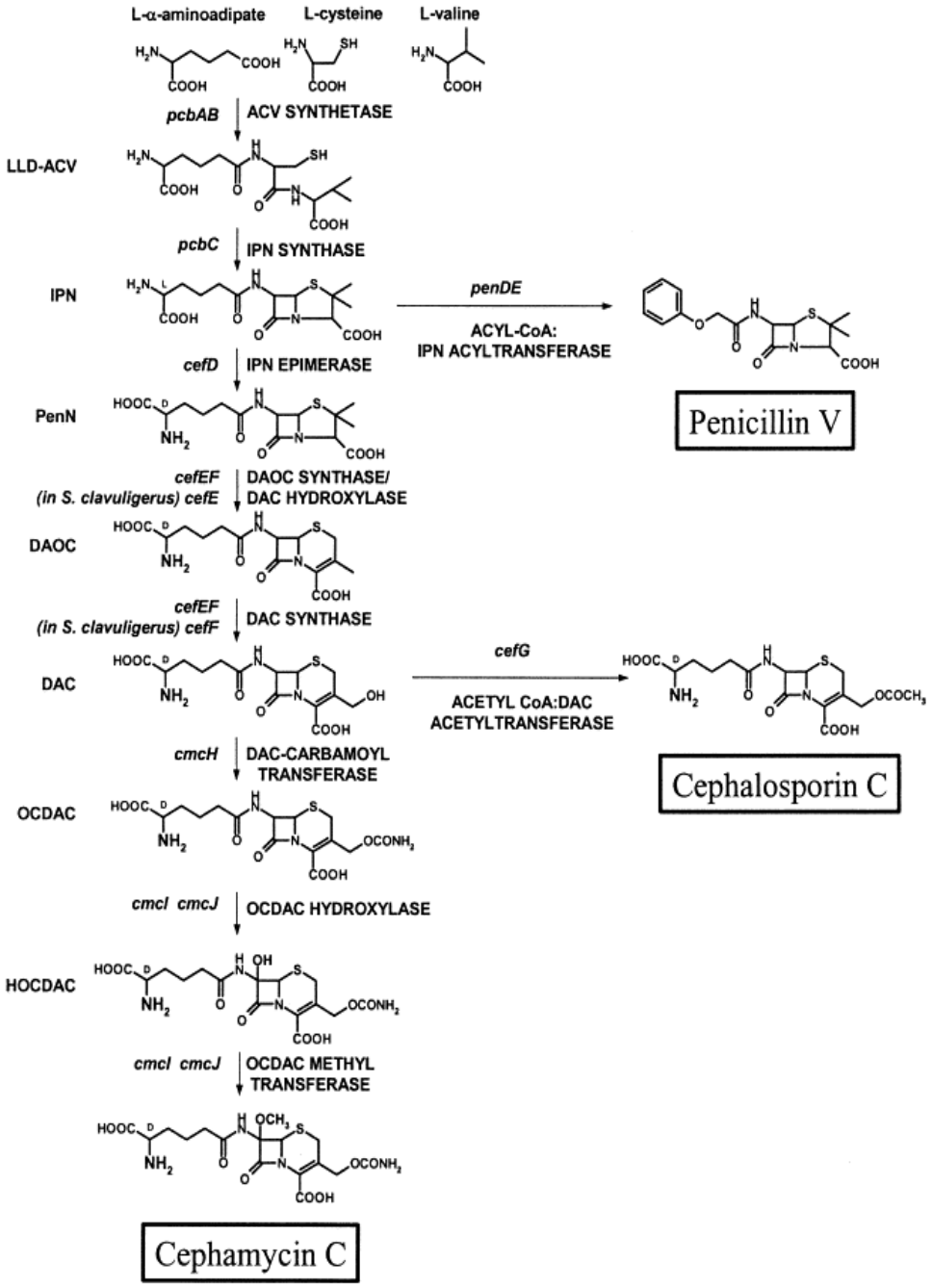


Figure 1.5. Biosynthesis of the β-lactams penicillin V, cephalosporin C and cephamycin C. Abreviations: : LLD-ACV, *α*-L-aminoadipyl-L-cysteinyl-D-valine; IPN, isopenicillin N; Pen N, penicillin N; DAOC, deacetoxycephalosporin C; DAC, deacetylcephalosporin C; OCDAC, O-carbamoyl- deacetylcephalosporin C; HOCDAC, 7-a-hydroxy-O-carbamoyl-deacetylcephalosporin C.

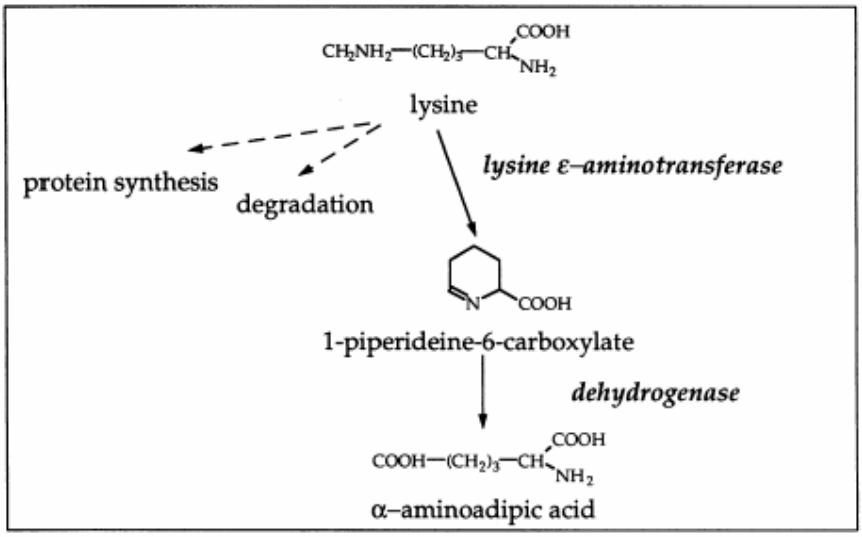


Figure 1.6. Biosynthesis of α-AAA from lysine in *S. clavuligerus* (Malmberg *et al*., 1993).

The next three steps of the pathway are identical to those existing in *Cephalosporium acremonium*, the cephalosporin C producer, but are not present in penicillin forming *Penicillum chysogenum.* The L-α-aminoadipyl lateral chain of isopenicillin N is isomerized to the D configuration by the isopenicillin N isomerase to give penicillin N, the five-membered thiazolidine ring of penicillin N is then expanded to a six-membered dihydrothiazinic ring common to all cephalosporins by the penicillin expandase (also named deacetoxycephalosporin C synthase). Finally, the deacetoxycephalosporin C formed is hydroxylated at C-3' by the deacetoxycephalosporin C hydroxylase to form deacetylcephalosporin C, the immediate precursor of cephalosporin C (Liras, 1999). Acetyl-CoA:DAC acetyltransferase (DAT) is responsible for the last step in the formation of cephalosporin C. In the biosynthesis of cephamycin C, the C-3 acetoxy group of DAC is replaced by a carbamyl group by DAC-carbaoyl transferase (DACCT) followed by hydroxylation and methylation of the C-7 catalyzed by 3'-carbomyl – DAC 7-hydroxylase and 7-hydroxy-3'-carbamoyl-DAC methyltransferase, a two protein system named P7 and P8 (Thykaer and Nielsen, 2003). The C-7 metoxyl

group of cephamycins confers to these antibiotics their characteristic β-lactamase resistance (Liras, 1999).

1.4. The genetic organization of the cephamycin C biosynthetic gene clusters

All of the structural genes necessary for the biosynthesis of cephamycin C in *S. clavuligerus* are organized into a cluster together with regulatory and resistance genes (Alexander *et al*., 2000) (Figure 1.7). The genes encoding three of the earliest enzymes in the biosynthetic pathway, LAT, ACVS, and IPNS are designated *lat*, *pcbAB*, and *pcbC* (Alexander and Jensen, 1998). The L-lysine 6-aminotransferase of *S. clavuligerus* was partially purified and characterized (Romero *et al*., 1997). The gene encoding this enzyme (*lat*) is located in the cephamycin C gene cluster both in *N. lactamdurans* (Coque *et al*., 1991) and *S. clavuligerus* (Madduri *et al*., 1991). The *S. clavuligerus* Δ-1-piperideine-6-carboxylate dehydrogenase, which catalyzes the conversion of P6C into α-AAA, was purified (de la Fuente *et al*., 1997). The *pcbAB* gene of *N. lactamdurans* and *S. clavuligerus* encoding the ACV synthetase, has been cloned and sequenced (Coque *et al*., 1991b; Doran *et al*., 1990b; Tobin *et al*., 1991; Yu *et al*., 1994). Nine *pcbC* genes have been studied from actinomycetes, fungi and Gram- negative bacteria (Aharonowitz *et al*., 1992). The uniform transcriptional orientation and short intergenic regions separating *lat*, *pcbAB*, and *pcbC* in *S. clavuligerus* suggested that these genes might be organized in an operon for coordinate expression (Alexander *et al*., 2000).

The genes encoding enzymes for the intermediate steps (*cefD-cefE*) are linked together in all cephamycin producers (Liras, 1999). Genes encoding isopenicillin N epimerases (*cefD*) from actinomycetes and gram-negative bacteria have been cloned (Kovacevic *et al*., 1990; Coque *et al*., 1993a; Kimura *et al*., 1996). The genes encoding penicillin N expandase (*cefE*) and DAOC 3'-hydroxylase (*cefF*) have been cloned in both *S. clavuligerus* and *N. lactamdurans* (Kovacevic *et al*., 1989; Kovacevic and Miller, 1991; Coque *et al*., 1993a; Coque *et al*., 1996a).

The genes for the last steps of the cephamycin C pathway (*cmcI-cmcJ* and *cmcH*) are linked, but present in different locations in the chromosomes of *S. clavuligerus* and *N. lactamdurans* (Liras, 1999). The proteins encoded by *cmcI* and *cmcJ* of *N. lactamdurans* (P7 and P8) were purified (Enguita *et al*., 1996a). Interaction between

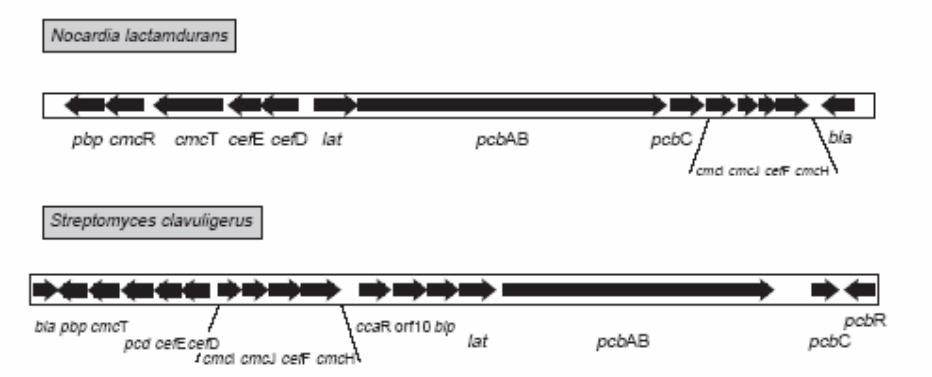


Figure 1.7. Cluster of genes encoding cephamycin C biosynthesis in *N. lactamdurans* and *S. clavuligerus* (Martin, 1998).

the two proteins of the system has been studied (Enguita *et al*., 1996b). The P7-P8 complex was co-purified by immunoaffinity chromotography from extracts of *N. lacdamdurans* and *S. Clavuligerus,* suggesting that both proteins form a complex 'in vivo' (Liras, 1999).

Finally, genes for a β-lactamase (*bla*), a penicillin-binding protein (*pbp*) and a transmembrane protein (*cmcT*) that appear to be involved in cephamycin exportation are clustered together with the biosynthetic genes in *S. clavuligerus* and *N. lacdamdurans* (Martin, 1998) (Figure 1.7).

Genes encoding β-lactamases (*bla*) are present in *N. lactamdurans* (Coque *et al*., 1993b), *S. clavuligerus* (Pérez-Llarena *et al*., 1997) and the cephabacin producer *Lysobacter lactamgenus* (Kimura *et al*., 1996). The *bla* gene, when amplified in high copy number, confers resistance to penicillin and cephalosporins but not to cephamycin. Penicillin-type intermediates and non-methoxylated cephalosporin compounds occur as intermediate or shunt products of the cephamycin pathway, and

small amounts of these intermediates are released into the extracellular fluid. Therefore, a cell wall-bound extracellular β-lactamase is a convenient tool for self-protection. Methoxylation of cephalosporin-type intermediates to cephamycin (β-lactamase resistant) might have therefore evolved as an additional mechanism to produce an antibiotic that is not degraded by its own β-lactamase (Martin, 1998). The presence of genes for extracellular β-lactamases in strains producing β-lactam antibiotics is intriguing, specially in *S. clavuligerus*, an organism that also produces two β-lactamase inhibitory proteins (BLIP and BLP) and the β-lactamase inhibitor clavulanic acid (Liras, 1999). An open question is whether the *bla* gene of the cephamycin clusters is merely an antibiotic-resistance gene or whether it modulates cephamycin biosynthesis (Martin, 1998).

Among gram-positive bacteria, the two main mechanisms of resistance to β-lactam antibiotics are production of β-lactamases and production of low-affinity PBPs. Low-affinity PBPs are modified versions of cell wall biosynthetic enzymes that fail to bind to the antibiotics as a result of their low binding affinity (Paradkar *et al*., 1996). Two genes encoding PBP proteins are located in the cephamycin C gene cluster of *S. clavuligerus* (Liras, 1999). Immediately downstream from *pcbC* and transcribed in the opposite direction is the *pcbR* gene (Paradkar *et al*., 1996). A second gene (*pbp74*) is present in the cephamycin gene cluster immediately downstream of the *bla* gene and transcribed in the opposite direction (Pérez-Llarena *et al*., 1998).

Perhaps the most striking result to emerge is the extremely high sequence similarity found among the prokaryotic and eukaryotic biosynthetic β-lactam genes and their occurrence in gene clusters. These findings are compatible with a horizontal gene transfer of the biosynthetic genes from the prokaryotes to the eukaryotes (Aharonowitz *et al*., 1992).

1.5. Regulation of β-lactam production in Actinomycetes

A regulatory gene (*ccaR*), located within the cephamycin gene cluster of *S. clavuligerus*, is linked to a gene (*blp*) encoding a protein similar to a β-lactamase-inhibitory protein. Disruption of *ccaR* resulted in the inability to produce cephamycin C and clavulanic acid, suggesting that the *ccaR* product is an activator. Complementation of the disrupted mutant with *ccaR* restored production of both secondary metabolites (Pérez-Llarena *et al*., 1997). CcaR specifically binds to the promoter region of the *lat* (Kyung *et al*., 2001). Santamarta *et al*. (2002) also demonstrated that CcaR interacts with the *cefD-cmcI* bidirectional promoter and also with its own promoter. CcaR also affects also clavulanic acid biosynthesis by an unknown mechanism (Santamarta *et al*., 2002).

Located at about 25 kb and in the opposite orientation of *ccaR* in the clavulanic acid cluster is a second regulatory gene, *claR*, which is also involved in the regulation of clavulanic acid-cephamycin C production (Paradkar *et al*., 1998; Perez-Redondo *et al*., 1998). The *car* gene encoding clavulanate-9-aldehyde reductase is linked to the regulatory gene *claR* encoding a LysR-like protein that positively controls clavulanic acid production and acts negatively on cephamycin C production. Expression of *car* and *claR* did not occur in *ccaR*-disrupted mutants (Pérez-Redondo *et al*., 1998).

The clavulanic acid gene cluster is directly adjacent to the cephamycin cluster in the chromosome of *S. clavuligerus*, *S. jumonjinensis*, and *S. katsurahamanus* (Ward and Hodgson, 1993). The fact that no known actinomycetes produce clavulanic acid alone, but there are actinomycetes that produce just cephamycin C or clavams suggests that the production of clavulanic acid evolved in an ancestral clavam and cephamycin producer as a response to the acquisition of β-lactamase-mediated resistance in bacteria inhabiting the same environmental niche and thus posing biological competition (Challis and Hopwood, 2003).

β-Lactam antibiotics are known to be controlled by carbon (Cortés *et al*., 1986), nitrogen (Aharonowitz and Demain, 1979) and in a lesser degree by phosphate

(Aharonowitz and Demain, 1977). Cephalosporin production by *Streptomyces clavuligerus* is mainly regulated by some type of carbon catabolite control. Increasing concentrations of preferred carbon sources, such as glycerol and maltose, decreased production of the antibiotics. When *S. clavuligerus* was grown on organic acids such as α-ketoglutarate or succinate which are relatively poor carbon sources, specific production was high and associated with growth. This is due to the slow hydrolysis of these carbon sources creating a situation of carbon limitation and releasing the culture from carbon source regulation (Aharonowitz and Demain, 1978).

The process of catabolite repression in the high-(GC) gram-positive organisms, such as *Streptomyces* spp., remains relatively unclear but it also appears to be mechanistically distinct from that occurring in gram-negative organisms. In gram-negative organisms, cAMP represents a key factor in regulation. Although cAMP has been detected in Streptomycetes, there is no correlation between cAMP levels and catabolite repression (Mc Mahon *et al*., 1997).

In Streptomycetes, the production of secondary metabolites is linked to the metabolism of nitrogen; therefore, nitrogen catabolite regulation has been investigated for these organisms for several years (Burkovski, 2003). It had been reported that the use of NH_4Cl as the sole nitrogen source or as an additive to asparagine depresses antibiotic production in *S. clavuligerus*. The negative effect is due to repression of L-α-aminoadipyl-L-cysteinyl-D-valine synthetase (ACVS), isopenicillin N synthase (cyclase), isopenicillin N epimerase and deacetoxycephalosporin C synthase (expandase) (Romero *et al*., 1997).

Phosphate control of the biosynthesis of antibiotics and many other types of secondary metabolites is also a well known phenomenon, although the molecular mechanisms by which this control is exerted is unknown (Sola-Landa *et al*., 2003). Aharonowitz and Demain (1977) showed that high concentration of inorganic phosphate inhibits cephalosporin production in *S. clavuligerus.* All available evidence suggest that inorganic phosphate concentrations control the pattern of

expression of a series of genes involved in the biosynthesis of different types of antibiotics in *Streptomyces* species (Sola-Landa *et al*., 2003).

1.6. Approaches for improvements in β-lactam production

Traditionally, β-lactam production has been improved by classical strain improvement programs. These programs have involved an iterative process where the production strains were exposed to random mutagenesis followed by analysis and selection of superior production strains (Thykaer and Nielsen, 2003). However, recent advances in actinomycete molecular biology and knowledge of antibiotic biosynthetic pathways and the gene clusters that encode them offer genetic engineering as an alternative approach to the improvement of strains in a targeted manner.

Veenstra *et al*. (1991) improved penicillin production by introducing additional copies of both the *pcbC* and *penDE* genes. The penicillin production was increased by up to 40% in the single copy strain *Penicillin chrysogenum* Wis54-1255 in this study. A similar study was performed by Theilgard *et al*. (2001) where different combinations of the three structural genes for the penicillin biosynthesis were introduced in *Penicillin chrysogenum* Wis54-1255: *pcbAB*, *pcbC, pcbC-penDE*, and *pcbAB-pcbC-penDE*. The largest increase of 176% in the specific penicillin productivity was observed in transformants with the whole penicillin gene cluster amplified.

The analysis of *Cephalosporium acremonium* transformants containing additional copies of *cefG* gene showed that a direct relationship exists between *cefG* copy number, *cefG* message levels, and cephalosporin C titers. This gene encodes for an enzyme possibly catalyzing a rate-limiting step in CPC production (Mathison *et al*., 1993). Gutierrez *et al*. (1997) also demonstrated that overexpression of the *cefG* gene alone resulted in a 2–3-fold increase in the cephalosporin C production in *A. chrysogenum.*

As mentioned above engineered strains of *Streptomyces* spp. have been constructed in which gene dosage has been increased by the introduction of additional copies of genes encoding pathway enzymes (Malmberg *et al*., 1993). Gene expression has also been increased by the introduction of positive- acting, pathway-specific regulatory genes on high-copy-number plasmids (Pérez-Llarena *et al*., 1997; Stutzman *et al*., 1992).

Malmberg *et al*. (1993) applied a metabolic engineering strategy which involved the introduction of an additional copy of *lat* gene on production of *O*-carbamoyl deacetylcephalosporin C and cephamycin C in *S. clavuligerus*. This manipulation resulted in a five fold increase in cephamycin C production. Pérez-Llarena *et al*. (1997) also demonstrated that amplification of the *ccaR* gene on a multicopy plasmid resulted in a two- to three fold increase in cephamycin and clavulanic acid yields.

Paradkar *et al*. (2001), by using gene replacement technology to eliminate competing and non-competing biosynthetic pathways, could convert a wild-type strain to a high-titer industrial strain. Cephamycin C production was blocked in a wild-type strain of clavulanic acid-producing organism *S. clavuligerus* by targeted disruption of the gene *lat*. Specific production of clavulanic acid increased in the *lat* mutants by 2- to 2.5-fold (Paradkar *et al*., 2001).

Metabolic engineering has proven to be a rational alternative to classical strain improvement and today the β-lactam industry applies the concept of metabolic engineering in parallel with classical strain improvement. The future studies will bring about many other examples of new and improved processes for the production of β-lactams (Thykaer and Nielsen, 2003).

1.7. The aspartate pathway

As mentioned earlier, the initial step in the biosynthesis of β-lactams is the condensation of the three amino acids L-α-aminoadipate (L-α-AAA), L-cysteine and L-valine to form the tripeptide α-aminoadipyl-cysteinyl-valine (ACV) catalysed by

the enzyme ACV synthetase. In fungi, α-AAA is formed as an intermediate of the lysine pathway but in actinomycetes it is synthesized from the lysine branch of the aspartate pathway. Therefore the carbon flow through the lysine-specific branch of the aspartate pathway is a rate-limiting step in the formation of cephamycin C (Aharonowitz *et al*., 1984).

The aspartate pathway uses L-aspartic acid as the precursor for the biosynthesis of the amino acids lysine, methionine, isoleucine, and threonine. This is an essential pathway in plants and microorganisms involving one-fourth of the building block amino acids that are required for protein synthesis. In addition, there are several important metabolic intermediates and products from this pathway including diaminopimelic acid (DAP), a key component required for cross-linking in bacterial cell wall biosynthesis, and dipicolinic acid, important for sporulation in gram-positive bacteria. During evolution, members of the animal kingdom have lost the enzymes that catalyze the reactions in this pathway and therefore the amino acids that are derived from aspartic acid are essential dietary components (Viola, 2001).

Several distinct genomic organizations and a diversity of regulatory mechanisms controlling the metabolic flux through this multibranched pathway have been identified in bacteria. The enzymes catalyzing the first steps of this core pathway, aspartokinase (Ask, EC2.7.2.4) and aspartate semialdehyde dehydrogenase (Asd, EC1.2.1.11) are well conserved functionally (Zhang *et al*., 1999). In *Escherichia coli* there are three isozymes of aspartokinase, regulated by lysine, methionine and threonine, respectively (Theze *et al*., 1974). *Bacillus subtilis* also has three aspartokinases: aspartokinase I is subjected to feedback inhibition by DAP, aspartokinase II is inhibited by L-lysine, while aspartokinase III is inhibited synergistically by L-lysine and L-threonine (Zhang *et al*., 1990).

Only one aspartokinase has been described in *Pseudomonas* (Cohen *et al*., 1969), *Corynebacterium glutamicum* (Kalinowski *et al*., 1991), *Amycolatopsis mediterranei* (Zhang *et al*., 1999) and *Amycolatopsis lactamdurans* (Hernándo-Rico *et al*., 2001). The regulation of aspartokinase in these bacteria is coordinated with respect to

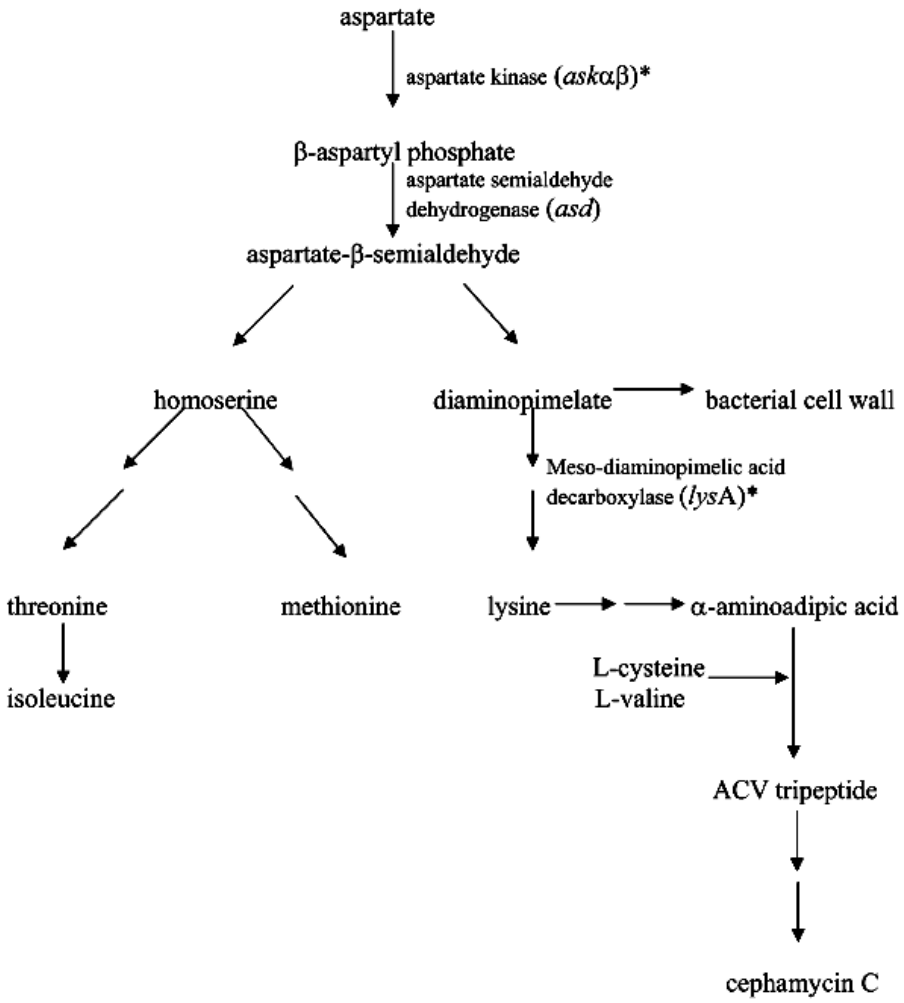


Figure 1.8. Biosynthetic pathway for the aspartate family amino acids and precursor flow to cephamycin C. Known control points in the pathway are shown by the asterics (Tunca *et al*., 2004).

different intermediates or end products of the aspartate pathway (Zhang *et al*., 1999). Zhang *et al*. (2000) demonstrated that no concerted type of inhibition was found for *A. mediterranei* aspartokinase. L-methionine and L-lysine act as competitive inhibitors, whereas Asks 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).

Although there are multiple forms of Ask, aspartate semialdehyde dehydrogenase (Asd) has been found to have only one form in many microorganisms except for several organisms. Moore *et al*. (2002) demonstrated that *Vibrio cholerae* has two *asd* genes. Publication of two complete *Streptomyces* genome sequences, *S. coelicolor* by Bentley *et al*. (2002) and *S. avermitilis* by Ikeda *et al*. (2003), demonstrated that these two microorganisms have also two *asd* genes.

The *ask* and *asd* genes are clustered in an operon in those mycobacteria, corynebacteria and bacilli, as well as in the actinomycete *Amycolatopsis* (Chen *et al*., 1993; Cirillo *et al*., 1994; Hernándo-Rico *et al*., 2001; Kalinowski *et al*., 1991; Zhang *et al*., 1999). However *asd* and *ask* in *S. akiyoshiensis* are not present in an operon, but are localized physically separate on the chromosome (Le *et al*., 1996).

The complete *ask* and *asd* of *S. clavuligerus* have been cloned and characterized for the first time and expressed functionally in an *E. coli* auxotrophic mutant (Tunca *et al*., 2004). The recombinant enzymes have been partially purified, kinetically characterized and their patterns of regulation by the end product amino acids have been also determined. The results of this study indicated that the *ask* and *asd* genes are also clustered in *S. clavuligerus* as in some other gram-positive bacteria. While partially purified recombinant Ask was inhibited about 70% by the mixture of lysine and threonine in this study, the inhibition of the parental Ask with the same amino acids was 94% in ammonium sulfate precipitated fraction, as revealed earlier by another group (Mendelovitz and Aharonowitz, 1982). Further kinetic analysis showed that lysine plus threonine acted as a noncompetitive inhibitor of the recombinant Ask, as can be expected from allosteric enzymes. End-product amino

acids and DAP had no significant effect on the activity of recombinant Asd (Tunca *et al*., 2004).

Cloning and characterization of other key enzymes of the aspartate pathway will pave way to understanding and genetically manipulating primary metabolite precursor flow to cephamycin C biosynthesis in *S. clavuligerus*.

1.8. The present study

As stated earlier the *ask* (aspartokinase)-*asd* (aspartate semialdehyde dehydrogenase) cluster which encodes the first two enzymes of aspartate pathway in *S. clavuligerus* has already been cloned, characterized and heterologously expressed in our laboratory (Tunca *et al*., 2004). The aim of the present study was to amplify either *ask-asd* cluster or *ask* gene alone in an autonomously replicating *Streptomyces* plasmid and to investigate the effects of multiple copies of these genes on cephamycin C biosynthesis since carbon flow through the lysine-specific branch of the aspartate pathway is rate limiting in cephamycin C formation.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains and plasmid vectors

The characteristics and sources of bacterial strains and plasmid vectors and constructs used in this study are given in Table 2.1. Spore suspensions of *Streptomyces* strains were prepared in 20 % glycerol according to the protocol described by Hopwood *et al.* (1985) and stored at –80 ^{o}C. Other microorganisms were streaked onto Nutrient Agar (Appendix A), grown at 37 ^{o}C and subcultured monthly. These cultures were also stored at –80 ^{o}C in nutrient broth (Appendix A) covered with 15 % glycerol. The *E. coli* vectors were Promega PGEM-T, Stratagene pBluescript II KS (+) (Figure 2.1) and pNST102 (Figure 2.3). *Streptomyces* plasmids pIJ702 (Figure 2.2.A) and pIJ486 (Figure 2.2.B) were used in cloning in *S. lividans* and *S. clavuligerus*.

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 the enzymes used as well as their suppliers are listed in Appendix C.

2.5. Protoplast preparation from *Streptomyces lividans*

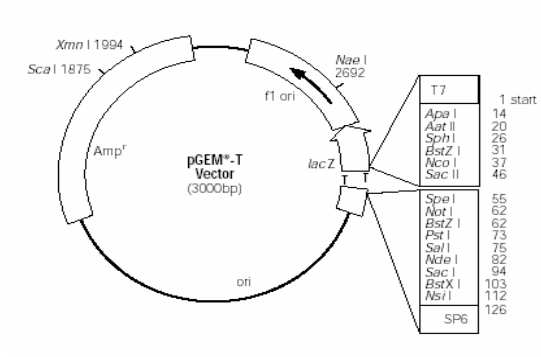
Protoplasts from *Streptomyces lividans* were prepared by the procedure of Hopwood *et al.* (1985) with minor changes. *S. lividans* was grown in 50 mL YEME (Protoplast Preparation Medium; Appendix A) for 18 hours. The mycelium was harvested and washed twice with 10.3 % sucrose. The pellet was resuspended in 5 mL of P buffer (Appendix B) containing 2mg/mL lysozyme. Following incubation at 30 ^{o}C for 30 minutes, the cultures were examined microscopically to determine extent of protoplasting. The protoplasts were filtered through non-absorbent cotton plugs, washed with P buffer, and pelleted twice by centrifugation at 3000 rpm for 10 minutes. The pellets were resuspended in a final volume of 1 mL of P buffer and stored as 120 µL aliquots at –80 ^{o}C.

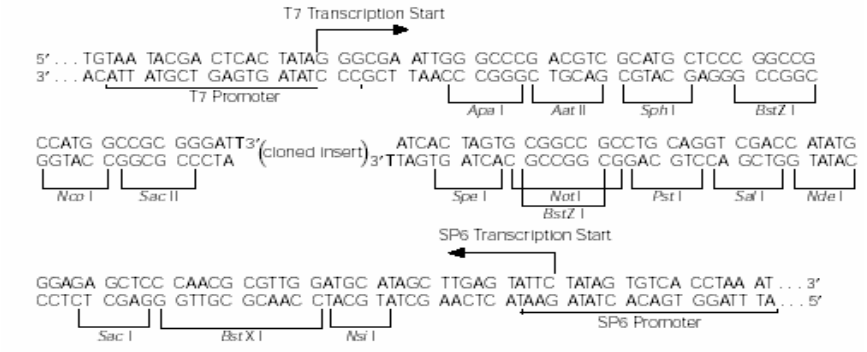
2.6. Protoplast preparation from *Streptomyces clavuligerus*

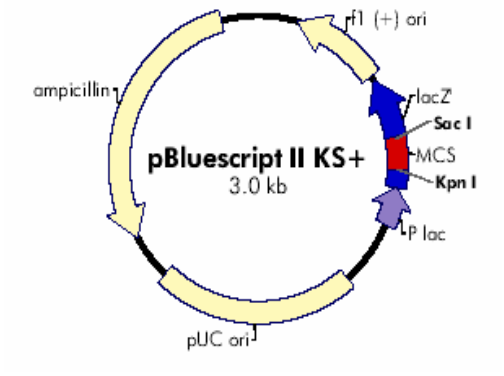
Protoplasts from *Streptomyces clavuligerus* were prepared by the procedure of Hopwood *et al.* (1985) with minor changes. *S. clavuligerus* was grown in 20 mL TSB+30 mL YEME (Protoplast Preparation Medium; Appendix A) at 26 ^{o}C for 24 hours. The mycelium was harvested and washed twice with 10.3 % sucrose. The pellet was resuspended in 2 mL of PE buffer (Appendix B) containing 1mg/mL lysozyme. Following incubation at 30 ^{o}C for 15 minutes, the cultures were examined microscopically to determine extent of protoplasting. The protoplasts were diluted with 2.5 mL of PE buffer, filtered through non-absorbent cotton plugs, washed with PE buffer and pelleted three times by centrifugation at 1000 g for 10 minutes. The pellets were resuspended in a final volume of 1 mL of PE buffer and stored as 50 µL aliquots at –80 ^{o}C.

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)
S. lividans 1326	Prototroph	Prof. J. Piret, Northeastern University, USA
S. clavuligerus TB 3585	Recombinant	This study
E. coli DH5α	F` ϕ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *sup*E44λ^{-} *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17	*E. coli* Genetic Stock Center
E. coli ET12567	F^{-} *dam* 13::Tn*9 dcm-6 hsdM hsdR lacYI*	Prof. Keith Chater, John Innes Centre, Colney, Norwich, UK
E. coli ESS	β-lactam supersensitive mutant	Prof. J. Piret, Northeastern University, USA
Plasmids		
pBluescript II KS (+)	Amp^{r} *lacZ*'	Stratagene
PGEM-T	Amp^{r} *lacZ*'	Promega
pIJ702	thio^{R}, *mel*	Prof. J. Piret, Northeastern University, USA (Katz *et al*., 1983)
pIJ486	Derivative of pIJ101, *tsr*, promoterless *aphII* gene, promoter-probe vector	Prof. Keith Chater, John Innes Centre, Colney, Norwich, UK
pNST102	pBluescript II KS + 3.5 kb *Bam*HI-*Bam*HI *S. clavuligerus ask-asd* cluster	Tunca *et al*., 2004
pBT KS1	pBluescript II KS + 1670 bp *Sal*I-*Eco*RI *S. clavuligerus ask* gene	This study
pBT KS2	pBluescript II KS + 1506 bp *Xba*I-*Eco*RV *S. clavuligerus ask* gene	This study
pTB486	pIJ486 + 1506 bp *Xba*I + *Hind*III *S. clavuligherus ask* gene	This study







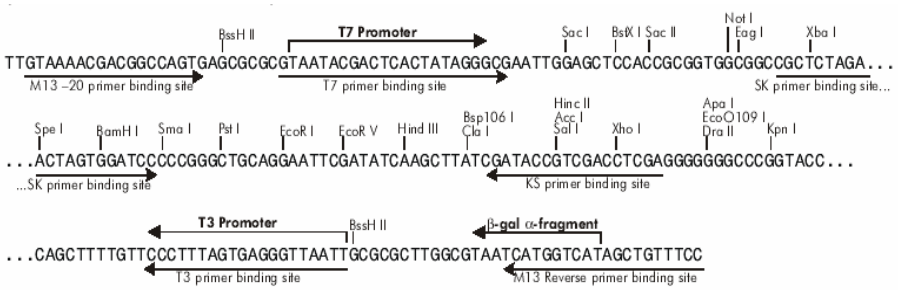
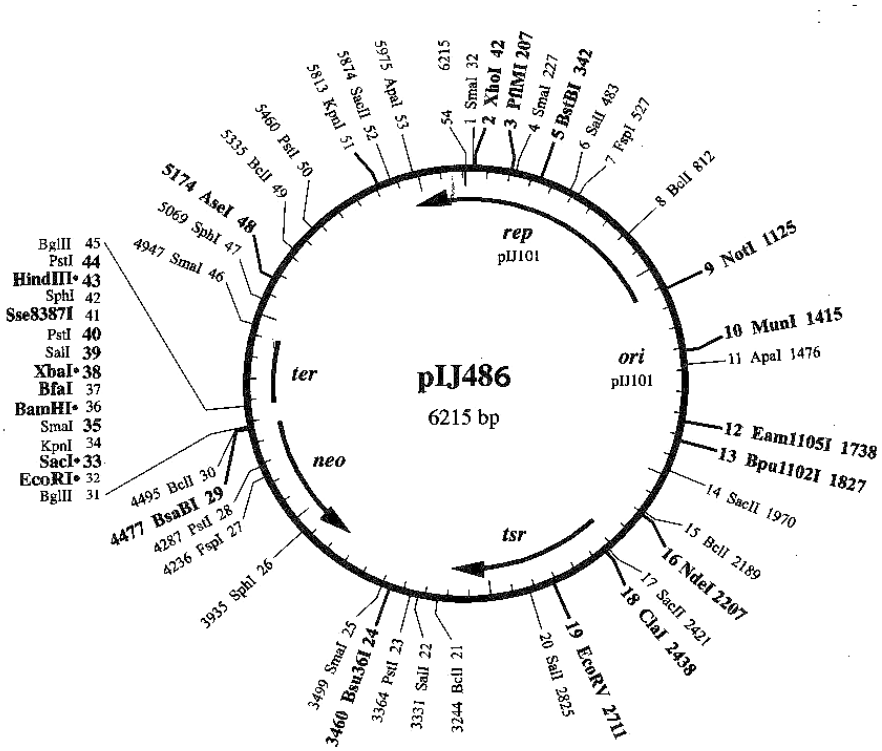


Figure 2.1. Maps and MCS sequences of pBlusecript II KS (+) and pGEM-T vectors.

a)



b)

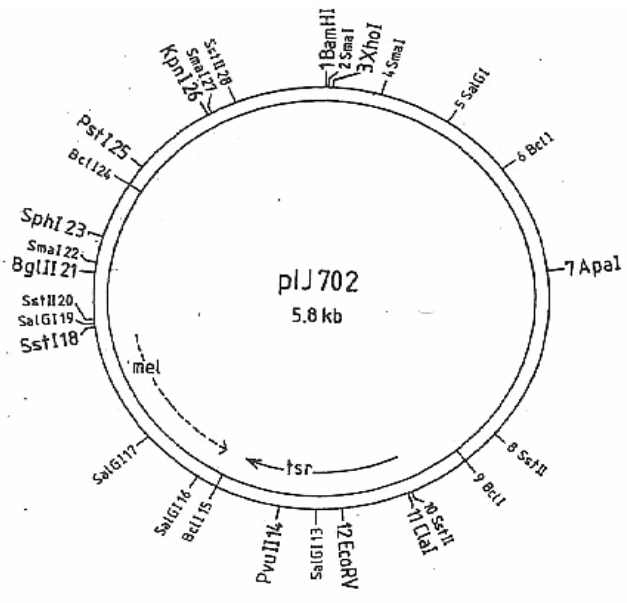


Figure 2.2. Structure of *Streptomyces* plasmids pIJ486 **(a)** and pIJ702 **(b).**

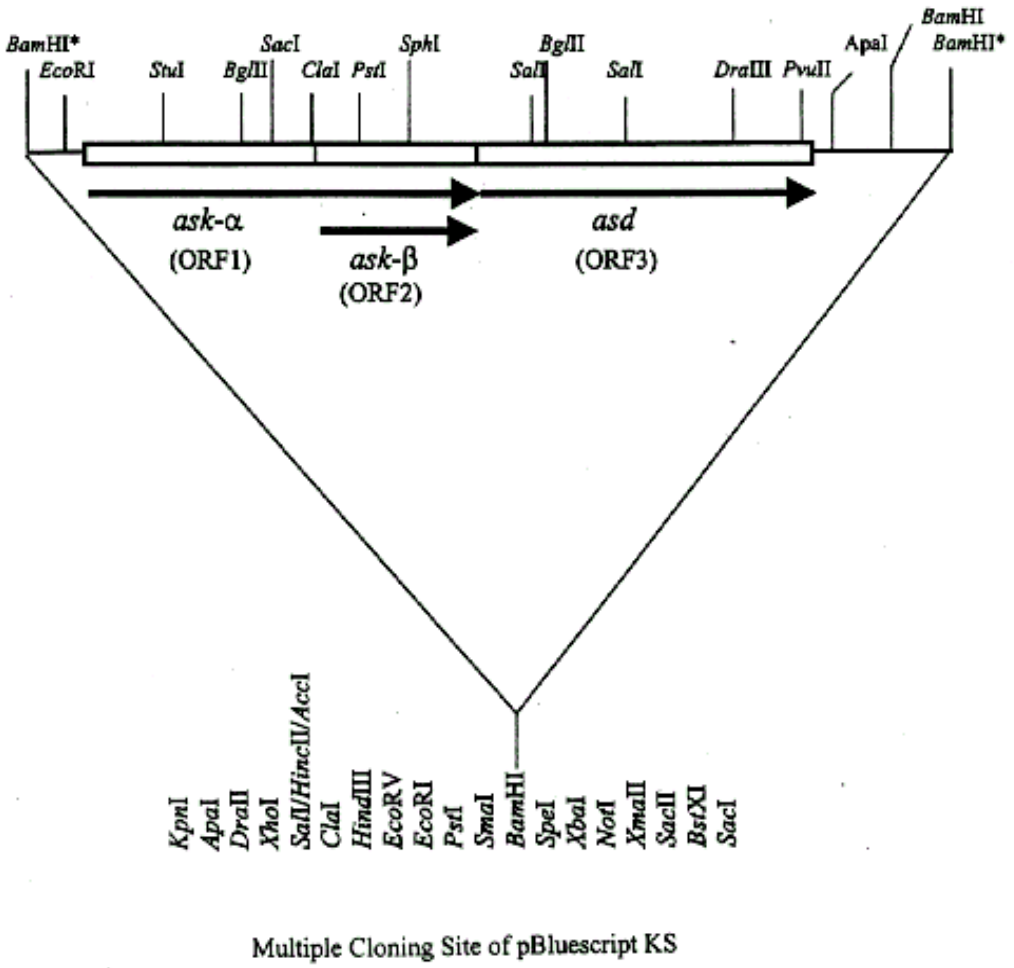


Figure 2.3. Map of selected restriction sites in the *S. clavuligerus* DNA insert of pNST102. The relative position and orientation of ORFs are indicated by bold arrows. *Restriction enzyme sites that lost during cloning (Tunca *et al*., 2004).

2.7. Transformation of *Streptomyces lividans* protoplasts

The procedure of Hopwood *et al.* (1985) was employed for transformation of *Streptomyces lividans* protoplasts. To 50 µL of protoplasts, plasmid DNA (2-5 µL; 0.05-1.0 µg) and 200 µL of 25 % PEG-1000 in P buffer were added and mixed by pipetting gently for three-four times. They were then allowed to

stand at room temperature for 1 minute. R2YE agar plates (Appendix A) dried for 2 hours in a laminar flow hood were spreaded with 125 µL of the transformation suspension and incubated at 30 ^{o}C. *S. lividans* cells were incubated for 18 hours, and then the plates were overlayered with soft nutrient agar (Appendix A) containing 50 µg/mL of thiostrepton. Incubation was continued at 30 ^{o}C for 2-4 days.

2.8. Transformation of *Streptomyces clavuligerus* protoplasts

The procedure of Kieser *et al*. (2000) was employed for transformation of *Streptomyces clavuligerus* protoplastas with modifications. To 50 µL of protoplasts, plasmid DNA (2-5 µL; 0.05-1.0 µg) and 500 µL of 50 % PEG-1000 in PE buffer without BSA were added and triturated gently for one time. They were then allowed to stand at room temperature for 1 minute and diluted with 1, 5 mL PE buffer. They were pelleted by centrifugation at 2000 g for 10 minutes. The pellets were resuspended in a final volume of 200 µL PE buffer. R2YE agar plates (Appendix A) dried for 2 hours in a laminar flow hood were spreaded with 200 µL of the transformation suspension and incubated at 26 ^{o}C. *S. clavuligerus* cells were incubated for 48 hours and then the plates were overlayered with soft nutrient agar (Appendix A) containing 8 µg/mL of thiostrepton. Incubation was continued at 26 ^{o}C for 3-4 days.

2.9. 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. 5- 50 mL of LB broth (Appendix A) was inoculated with *E. coli* from a fresh LB agar plate in a 250 ml flask and incubated overnight with shaking at 37 ˚C to obtain a stationary phase culture. 300 µL from this seed culture was inoculated into a fresh flask containing 50 mL LB

broth. The culture was incubated for 2- 2.5 hours at 37 ˚C with vigorous shaking around 300 cycles/minute in an orbital shaker to obtain an exponentially-growing culture. Then the culture was asepticaly split into two sterile pre-chilled 40 mL screwcap centrifuge tubes and stored on ice for 10 minutes. 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 resuspended in 5 mL ice-cold 10 mM $CaCl_2$ 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 resuspended very gently in 1 mL ice-cold 75 mM $CaCl_2$. The competent cells were stored at – 80 ˚C.

2.10. 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 prechilled Eppendorf 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. 0.9 mL 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.

2.11. Plasmid isolation from *Streptomyces*

Qiagen Plasmid Purification Kits (Qiagen Inc., Valencia, CA) were used for isolation of *Streptomyces* plasmid DNA. The first steps of the manufacturer's instructions were slightly modified. One square cm of cell mass was transferred from an R2YE agar plate into a 500 mL baffled flask containing 100 mL YEME (Appendix A), 10 µL $MgCl_2.6H_2O$ (2.5 M) and 1.25 mL 20 % glycine and incubated at 30 ^oC by continuous agitation on a rotary shaker at

250 rpm for 2 days. Then each culture was centrifuged at 6000 rpm for 20 minutes and the pellet was washed once with 10.3% sucrose. After a second centrifugation (6000 rpm, 20 min.), the pellet was frozen at –20 ^{o}C overnight. Next day, the cells were resuspended in 1.5 mL P1 buffer (from the kit) by vortexing until cell clumps were no longer visible, then 3 µg/mL lysozyme was added and the sample was incubated at 37^{o}C for 30 minutes. After addition of 1.5 mL P2 buffer, incubation was continued for at least 10 minutes at room temperature. From this point on, the procedure was as described by the manufacturer.

Streptomyces plasmid DNA was also prepared by the small scale plasmid DNA isolation method as described by Hopwood *et al.* (1985). Each cell was grown as patches or sectors (Figure 2.3) on selective medium, R2YE with selective antibiotic. About 1 square cm of cell mass was scraped with a sterile flat tootpick and put into an Eppendorf tube containing 50 µL TSE solution supplemented with 2 mg/mL lysozyme (Appendix B). After mixing the content of the tube, the mixture was incubated at 37 ^{o}C for 30 minutes. 3/5 volume of Lysis solution (Appendix B) was then added and immediately mixed by using vortex. The mixture was incubated at room temperature for 10 minutes to lyse cells, then at 70 ^{o}C for 10 minutes to denature DNA. The tube was then coolled rapidly by placing it in cold water. An equal volume of phenol-chloroform (water saturated, Appendix B) was added to the tube quickly and vortexed hard until the mixture became homogenous and milky white. Finally the phases were separated by spinning the mixture for 5 minutes at 13 000 rpm. A sample of supernatant (10 µL) was loaded directly on a gel to screen plasmid sizes.

Figure 2.4. A *Streptomyces* culture patched on an agar plate.

2.12. 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 manufacturers.

E. coli plasmid DNA was also prepared by using the plasmid minipreps 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 Eppendorf 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. Then, tubes were cooled rapidly in cold water. An equal amount of phenol-chloroform solution was added (water-saturated, Appendix B), vortexed hard until 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 loaded directly on an agarose gel for electrophoresis.

2.13. Manipulation of DNA

2.13.1. Restriction endonuclease digestions

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

2.13.2. Agarose gel electrophoresis

Agarose gels were used for DNA analysis at a concentration of 0.8-1.0 %. The final concentration of ethidium bromide in the gels was 0.5 μg/mL. Gels were run for 2-3 hours (at 6-9 Volts/cm). 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 marker (Appendix C) was used to determine the molecular weights of DNA bands.

2.13.3. Isolation 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. DNA in such gels was recovered according to the Qiagen's instructions. After recovery, an aliquot was run on agarose gel to assess the yield.

2.13.4. Ligations

Ligation reactions of inserts with pBluescript II KS (+), pIJ702 and pIJ486 vectors were performed as follows: 1 µL (10 units) T4 DNA ligase (MBI Fermentas), 1x ligation buffer (supplied by the manufacturer), with the insert to vector molar ratio of 3:1 to 5:1 were mixed and volume was completed to 20 µL with H_2O. Reaction mixtures were incubated at 4 ^oC or 16 ^oC overnight.

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 H_2O. Ligation was carried out as overnight incubation at 16 ^oC.

2.14. Primer design

Primers were designed in this study according to nucleotide sequence of the *S. clavuligerus ask-asd* operon (Tunca *et al*., 2004; Figure 2.5). The nucleotide sequences of the primers and expected product size are given in Table 2.2. The designed primers were synthesized by the Iontek Company (İstanbul, Turkey).

Table 2.2. Primers used for the amplification of the *ask* gene.

Name of the primer	Sequence of the primer	Expected product size (bp)
ASKFW	5' TTCTAGAGTTCGTCCGGCTGCCGGT 3'	1506
ASKREV	5'GGATATCCTACCGCCCACTTCCCGA 3'	1506

ASKFW

```
   1 cgtcccggcg ttcgtccggc tgccggtgga tgcgcaccgc cggaattccc gtcacggccc
  61 gccccggccg cgccggtgag gtcggtcacc gatgtgctgg acacgccgga cagcctagtc

 121 gagcaggcca ttcgcagtgg tcgcggccac acgacggcac gcccggacgg cccgatcccg
 181 gtaggctcac cgactgagtc gaattcgatg gagcgggctc ggcgcccgct cggcgaggag

 241 gttcgggccg tggcgctcgt agtccagaag tacggcggat cgtcgttgga gagcgctgat
 301 cggatcaagc gcgtggccga gcggatcgtg gccacgaaaa aggccgggaa cgaggtggtg

 361 gtggtctgct cggcgatggg cgacaccacc gacgaactgc tcgacctggc ccagcaggtg
 421 aacccggttc cgccggagcg ggagatggac atgctgctca ccgcgggtga gcgtatctcc

 481 aacgcgctgg tcgcgatggc catctccgcc cagggcgcgc aggcctggtc gttcaccgga
 541 tcgcaggcgg gggtggtgac cacggccgtg cacggcaacg cgcggatcat cgacgtcacc

 601 ccgagccggg tcagcgaagc actggaacag ggttacgtcg cgctggtcgc cgggttccag
 661 ggcgtggcgc aggacaccaa ggacatcacc acgctcggcc gcggtggctc ggacaccacc

 721 gcggtggcgc tggccgcggc gctcaacgcc gacgtgtgcg agatctactc cgatgtggac
 781 ggtgtgtact cggccgaccc ccgcatcgtg ccggacgcgc gcaagctgga ttccgtgccg

 841 tacgaggaaa tgctggagct cgcggcgagc ggctcgaaga tcctgcacct gcggtcggtg
 901 gagtacgcgc ggcgctacgg cgtgccgatc cgagtccgtt cttcctacag tgacaagccg

 961 ggcacgacgg tgaccggatc gatcgaggag atccccgtgg aacaagcgtt gatcaccggt
1021 gtggcgcacg accgctccga agccaagatc acggtgaccg gggtgccgga ccacgccggt

1081 gccgccgccc ggatcttccg ggtgatcgcc gacgccgaga tcgacatcga catggtgctg
1141 cagaacgttt ccaacacctc gtcggggcgc acggacatca ccttcacgct gtcgaaggcc

1201 aacggcccca aggcggtcgc gtcgctggag aagatcaagg aggagctggg cttctcctcg
1261 gtgctctacg acgaccacgt cggcaaggtc tccctggttg gcgccggcat gcgctcgcac

1321 cccggggtca ccgccacctt ctgcgaagcc ctgtccaagg tcggggtgaa catcgagatc
1381 atcaacacct ccgagatccg catctccgtg ctgatccggg acgcgcagct cgacgacgcg

1441 gtgcgcgcca tccatgacgc attcgaactg ggcggcgacg aagaagccgt cgtctacgcg
1501 ggaagtgggc ggtagaaatg gctcctgttc tggctctggt cggtgccacc ggcgcggtgg
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ASKREV

```
1561 gcaccgtgat gatcgacatc atcaacaacc gggaaaccgt gccgtggggc gaaatccggc
1621 tgatcgcgtc ggcgcgctcg gcgggcaaga agctcaccgt gcgcggcgag gagctgaccg

1681 tcatcgagct gaccgcggaa gccttcgacg gcgtcgacgt ggcgatgttc gacgtgcccg
1741 acgagatctc cgcggaatgg gcgccggtcg cggcggcccg tggcgcggtg gccgtggaca

1801 actccggcgc cttccggatg gacgacgacg tgccgctggt ggtccccgag gtcaacgcgg
1861 acaaggtcgg cgagcgcccg cgcggcatca tcgcgaaccc gaactgcacc acgctgtcga

1921 tgatggcggc gctgggcgcg ctgcaccgcg agttcgagct gaaggaactc gtggtcgcct
1981 cgtaccaggc ggtttccggt gctggcaagg aaggcgtcga ccggctgtac gccgaactcg

2041 aagcggtcgc gggcaagccg gtcggggtgt ccgccggtga cgtgcgcaag acgctggagg
2101 cggccggtct gtccatttcg gactccccgt tcccggcgcc gctggcgttc aacgtggtgc

2161 cctcggccgg ttcctacaag ggcgacggct ggtactccga agaactcaag gtgcgcaacg
2221 aatcccgcaa gatcctgggc atcccggacc tgaaggtctc ggcgacctgc gtgcgcgtcc

2281 cggtggtcac cacgcattcg ctggccgtgc acgccacctt cgcgcgcgag gtcaccgtgg
2341 aggaagcgca caaggtgttc gaagcgcagc cgaccatcgt gctggtggac gacccggaga

2401 acggcgtgtt cccgacgccc gccgaggtcg tcggcgagga cccgacctac gtcggccggg
2461 tgcgccaggc gctggacttc ccgaacacgc tggacttcct tcgtgtgcgg ggacaacctg

2521 cgcaaggggc ggcggctgaa cacctacgag atgcggagac gctcgccccg cagctgggct
2581 gaccggttcg tctgggaagg ccgtgggtgc ccctcaccca cggccttttc cgcgcccgga
2641 tcaggggcgg cgggcccggc ggcaggcttc gccgcgggcg agc
```

Figure 2.5. Nucleotide sequence of *S. clavuligerus ask-asd* operon (Gene Bank AY112728). The sequences used in primer design are boxed and indicated bt the arrows.

2.15. Polymerase chain reaction

PCR was performed in a total volume of 50 µL total volume. PCR mixtures contained 28 µL of dH_2O, 5 µL of 10 x PCR buffer without Mg^{+2}, 6 µL $MgCl_2$, 1 µL primer (50 pmol of each), 1µL 10 mM dTTP, 1µL 10 mM dATP, 1µL 10 mM dCTP, 1µL 10 mM dGTP, 50 ng of pNST102 vector plasmid as template, 2 µL of DMSO and 1 Unit Taq Polymerase. The PCR cycles and conditions were as follows: PCR for the amplification of 1520 bp *ask* gene was carried out at 30 cycles. Initial denaturation was performed at 95 ˚C for 10 minutes. All cycles were carried out as 1 min at 95 ˚C, 1 min at 63 ˚C and 2 min at 72 ˚C for denaturation, annealing and extension, respectively. Reaction mixtures were run in a 0.9 % agarose gel. PCR products were extracted from the gel as mentioned in Section 2.13.3. MBI Fermentas PCR kit was used in this procedure.

2.16. Construction of growth curves

S. clavuligerus spores were inoculated into 250 mL triple-baffled flasks containing 50 mL of Trypticase Soy Broth (TSBS) medium (Appendix A) (30 g/L, pH 7.2). After optical density OD_{595} of a suspension of broken mycelia reached to ~0.3, 2.5 mL of this seed culture was inoculated into 500 mL triple-baffled flasks containing 100 mL of TSBS medium. Incubation was performed in an orbital incubator at 250 rpm over 96 hours at 28 ˚C. Samples were taken at time intervals (6 h) and supernatants were used to determine cephamycin C production.

Cultural growth was monitored by measuring the optical density of a suspension of broken mycelia at 595 nm (OD_{595}) according to the procedure of 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 for 30 seconds. The OD_{595} of the resulting suspension was then measured. The cultures were diluted appropriately in order to measure within the range where the turbidities of the culture are proportional to the dry cell weights (DCW) in our calibration curve.

2.17. Determination of DCW and construction of the DCW versus OD_{595} calibration curve

Cellulose filter membranes of 0.45 µm pore size were dried in an oven at 85 ˚C for overnight. Then they were weighed and stored in a dessicator. A sample of 48 hours culture was diluted to 5X, 10X, 15X, 20X and 40X. 10 mL of these 5 dilutions along with an undiluted sample were poured separately into the holding reservoir fitted on the dried, pre-weighed filter membrane. A vacuum was applied to pull the liquid through the membrane. The reservoir was rinsed with a few mL of water. The cell paste was then dried in an oven at 85 ˚C for 24 hours and stored in a dessicator. Filter plus the cell paste measurement was made and finally the difference in the weight was taken as dry cell weight (mg/mL). The OD_{595} of the samples with the same dilutions was measured according to procedure given by Malmberg *et al*. (1993). DCW versus OD_{595} calibration curve is given in Figure 2.6.

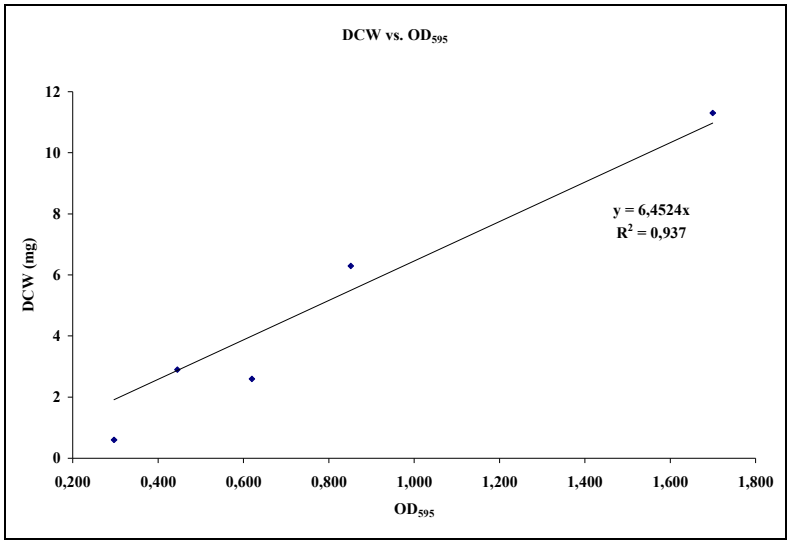

Figure 2.6. DCW versus OD_{595} calibration curve.

2.18. Cephamycin C bioassay

Cephamycin C bioassay was performed according to the procedure of Jensen *et al.* (1982) with minor changes. It involved the use of the agar-diffusion method with *E coli* ESS (a β-lactam-supersensitive mutant) as the indicator organism. 10 mL of soft NB agar (NB + 0.5% (w/v) agar) (Appendix A) containing 1.5 mL *E. coli* ESS glycerol stock (diluted 1:1 with 50% (w/v) glycerol) was poured into a bioassay plate containing 15 mL NB agar (NB + 1.5 (w/v) agar) (Appendix A). Oxoid bioassay disks (England) were placed on the surface of a sterile, empty petri plate cover. 20 µL of culture supernatant was impregnated into the disks. 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 after overnight growth. Cephamycin C production by both parental and recombinant *S. clavuligerus* cells was determined by using the cephamycin calibration curve given in Figure 2.7.

2.19. Construction of cephamycin C bioactivity calibration curve

For preparing the cephamycin C bioactivity calibration curve, cephalosporin C was used as the standard. Cephamycin C calibration curve was constructed using the agar-diffusion method with *E coli* ESS-2231 (a β-lactam-supersensitive mutant) as the indicator organism. 10 mL of soft NB agar (NB + 0.5% (w/v) agar) (Appendix A) containing 1.5 mL *E. coli* ESS glycerol stock (diluted 1:1 with 50% (w/v) glycerol) was poured into a bioassay plate containing 15 mL NB agar (NB + 1.5 (w/v) agar) (Appendix A). Oxoid bioassay disks (England) were placed on the surface of a sterile, empty petri plate cover. Then 20 µL of 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180 and 200 µg/mL of cephalosporin C solution (Appendix B) was spotted on the disks. After the soft agar was solidified, the disks were placed on the agar surface and plates were incubated overnight at 37 ˚C. One unit of β-lactam produced an inhibition zone equivalent to that formed by 1 g of cephalosporin C. Zones of inhibition of growth were measured as millimeter after overnight growth.

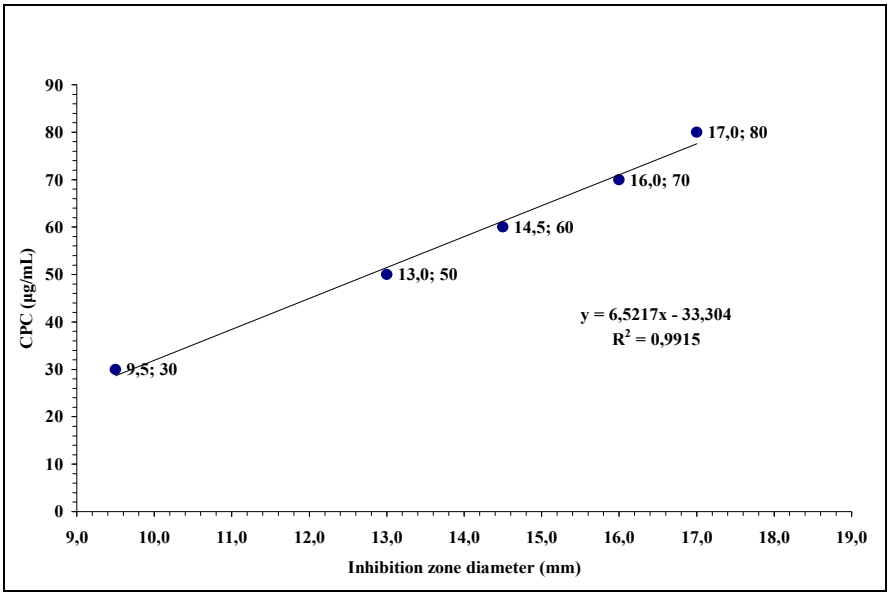

Figure 2.7. Cephamycin C bioassay calibration curve.

2.20. Measurement of aspartokinase (Ask) activity

Measurement of aspartokinase (Ask) activity was performed according to the procedure of Follettie *et al.* (1993) with minor changes. Both parental and recombinant *Streptomyces clavuligerus* cells were grown in 50 mL Trypticase Soy Broth (TSBS) medium (Appendix A) (30 g/L, pH 7.2) at 28 ˚C for 30 and 54 hours and harvested by centrifugation (8 000 rpm, 15 min, 4 ˚C) in a Sigma centrifuge. After the cell pellets were washed with 25 mL of 50 mM cold phosphate buffer (Appendix B) (pH 7.5), crude extracts were prepared by resuspending the cell pellets in 5 mL of 10 mM sonication buffer (Appendix B) (pH 7.5). For disruption, cells were sonicated for 3 min. with 1 min. intervals (10 W with 50% pulsing). Cell debris was subsequently removed by centrifugation.

Ask 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) (pH 7.6). 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. Crude protein extract was added, the reaction mixture was incubated for 60 min. at 30 ˚C, and the reaction was terminated by the addition of 0.75 mL of ferric chloride solution (Appendix B). After centrifugation for 5 min. to remove protein debris, the 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 2.8. Background activity measured in the absence of added substrate was subtracted and Ask specific activity was reported as nanomoles of aspartate-hydroxymate formed per milligram protein per minute.

2.21. Determination of protein concentration

Protein concentrations were measured by the Bradford quantification method (1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 at 595 nm when binding to protein occurs. 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. Bovine serum albumin (BSA) was used as the standard for preparation of protein calibration curve. Volumes of 40, 60, 80, and 100 µL of 1 $mg.mL^{-1}$ BSA were added to tubes and volumes were adjusted to 500 µL with water. 500 µL of distilled water was added into a tube as reagent blank. 4.5 mL of assay reagent was added to each tube and mixed gently, but thoroughly. A standard curve of absorbance versus milligrams protein was prepared and the amounts of proteins were determined from the slope of the curve (Figure 2.9).

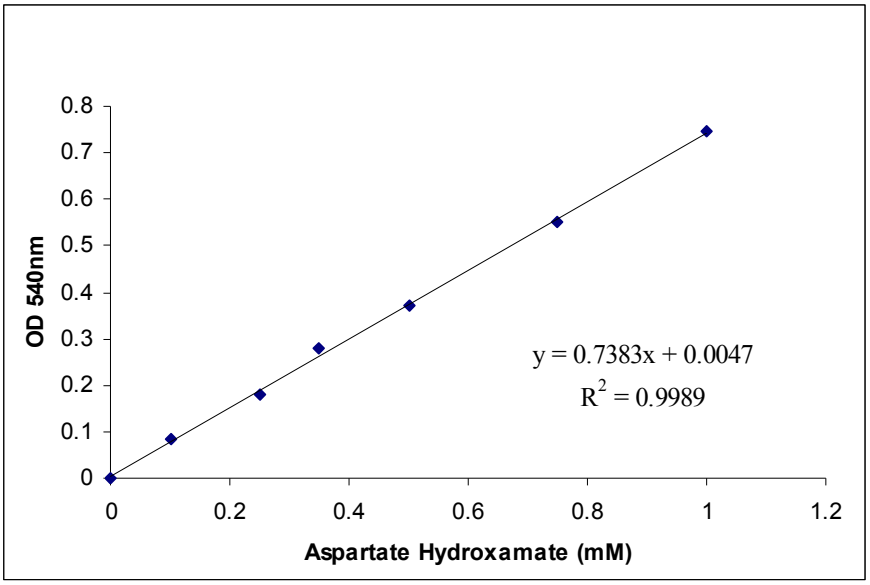

Figure 2.8. Absorbance as OD_{540} versus aspartyl hydroxymate concentration (mM) calibration curve.

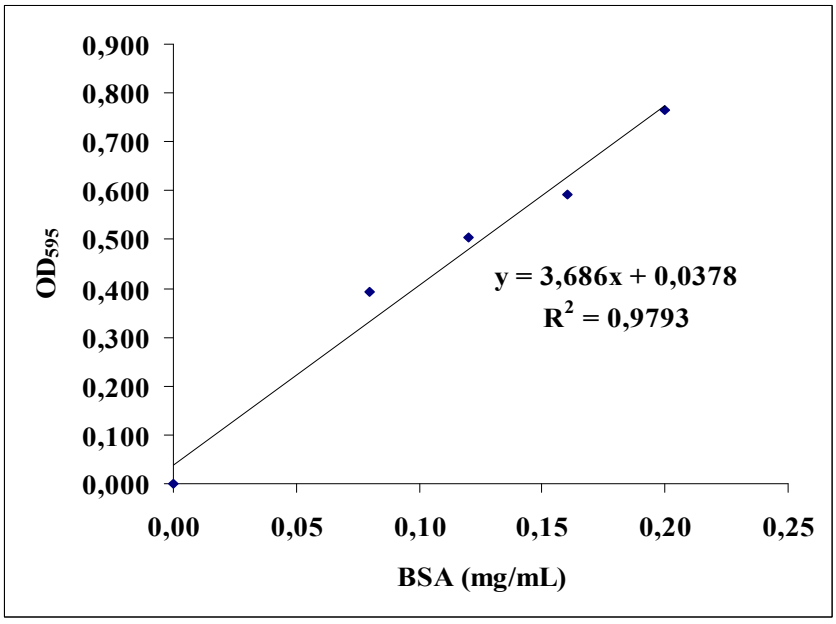

Figure 2.9. Protein determination calibration curve.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Insertion of *ask-asd* into a *Streptomyces* plasmid pIJ702 as the cloning vector and transformation of *S. lividans* and *S. clavuligerus* with recombinant plasmid

The aim of this study was to subclone the *ask-asd* genes of *S. clavuligerus* into a *Streptomyces* vector, namely pIJ702 and expression of the cloned genes in *Streptomyces* under their own promoter.

The *Streptomyces* plasmid pIJ702 has been used widely in cloning experiments (Lee *et al.*, 1986). One useful feature of pIJ702 is the presence of the genetic elements that facilitate screening of clones. Selection of transformants is based on either their resistance to the antibiotic thiostrepton (tsR^+) or insertional inactivation of the melanin gene (*mel*$^-$) (Gusek and Kinsella, 1992) (Figure 2.2.b). Earlier attempts in our laboratory to clone *ask-asd* cluster into *S. clavuligerus* using this vector resulted in either the loss of the insert and/or plasmid rearrangement in recombinants (Tunca, 2002). In these studies, the *ask-asd* cluster having their own promoter was inserted downstream of the promoter of melanin (*tyr*) gene. The consistent difficulty encountered was thought to be related with cloning into *mel* gene of pIJ702 and expression of the cluster under multiple tandem promoters. In the present study,

cloning into *Bam*HI-*Kpn*I site of pIJ702 was planned since this kind of manipulation was thought to prevent expression from another promoter.

Since both *Bam*HI sites of the pNST102 vector were lost during cloning of the *ask-asd* insert into pBluescript KS at the unique *Bam*HI site, it was not possible to take out the exact insert from pNST102 with *Bam*HI digestion. Nucleotide sequencing of the insert showed that there was one *Bam*HI site within the insert at the downstream of the *asd* gene (Tunca, 2002) (Figure 2.3). The *ask-asd* insert was excised from pNST102 by *Bam*HI-*Kpn*I (at the MCS of the vector; Figure 2.3) digestion (Figure 3.1). After extraction from the gel, the fragments were treated with alkaline phosphatase in several dilutions (Figure 3.3). The pIJ702 vector was also digested with the same enzymes and extracted from the gel (Figure 3.2). Ligation reaction was checked on an agarose gel before each transformation (Figure 3.4). The constructs were used to transform both *S. lividans* 1326 as an intermediate and easily transformable host which presents less severe restriction barriers than *S. clavuligerus* to foreign DNA and *S. clavuligerus* as the target host. In every of several attempts, pIJ702 was recovered without any insert despite high transformation efficiencies (Figure 3.5).

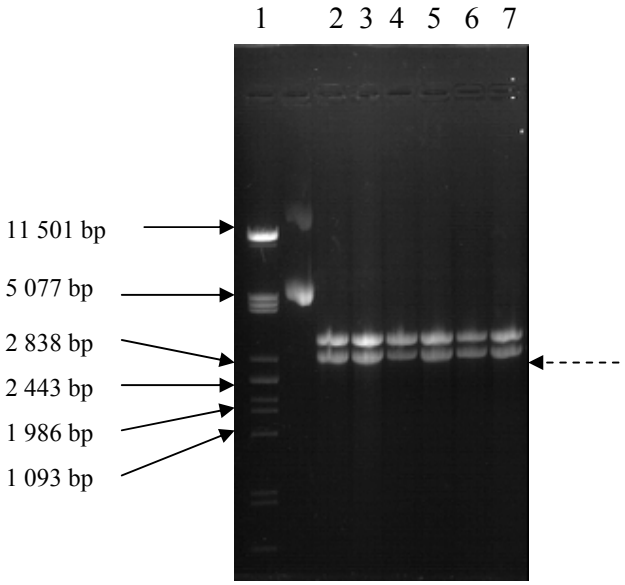

Figure 3.1. Digestion of pNST102 with *Bam*HI and *Kpn*I.

1) Lambda *Pst*I marker, 2-7) pNST102 *Kpn*I + *Bam*HI digest. Fragment that carries *ask-asd* insert is shown by dashed arrow.

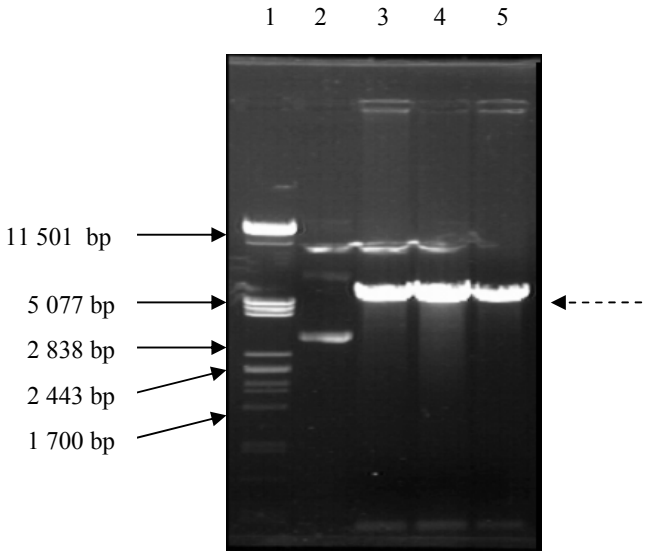

Figure 3.2. Digestion of pIJ702 with *Bam*HI and *Kpn*I

1) Lambda *Pst*I marker, 2) pIJ702, 3, 4, 5) pIJ702 *BamH*I + *Kpn*I digest. Dashed arrow shows *Bam*HI-*Kpn*I digested pIJ702 fragments

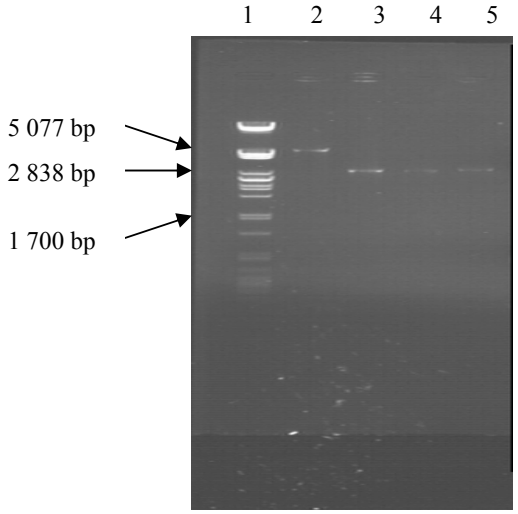

Figure 3.3. Relative concentrations of insert and vector before ligation.

1) Lambda *Pst*I marker, 2) pIJ702 *Bam*HI + *Kpn*I digest, 3) *ask-asd* insert without alkaline phoshatase treatment, 4) *ask-asd* insert treated with 1:1 diluted alkaline phosphatase, 5) *ask-asd* insert treated with 1:100 diluted alkaline phosphatase.

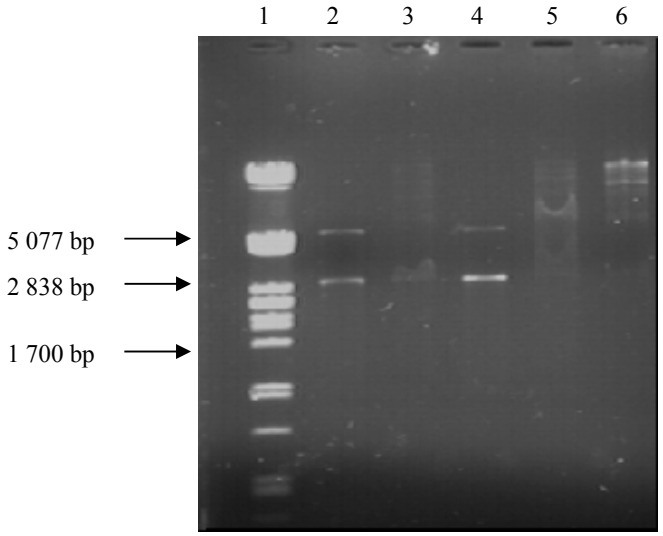

Figure 3.4. Ligation of *ask-asd* cluster to pIJ702 vector.

1) Lambda *Pst*I marker, 2, 4) *ask-asd* insert and pIJ702 vector before adding ligase enzyme, 3) Ligation of *Bam*HI-*Kpn*I *ask-asd* fragment from pNST102 treated with 1:1 diluted alkaline phosphatase to pIJ702 digested with the same enzymes, 5) Ligation of the same fragment treated with 1:100 diluted alkaline phosphatase to pIJ702 vector, 6) Ligation of the same fragment without alkaline phosphatase treatment to pIJ702 vector.

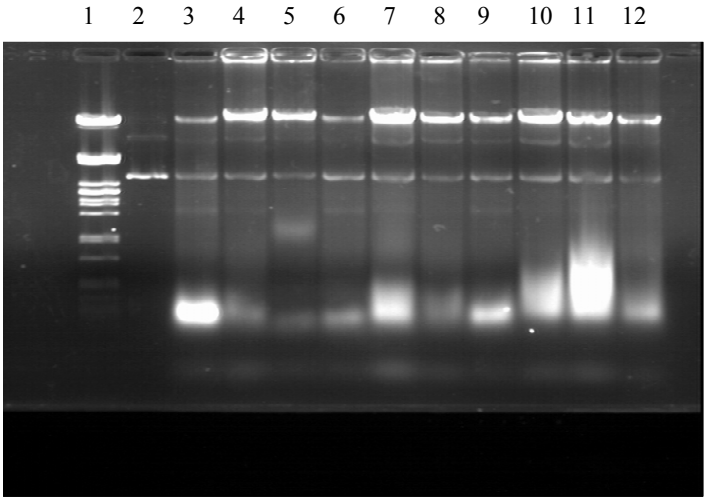

Figure 3.5. Plasmid isolation from different putative *Streptomyces* recombinants.

1) Lambda *Pst*I marker, 2) pIJ702, 3-12) Plasmids from different transformants.

3.2. Insertion of *ask* only into pIJ702 after amplification in a methylation deficient *E. coli* host and transformation of *S. lividans* and *S. clavuligerus* with recombinant plasmid

Increasing the stability of the recombinant vector in *Streptomyces* by some manipulations was our second strategy. This strategy included reducing the size of the insert fragment and amplification of the resulting recombinant in a methylation-deficient *E. coli* strain before transforming the *Streptomyces* host to avoid methyl-specific restriction system in *Streptomyces*. We decided to reduce the size of the operon by removing most of the *asd* fragment by restriction enzyme digestion. The reason for this manipulation was that there have been no experimental results about a key role of Asd enzyme on pathway activity. In our previous studies, end-product amino acids of aspartate pathway and DAP had no significant effect on Asd activity (Tunca *et al*., 2004).

Nucleotide sequencing of the insert showed that there was one *Eco*RI site at the upstream of the *ask* gene and two *Sal*I sites within the *asd* gene at the downstream of the *ask* gene (Tunca, 2002) (Figure 2.3). Therefore, we decided to use *Eco*RI and *Sal*I for reducing the insert size. Accordingly, pNST102 was digested with both *Eco*RI and *Sal*I and from the two fragments of DNA seen in the gel, the smaller one (1670 bp) being the *ask* gene was extracted from the gel, thus getting rid of the bigger one which was the rest of the recombinant plasmid (Figure 3.6). The *E. coli* pBluescript II KS (+) vector also was digested with the same enzymes (at the MCS of the vector; Figure 2.3) and extracted from the gel. The *ask* gene (1670 bp) that contained *Eco*RI and *Sal*I sites was ligated to the gel extracted pBluescript II KS *Eco*RI-*Sal*I vector. *E. coli* ET12567 competent cells were then transformed with the ligation mixture and recombinant colonies (white ones) were detected on LB agar containing ampicillin (100 µg/mL), X-gal (40 µg/mL) and IPTG (40 µg/mL).

Screening of a few white colonies revealed three recombinant plasmids (Figure 3.7). Restriction enzyme analysis of first recombinant plasmid verified the 1670 bp *ask*

*Eco*RI-*Sal*I insert and the rest of the 2974 bp pBluescript II KS vector (Figure 3.8). The size of the newly formed vector, designated pBT KS1, was 4644 bp as expected.

The *ask* insert was excised from pBT KS1 by *Bam*HI-*Kpn*I (at the MCS of the vector; Figure 2.3) digestion (Figure 3.9). After extraction from the gel, it was ligated to pIJ702 which was digested with the same enzymes. The constructs were used to transform both *S. lividans* and *S. clavuligerus*. Again in every of several attempts, pIJ702 was recovered without any insert despite high transformation efficiencies (Figure 3.10).

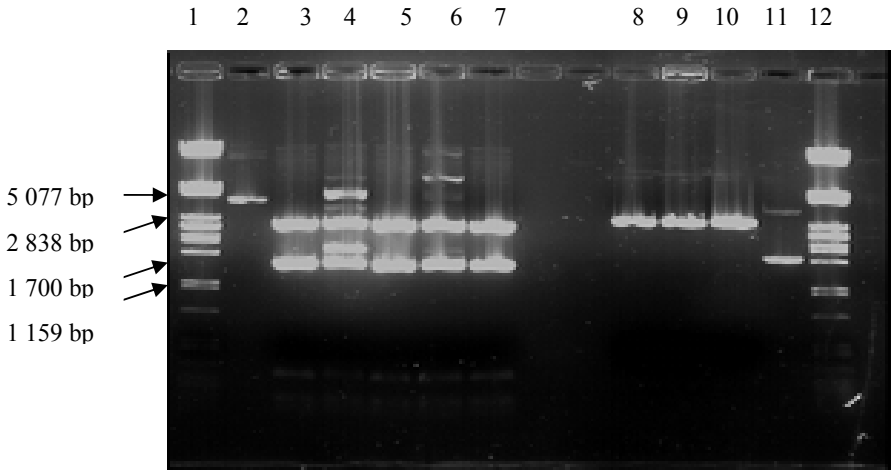

Figure 3.6. Digestion of pNST102 and pBluescript II KS (+) with *Eco*RI and *Sal*I.

1) Lambda *Pst*I marker, 2) pNST102, 3-7) pNST102 *Eco*RI + *Sal*I digest, 8-10) pBluescript II KS (+) *Eco*RI + *Sal*I digest, 11) pBluescript II KS (+), 12) Lambda *Pst*I marker.

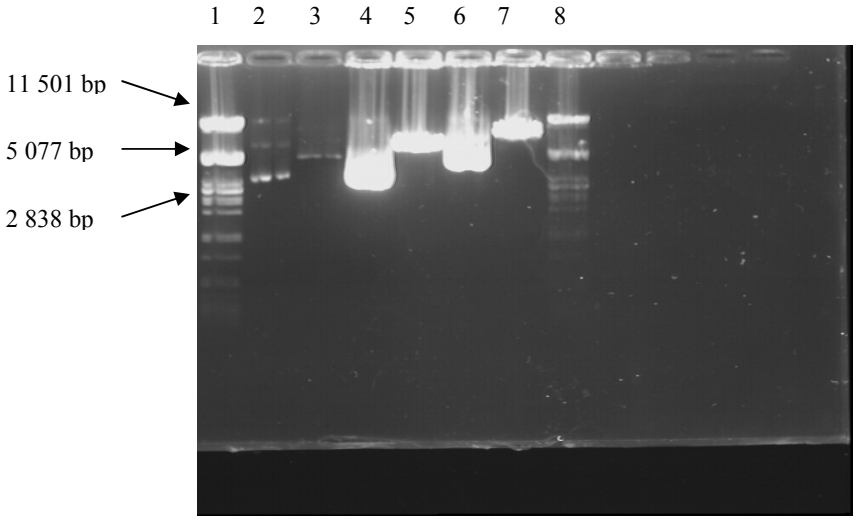

Figure 3.7. Plasmid isolation from different *E. coli* recombinants.

1) Lambda *Pst*I marker, 2) Plasmid from recombinant 1, 3) Plasmid from recombinant 1 *Eco*RI digest, 4) Plasmid from recombinant 2, 5) Plasmid from recombinant 2 *Eco*RI digest, 6) Plasmid from recombinant 3, 7) Plasmid from recombinant 3 *Eco*RI digest, 8) Lambda *Pst*I marker.

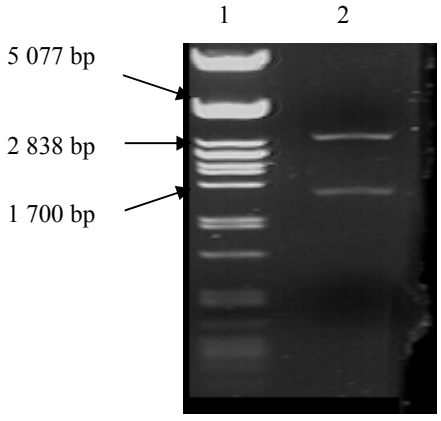

Figure 3.8. Digestion of pBT KS1 with *Eco*RI and *Sal*I to confirm the presence of *ask* insert.

1) Lambda *Pst*I marker, 2) pBT KS1 *Eco*RI + *Sal*I digest.

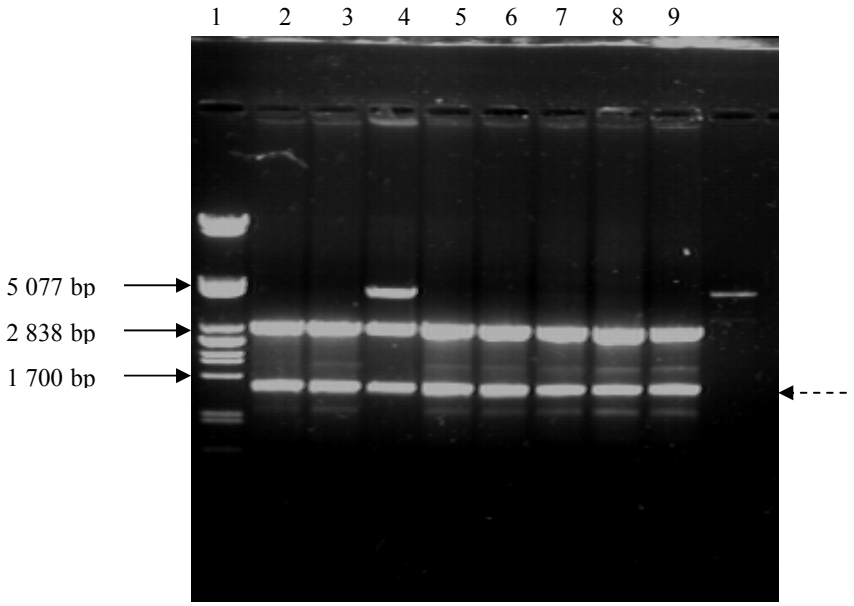

Figure 3.9. Excision of *ask* insert from pBT KS1 with *Bam*HI and *Kpn*I.
1) Lambda *Pst*I marker, 2-9) pBT KS1 *Bam*HI + *Kpn*I digest. Ask insert is shown by the dashed arrow.

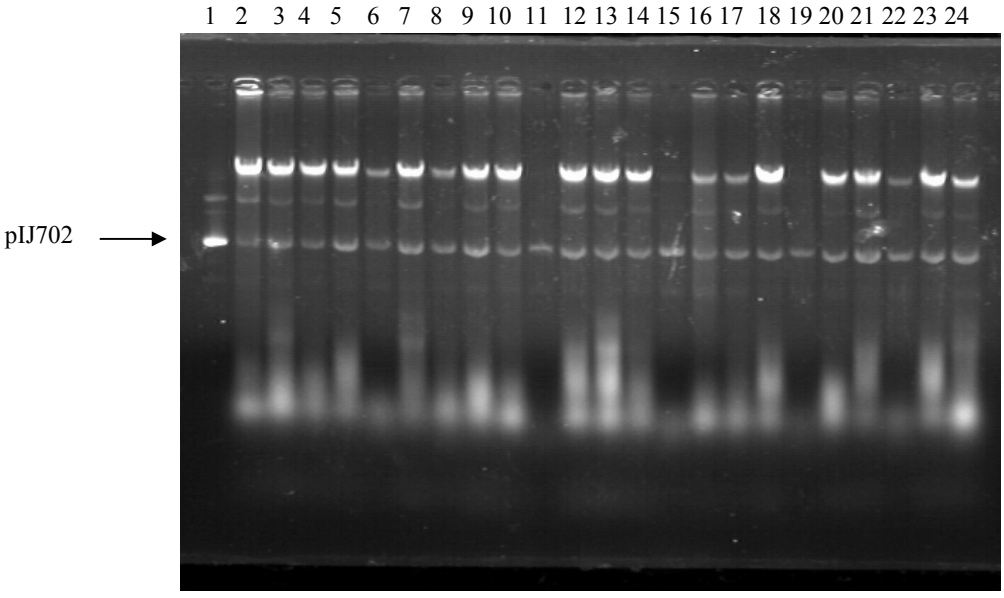

Figure 3.10. Plasmid isolation from different *Streptomyces* recombinants.

1) pIJ702, 2-24) Plasmids from different transformants.

pIJ702 is one of the most widely used vectors for gene cloning and expression in *Streptomyces*, however, structural instability of recombinant pIJ702 and its derivatives were reported earlier (Lee *et al*., 1986; Yang *et al*., 2001). There arose occasions where this vector is problematic (Gusek and Kinsella, 1992). A recombinant plasmid pWCL1 containing *Streptomyces* plasmid pIJ702, *E. coli* plasmid pUC12, and hepatitis B viral surface antigen (HBsAg) gene was stably maintained in *E. coli*, but exhibited structural instability in *S. lividans* 1326. The deletions were found ranging from 2.75 to 5.65 kb and most of them occurred within the melanin (*mel*) gene of pIJ702, resulting in the loss of part of the *mel* gene sequence plus the insert. The removal of the pUC12 sequence from pWCL1 eliminated the instability. However, pUC12 alone inserted in either orientation on pIJ702 also caused the deletion in *S. lividans* 1326. The results indicated that the structural instability of hybrid plasmid of pIJ702 dependent on the interaction between the *mel* sequence and the inserted sequence (Lee *et al*., 1986). Yang *et al* (2001) cloned glucose isomerase gene from *Streptomyces diastaticus* into *mel* gene (*Pst*I-*Bgl*II) of pIJ702 and selected the recombinants as based on insertional inactivation of melanin phenotype, i.e. white colonies. They also reported the existence of pIJ702 deletion, presumably owing to the interaction between the melanin and cloned gene sequence. They could not much understand the molecular mechanism of these deletions, but it might involve an inverted repeat sequence on either side of the *Bgl*II site. In the present study, we neither used *mel* gene to clone within, nor we had such inverted repeat sequences in our insert. Moreover, the *ask-asd* insert was quite stable in *E. coli* (Tunca *et al*., 2004). Thus, our study constitutes the first report on recombinant pIJ702 instability when homologous fragment was cloned in between *Bam*HI and *Kpn*I sites, i.e. a site quite far from *mel* gene.

3.3. Cloning of *ask* gene in *S. clavuligerus* using pIJ486 as the vector

When our attempts to subclone *ask-asd* cluster and *ask* gene into pIJ702 gave no positive results, we therefore decided to use another multicopy *Streptomyces* vector which was pIJ486 containing promoterless *neo* (neomycin) and functional *tsr*

(thiostrepton) genes. The selection of transformants is based on their resistance to the antibiotic thiostrepton.

We used PCR approach to amplify the *ask* gene. For this purpose, a set of primers was designed according to nucleotide sequence of the *S. clavuligerus ask-asd* operon (Figure 2.5). PCR with primers Askfw and Askrev (Table 2.2) yielded a 1506 bp fragment with *Xba*I and *Eco*RV sites by using pNST102 plasmid DNA as template.

After purification from the gel, *ask* gene was cloned into the pGEM-T vector and amplified in *E. coli* DH5α cells. Figure 3.11 shows the recombinant plasmids obtained from the ligation of pGEM-T and 1506 bp *ask* insert with *Xba*I-*Eco*RV sites. One of the plasmids which designated pGEM-T1 was digested with *Xba*I and *Eco*RV to release the 1506 bp insert (Figure 3.12).

Since MCS of pIJ486 does not contain *Eco*RV site, we could not subclone our *Eco*RV-*Xba*I insert into MCS of pIJ486 vector directly. To overcome this problem, we decided to subclone of *Eco*RV-*Xba*I insert first into the MCS of pBluescript II KS (+), then to extract the insert with suitable unique restriction sites which do already exist in the MCS of pIJ486 vector. For this purpose pGEM-T1 was digested with both *Eco*RV and *Xba*I and the *ask* gene was extracted from the gel. The *E. coli* pBluescript II KS (+) vector also was digested with the same enzymes (at the MCS of the vector; Figure 2.3) and extracted from the gel. The *ask* gene (1506 bp) that contained *Eco*RV and *Xba*I sites was ligated to the gel extracted pBluescript II KS *Eco*RV-*Xba*I vector. Then methylation-deficient mutant *E. coli* ET12567 competent cells were transformed with the ligation mixture and recombinant colonies (white ones) were detected on LB agar containing ampicillin (100 µg/mL), X-gal (40 µg/mL) and IPTG (40 µg/mL).

Screening of a few white colonies revealed three recombinant plasmids (Figure 3.13). Restriction enzyme analysis of one of the recombinant plasmids verified the 1506 bp *ask Eco*RV-*Xba*I insert and the rest of the 2965 bp pBluescript II KS vector

(Figure 3.14). The size of the newly formed vector, designated pBT KS2, was 4471 bp as expected.

The *ask* insert was excised from pBT KS2 by *Xba*I-*Hind*III (at the MCS of the vector; Figure 2.3) digestion (Figure 3.15). After extraction from the gel, it was ligated to pIJ486 which was digested with the same enzymes. The constructs were used to transform *S. clavuligerus*. The size of the plasmid from one of the transformants was bigger than the size of pIJ486 (Figure 3.16).This plasmid was digested with *Xba*I and *Hind*III to release and verify the *ask* insert (Figure 3.17). PCR with primers Askfw and Askrev (Table2.2) also verified the 1506 bp *ask* fragment by using the newly formed recombinant vector, designated pTB486, as template (Figure 3.18).

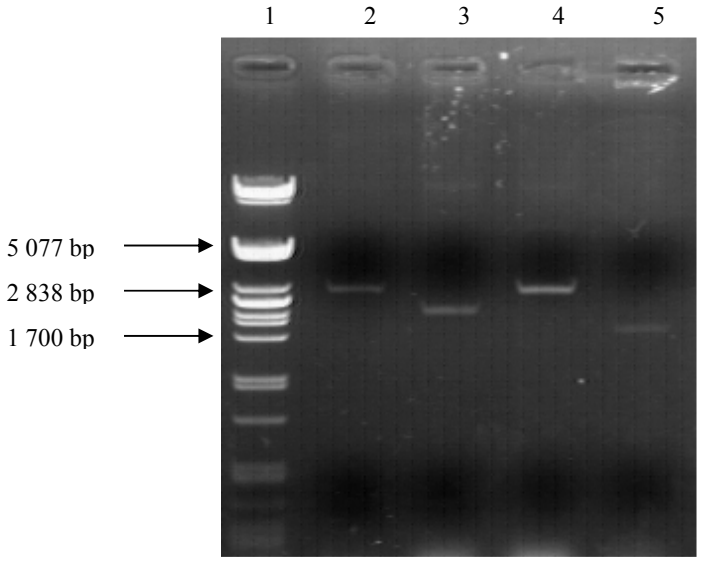

Figure 3.11. Analysis of the plasmids of the putative *E. coli* recombinants carrying the 1506 bp *ask* amplicon.
1) Lambda *Pst*I marker, 2-4) Recombinant pGEM-T vectors, 5) Native pGEM-T vector.

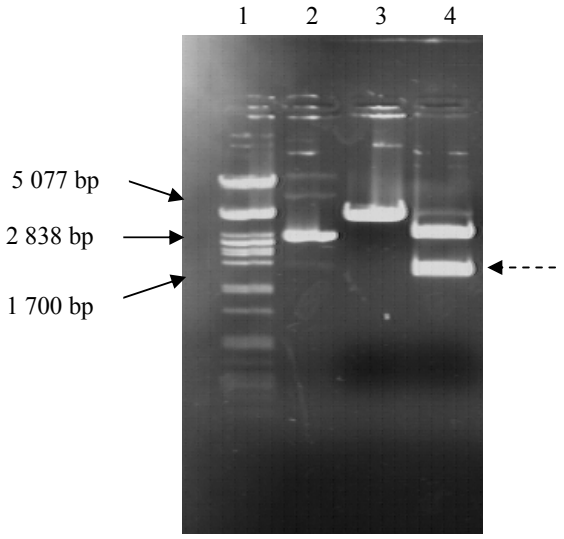

Figure 3.12. Verification of the cloning of the *ask* amplicon by digestion with *Eco*RV and *Xba*I.

1) Lambda *Pst*I marker, 2) Recombinant pGEM-T1 vector, 2) Recombinant pGEM-T1 vector digested with *Eco*RV, 3) pGEM-T1 *Eco*RV + *Xba*I digest. Ask insert is shown by the dashed arrow.

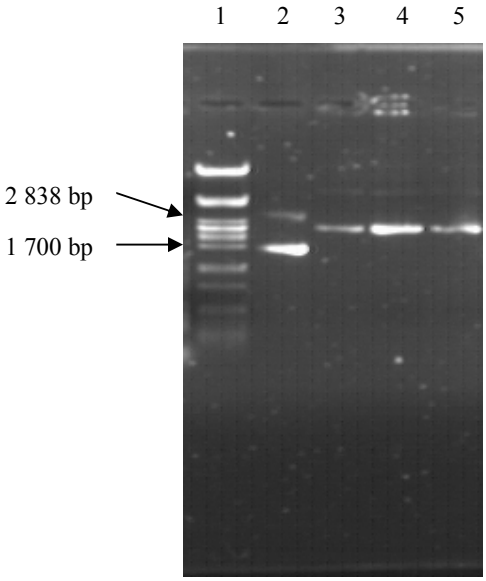

Figure 3.13. Subcloning of *ask* into pBluescript II KS (+) vector and analysis of putative *E. coli* recombinants.

1) Lambda *Pst*I marker, 2) pBluescript II KS (+), 3-5) Recombinant pBluescript II KS (+) vectors.

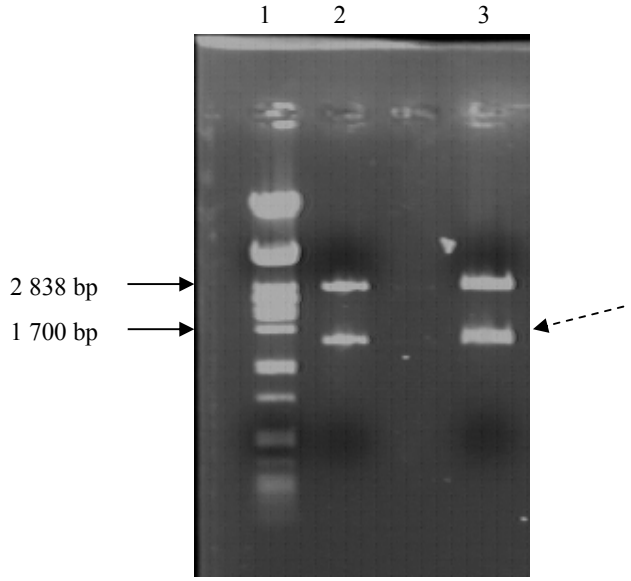

Figure 3.14. Verification of the subcloning by digesting a recombinant plasmid (pBT KS2) with *Eco*RV-*XbaI*I insert.

1) Lambda *Pst*I marker, 2) pBT KS2 *Eco*RV + *Xba*I digest. Ask insert is shown by the dashed arrow.

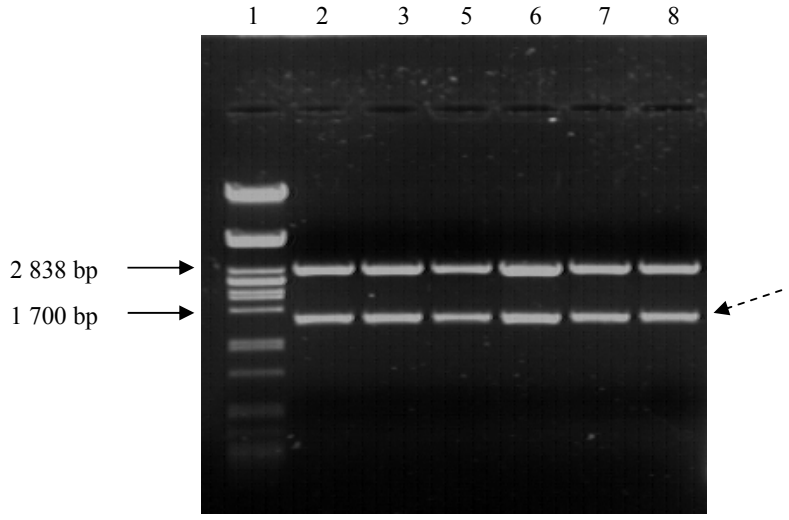

Figure 3.15. Digestion of pBT KS2 with *Hind*III and *Xba*I.

1) Lambda *Pst*I marker, 2) pBT KS2 *Hind*III + *Xba*I digest. Ask insert is shown by the dashed arrow.

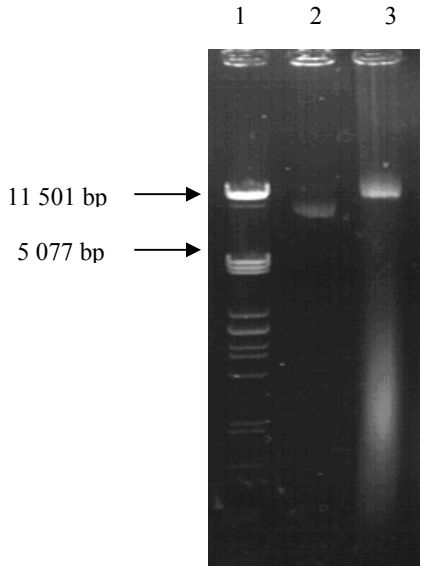

Figure 3.16. Plasmid isolation from putative *S. clavuligerus* recombinants.
1) Lambda *Pst*I marker, 2) pIJ486, 3) Recombinant pIJ486 from *S. clavuligerus* transformant.

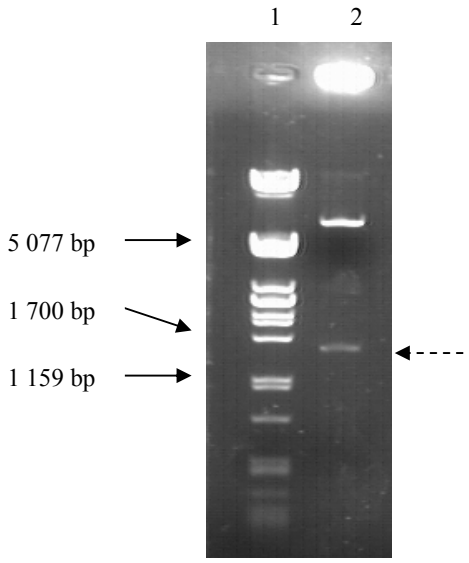

Figure 3.17. Digestion of pTB486 with *Xba*I and *Hind*III to verify the cloning of *ask* gene in *S. clavuligerus*.
1) Lambda *Pst*I marker, 2) pTB486 *Xba*I + *Hind*III digest. Ask insert is shown by the dashed arrow.

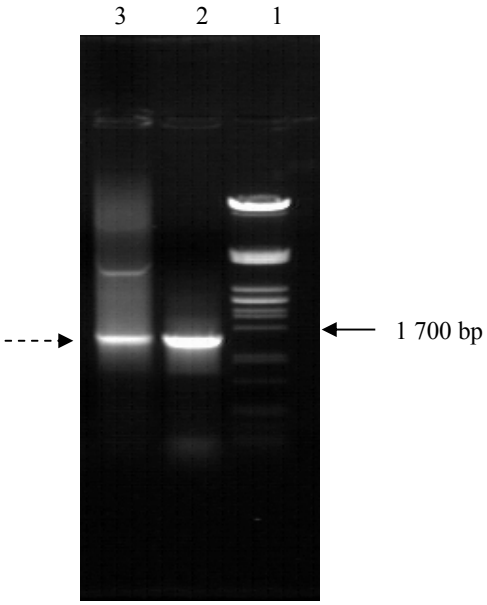

Figure 3.18. PCR product obtained with primers Askfw and Askrev to further verify the cloning of *ask* gene in *S. clavuligerus*. 1) Lambda *Pst*I marker, 2) 1506 bp *ask* gene from pTB486 as template, 3) 1506 bp *ask* gene from pNST102 as template, positive control.

3.4. Expression in *S. clavuligerus* and determination of the effects of *ask* multiple copies on cephamycin C production

S. clavuligerus protoplasts were transformed with above mentioned recombinant vector (pTB486). After stable recombinants were obtained, growth and cephamycin C production curves were prepared for both parental (NRRL 3585) and one of the recombinant (TB 3585) *S. clavuligerus* strains (Figure 3.19 and Figure 3.20). Ask activity was also measured for both parental and recombinant *S. clavuligerus* for 30^{th} and 54^{th} hours. Ask specific activity was reported as nanomoles of aspartyl-hydroxymate formed per milligram protein per minute. Cephamycin C specific production was reported as μU cephamycin C/mg DCW. Although recombinant *S. clavuligerus* cells showed 5.5 fold increased level of Ask activity as compared to that of the parental type (Figure 3.21), they exhibited only 1.1 fold increase in the level of cephamycin C between 30^{th} and 54^{th} hours of fermentation (Figure 3.22).

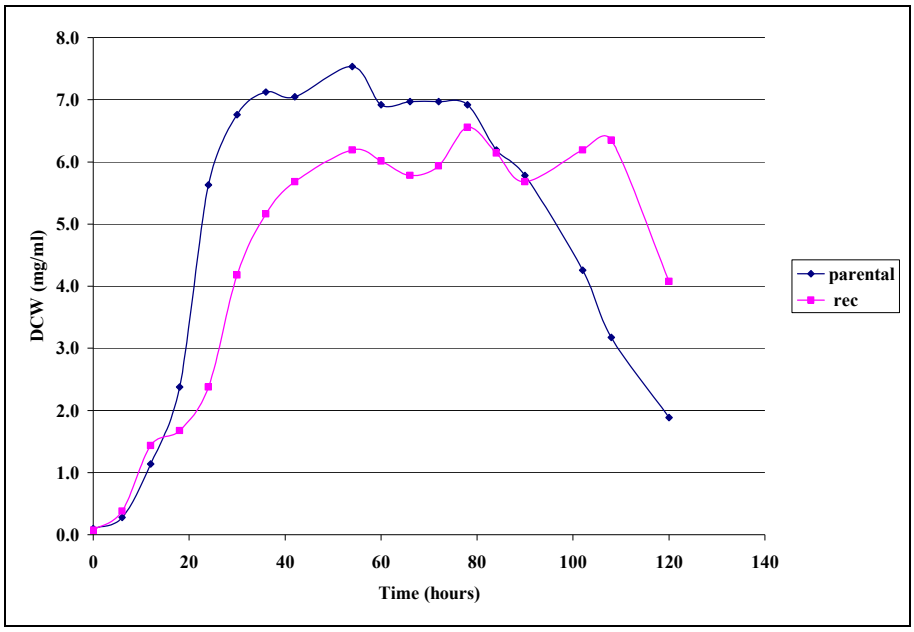

Figure 3.19. Growth curves of parental (NRRL 3585) and recombinant (TB 3585) *S. clavuligerus* cultures.

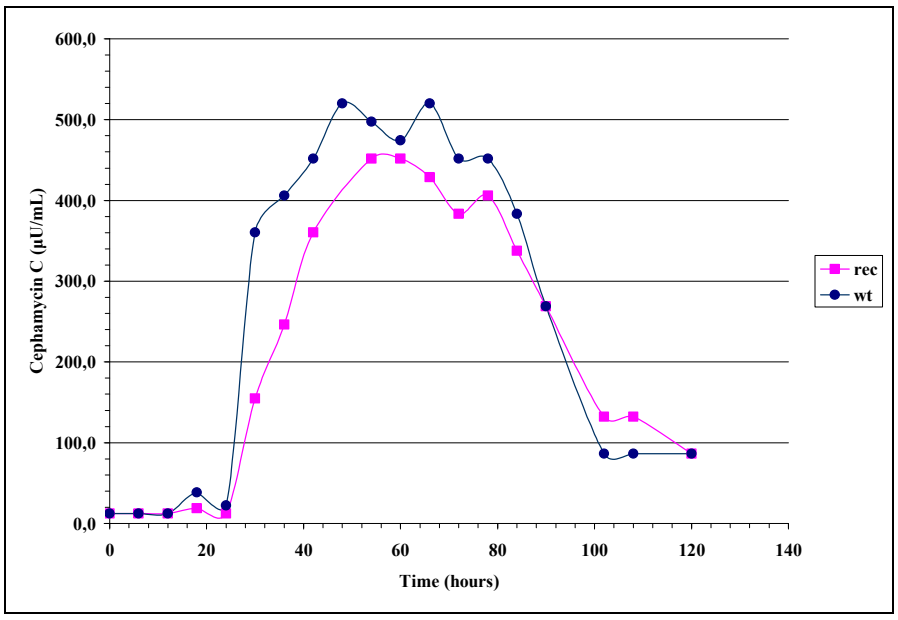

Figure 3.20. Cephamycin C production by both parental (NRRL 3585) and recombinant (TB 3585) *S. clavuligerus* cells.

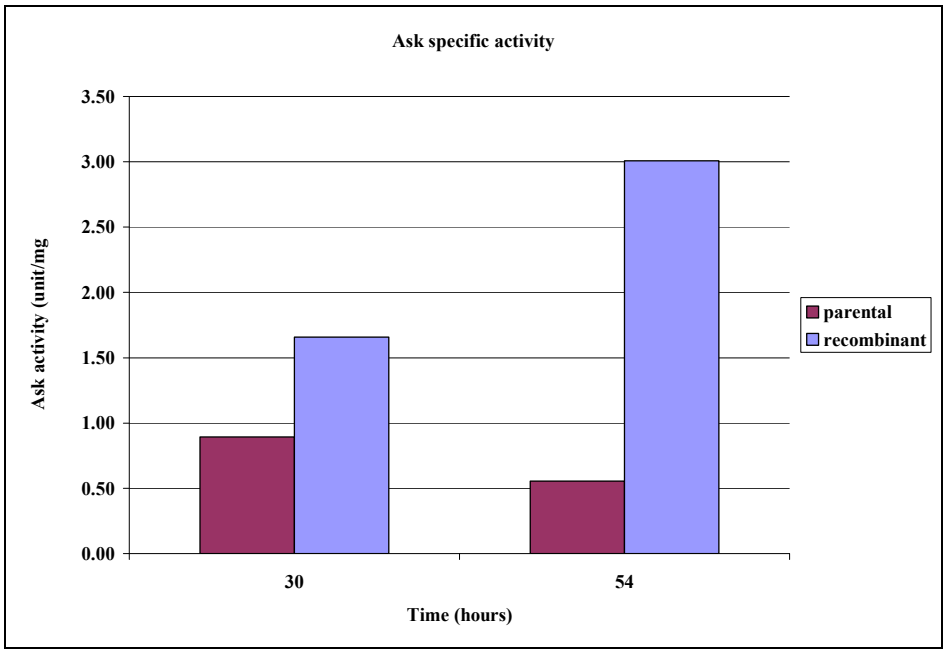

Figure 3.21. Specific activity of Ask enzyme in parental (NRRL 3585) and recombinant (TB 3585) strains.

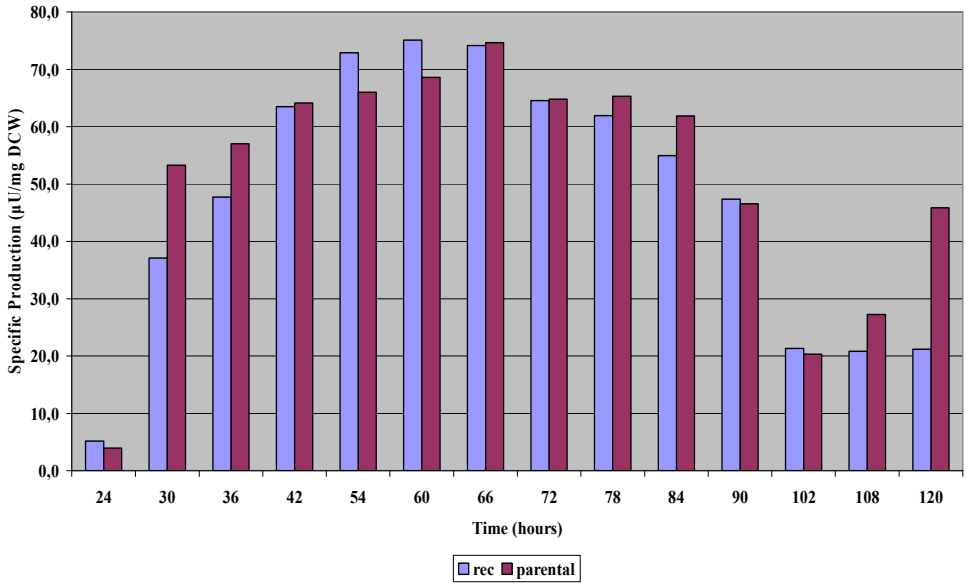

Figure 3.22. Specific production of cephamycin C by parental (NRRL 3585) and recombinant (TB 3585) strains.

Multiple copies of *ask* gene were indeed expected to increase the activity of aspartokinase enzyme more than 5.5 fold within the cell. Also, increase in Ask activity was not accompanied with a remarkable increase in cephamycin C biosynthesis in recombinant *S. clavuligerus* cells. These findings suggested concerted feedback inhibition of Ask by threonine and lysine which might have also prevented the accumulation of lysine which is the precursor of cephamycin C. Moreover, a general increase in pathway activity must have yielded the other end products (L-methionine, L-isoleucine) which repress *ask* gene and contribute to overall negative regulation. An analysis of the intracellular lysine pool in parental and recombinant strains by HPLC will be quite useful to test this hypothesis. In addition, transcriptional analysis of *ask* gene in both recombinant and parental *S. clavuligerus* cells via Northern blot analysis may give us direct information about the expression levels of the *ask* gene.

Biosynthesis of the cephamycin C like other β-lactams begins with the non-ribosomal condensation of L-α-AAA, L-cysteine and L-valine to form the tripeptide α-aminoadipiyl-cysteinyl-valine (ACV). Thus, even when we could have increased the abundancy of L-α-AAA, there could have been insufficiency of the other precursor amino acids, L-cysteine and L-valine. This possibility can be tested by externally adding these two amino acids to the medium when culturing the recombinant.

It should be particularly noted that recombinant *S. clavuligerus* cells showed much poorer growth when compared to parental type (Figure 3.19). Interference of growth by the presence of multi-copies of Ask was proved by testing the growth profile in liquid culture of another recombinant colony (data not shown). Aspartokinase enzyme catalyzes the initial reaction of aspartate pathway. The metabolic reactions of this pathway are finely balanced and metabolic intermediates other than those necessary for cell survival rarely accumulate as in other primary metabolic reactions. There are several important metabolic intermediates and products from aspartate pathway including diaminopimelic acid which is a key component required for cross-

linking in bacterial cell wall biosynthesis, and dipicolinic acid, important for sporulation in gram-positive bacteria. It can therefore be speculated that increasing the copy number of *ask* using a high copy number plasmid might have caused pleiotropic effects on the developmental cycle of *S. clavuligerus,* affecting cellular metabolism and causing a poor growth.

As mentioned before, the Ask activity of *S. clavuligerus* was shown to be under concerted feedback inhibition by two end products, lysine plus threonine. It is also known that carbon flow through the lysine branch of the aspartate pathway is the rate limiting step in cephamycin C biosynthesis. Therefore, mutations in the allosteric sites of the Ask enzyme may prevent concerted feedback inhibition and lead to increased cephamycin C biosynthesis. Isolation of deregulated *S. clavuligerus* Ask mutants (cephamycin C overproducers) and identification of the point mutation(s) accounting for resistance to feedback inhibition is an ongoing study in our laboratory. After isolation and characterization of *S. clavuligerus* mutants resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine (AEC), the mutation in the *ask* gene of such deregulated mutants will be determined via DNA sequencing. On the basis of this information, it would also be possible to make site-directed mutagenesis to obtain recombinant *ask* gene with specific alteration(s) on allosteric sites. Further studies in our laboratory will focus on homologous expression of such an altered *ask* gene and determination of its multiple copies on cephamycin C biosynthesis.

Another ongoing study in our laboratory involves disruption of the homoserine dehydrogenase gene (*hom*) which encodes for the first enzymatic step of the other branch of the aspartate pathway leading to L-methionine, L-isoleucine, and L-threonine. For this purpose, previously cloned *hom* gene has been blocked via insertion of a kanamycin resistance cassette into the gene. The disrupted gene has been transferred to *S. clavuligerus* cells in a *Streptomyces* vector. The selection of *hom*$^-$ mutants resulting from the homolog recombination between the chromosomal *hom* gene and the disrupted *hom* gene on the plasmid is being undertaken. It is expected that blocking of the *hom* gene in *S. clavuligerus* will result in an increase the carbon flow toward to lysine by closing the other branch, thereby increasing the

amount of cephamycin C biosynthesis. Another approach for increasing the cephamycin C biosynthesis would be the use of such *hom*$^-$ mutants as cloning hosts for homolog expression of deregulated *ask* gene on a multicopy plasmid.

The new methods of molecular genetics and completed genome projects have provided a wealth of information on the genetic potential of *Streptomyces* in regard to secondary metabolism. The information provided will greatly enhance our ability to understand complex regulatory mechanisms involved in the biosynthesis of secondary metabolites and enable us to modify strains to overexpress specific genes, to block unwanted ones and direct carbon flux to the desired end products. Genomics and proteomics are now the most effective tools to investigate the biology of *Streptomyces* and also to manipulate them for commercial purposes. Therefore, in addition to the classic molecular techniques, the use of genomics, transcriptional analysis and proteomics in a combined manner will open up new eras in the study of *Streptomyces* especially with respect to biotechnological applications.

CHAPTER 4

CONCLUSION

* After cloning into the multicopy *Streptomyces* vector pIJ702, the aspartokinase (*ask*)-aspartate semialdehyde dehygrogenase (*asd*) operon was used to transform both *S. lividans* as an intermediate and easily transformable host and *S. clavuligerus* as the target host. However, all our attempts to obtain *ask-asd* cluster cloned in *Streptomyces* failed because of the instability of recombinant plasmid.

* Only the *ask* gene fragment was amplified in a methylation-defficient mutant *E. coli* (ET12567) for the aim of increasing recombinant stability both by reducing the insert size and avoiding the methyl-specific barrier in *Streptomyces*. The *ask* gene amplified in this way was cloned in pIJ702 and then transferred to both *S. lividans* and *S. clavuligerus*. Again in every of several attempts, pIJ702 was recovered without any insert from the putative recombinants. The consistent diffuculty experienced in the recovery of a stable recombinant suggested structural instability of pIJ702.

* Another multicopy *Streptomyces* vector, pIJ486, was used to clone and homologously express *ask* gene in *Streptomyces clavuligerus*. Ask gene was cloned to this vector and *S. clavuligerus* protoplasts were transformed with this recombinant plasmid. After stable recombinants were succesfully obtained, the effects of the multiple copies of *ask* gene on cephamycin C biosynthesis were determined.

* Although recombinant *S. clavuligerus* cells showed a 5.5 fold increased level of Ask activity as compared to the parental strain, they displayed only 1.1 fold increase in specific production of cephamycin C. This result suggested concerted feedback

inhibition of Ask by threonine and lysine as well as its repression by L-methionine and L-isoleucine which might have altogether prevented the accumulation of lysine as an important precursor of cephamycin C biosynthesis. Insufficiency of the other precursor amino acids, L-cysteine and L-valine, might have also might put a barrier on overproduction of the antibiotic by the recombinant.

* Recombinant *S. clavuligerus* cells showed a poorer growth as compared to the parental type. Overexpression of the cloned *ask* gene might have caused a metabolic burden which could account for lowered growth.

* Analysis of the intracellular lysine pool by HPLC and transcriptional analysis of *ask* gene in both recombinant and parental *S. clavuligerus* cells via Northern blot analysis are now being performed to get information about the nature of the barrier against overproduction in both cell types.

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APPENDIX A

COMPOSITION AND PREPARATION OF CULTURE MEDIA

1. Media

1.1. Liquid Media

Luria Broth (LB)

	$g.L^{-1}$
Luria Broth	25

Sterilized at 121 °C for 15 minutes

Nutrient Broth (NB)

	$g.L^{-1}$
Nutrient Broth	8

Sterilized at 121 °C for 15 minutes

Tryptone Soy Broth (TSB)

	$g.L^{-1}$
Tryptic Soy Broth	30

Sterilized at 121 °C for 15 minutes

Tryptone Soy Broth With Sucrose (TSBS)

	$g.L^{-1}$
Tryptic Soy Broth	30
Sucrose	100

Sterilized at 121 °C for 15 minutes

Yeast Extract Malt Extract Medium (YEME) (Hopwood *et al*., 1985; modified**)**

	$g.L^{-1}$
Yeast Extract	3
Malt Extract	3
Bacto-peptone	5
Glucose	10
Sucrose	340

Sterilized at 121 °C for 15 minutes

After sterilization following sterile components were added to the medium:

$MgCl_2.6H_2O$ (2.5M)	2 mL/L
Glycine (20%)	25 mL/L

1.2. Solid Media

LB Agar

	$g.L^{-1}$
Luria Broth	25
Agar	15

Sterilized at 121 °C for 15 minutes

NB Agar

	$g.L^{-1}$
Nutrient Broth	8
Agar	15

Sterilized at 121 °C for 15 minutes

Soft NB Agar	$g.L^{-1}$
Nutrient Broth	8
Agar	5

Sterilized at 121 °C for 15 minutes

R2YE Medium (Hopwood *et al*., 1985; modified**)**

	g.L^{-1}
Sucrose	103
K_2SO_4	0.25
$MgCl_2.6H_2O$	10.12
Glucose	10
Yeast Extract	5
Casaminoacids	0.1
Distilled water added to	800 mL

Sterilized at 121 °C for 15 minutes

Before sterilization, the medium was splitted into 160 mL aliquots.

Sterilized at 121 ^{o}C for 15 minutes.

After sterilization, sterilized components below were added to each 160 mL aliquot:

KH_2PO_4 (0.5 %)	2 mL
$CaCl_2.2H_2O$(3.68 %)	16 mL
L-Proline(20 %)	3 mL
TES Buffer(5.73 %, pH 7.2)	20 mL
* Trace elements solution	0.4 mL
NaOH (1 N)	1 mL

*Trace elements solution

	mg/L
$ZnCl_2$	40
$FeCl_3.6H_2O$	200
$CuCl_2.2H_2O$	10
$MnCl_2.4H_2O$	10
$Na_2B_4O_7.10H_2O$	10
$(NH_4)_6Mo_7O_{24}.4H_2O$	10

APPENDIX B

BUFFERS AND SOLUTIONS

P Buffer

	g
Sucrose	103
K_2SO_4	0.25
$MgCl_2.6H_2O$	2.02
*Trace Elements Solution	2 mL
Distilled water to	800 mL

The buffer was dispensed into separate bottles so that each bottle contains 80 mL of the mixture before sterilization.

Sterilized at 121 ^oC for 15 minutes.

Before use following sterile components were added into 80 mLbuffer in order:

KH_2PO_4 (0.5%)	1 mL
$CaCl_2.2H_2O$ (3.68)	10 mL
TES Buffer (5.73%, pH 7.2)	10 mL

*Trace Elements Solution

Component	mg/L
$ZnCl_2$	40
$FeCl_3.6H_2O$	200
$CuCl_2.2H_2O$	10
$MnCl_2.4H_2O$	10

$Na_2B_4O_7.10H_2O$	10
$(NH_4)_6Mo_7O_{24}.4H_2O$	10

PE Buffer

	$g.L^{-1}$
Sucrose	171
K_2SO_4	0.1

Completed to 784 mL with distilled water.

Sterilized at 121 ^oC for 15 minutes.

Before use following sterile components were added

*Trace Elements Solution	1 mL
TES (5.72%, pH 7.2)	25 mL
190 mL $CaCl_2.2H_2O$	190 mL

* Trace Elements Solution

Component	mg/L
$ZnCl_2$	40
$FeCl_3.6H_2O$	200
$CuCl_2.2H_2O$	10
$MnCl_2.4H_2O$	10
$Na_2B_4O_7.10H_2O$	10
$(NH_4)_6Mo_7O_{24}.4H_2O$	10

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, protected from light.

50 mM Phosphate Buffer

	$g.L^{-1}$
KH_2PO_4	6,8
KOH	1,9

pH adjustment was done to with
*50 mM KH_2PO_4 solution (without added KOH) to lower the pH
*0.1 M KOH, added dropwise, to raise the pH

Sonication Buffer

150 mM KCl
5% Glycerol (v/v)
1 mM EDTA
1 mM PMSF in isopropanol
Completed to 100 mL with 50 mM phosphate buffer (pH 7.5)

Ferric Chloride Solution

10% (w/v) $FeCl_3.6H_2O$
3.3% (w/v) TCA
0.7 N HCl
Completed to 100 mL with dH_2O and protected from light

Assay Mixture (pH 7.6)

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) (50 X)

Tris Base	242 g
Glacial Acetic Acid	57.1 mL
EDTA (0.4 M, pH 8.0)	125 mL
Distilled water added to	1000 mL

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 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.

APPENDIX C

CHEMICALS AND THEIR SUPPLIERS

Chemicals

Agar	Merck
Agarose	Prona
Ampicillin	Sigma
L-Aspartate β-hydroxymate	Sigma
ATP	Sigma
Bacto-peptone	Difco
BSA (Bovine Serum Albumin)	Sigma
$CaCl_2.2H_2O$	Merck
Casaminoacids	Difco
Cephalosporin C (CPC)	Sigma
Chloroform	Merck
Coomassie Brilliant Blue G-250	Merck
$CuCl_2.2H_2O$	Sigma
Dimethylformamide	Merck
EDTA	AppliChem
Ethanol	Botafarma
Ethidium bromide	Sigma
$FeCl_3.6H_2O$	Sigma
Glacial Acetic Acid	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
HCl	Fluka

HEPES	Sigma
Hydroxylamine	Fisher
IPTG	MBI Fermentas
Isopropanol	Merck
K_2SO_4	Merck
KCl	Merck
KH_2PO_4	Merck
KOH	Merck
L-aspartate	Sigma
L-Proline	Sigma
Luria Broth	Q-Biogene
Malt Extract	Difco
$MgCl_2.6H_2O$	Merck
$MnCl_2.4H_2O$	Merck
$Na_2B_4O_7.10H_2O$	Sigma
$(NH_4)_6Mo_7O_{24}.4H_2O$	Sigma
NaOH	Merck
Nutrient Broth	Merck
PEG-1000	Merck
Phenol	Merck
PMSF (Phenylmethylsulfonylfluoride)	Sigma
SDS	Merck
Sucrose	Merck
TCA (Trichloroacetic acid)	Fluka
TES Buffer	AppliChem
Thiostrepton	Sigma
Tris Base	Merck
Tris-HCl	Merck
Tryptic Soy Broth	Oxoid
X-Gal	MBI Fermentas
Yeast Extract	Difco
$ZnCl_2$	Sigma

Enzymes

Lysozyme	Q-Biogene
T4 DNA Ligase	MBI Fermantas
Calf-intestinal alkaline phosphatase	Promega
*Kpn*I	MBI Fermantas
*Bam*HI	MBI Fermantas
*Eco*RI	New England
*Sal*I	MBI Fermantas
*Eco*RV	MBI Fermantas
*Xba*I	MBI Fermantas
*Hind*III	New England
Yellow/Tango Buffer	MBI Fermentas
NE2 Buffer	New England
BSA	New England
Taq DNA polymerase	MIB Fermentas

Size Markers

*Pst*I digested /Lambda DNA	MBI Fermentas

Kits

DNA Extraction Kit	Qiagen
Plasmid isolation Kit	Qiagen
p-GEM T Vector	Promega