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**TARGETED DISRUPTION OF HOMOSERINE DEHYDROGENASE GENE  
IN STREPTOMYCES CLAVULIGERUS AND ITS EFFECTS ON  
CEPHAMYCIN C PRODUCTION**

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IN *STREPTOMYCES CLAVULIGERUS* AND ITS EFFECTS ON  
CEPHAMYCIN C PRODUCTION**

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Approval of the Graduate School of Natural and Applied Sciences

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## ABSTRACT

### TARGETED DISRUPTION OF HOMOSERINE DEHYDROGENASE GENE IN *STREPTOMYCES CLAVULIGERUS* AND ITS EFFECTS ON CEPHAMYCIN C PRODUCTION

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The members of the genus *Streptomyces* are well-known for their capacity to synthesize a vast repertoire of secondary metabolites, including many useful antibiotics and proteins. *Streptomyces clavuligerus* is the producer of the medically important  $\beta$ -lactam antibiotics such as cephamycin C and the potent  $\beta$ -lactamase inhibitor clavulanic acid. The aspartate pathway of *S. clavuligerus* is an important primary metabolic pathway providing substrates for  $\beta$ -lactam synthesis. This pathway uses L-aspartic acid as the precursor for the biosynthesis of the amino acids L-lysine, L-methionine, L-isoleucine, L-threonine and several important

metabolic intermediates. L- $\alpha$ -aminoadipic acid ( $\alpha$ -AAA) required for  $\beta$ -lactam synthesis is a catabolic product of L-lysine produced from the lysine branch of the aspartate pathway. The carbon flow through the L-lysine-specific branch of aspartate pathway is limiting for the formation of cephamycin C. Formation of L-homoserine from aspartate semialdehyde (ASA) is the first step of the other branch of the aspartate pathway leading to L-threonine, L-isoleucine and L-methionine synthesis and is catalyzed by homoserine dehydrogenase (HSD, EC 1.1.1.3). Regulation of the activity or biosynthesis of the HSD of *S. clavuligerus* determines the availability of ASA for the biosynthesis of L-lysine and  $\alpha$ -AAA.

The gene encoding for homoserine dehydrogenase (*hom*) was previously cloned from *S. clavuligerus* NRRL 3585 and characterized in our laboratory. In this study, the *hom* gene was disrupted via insertion of a kanamycin resistance cassette into this gene which was subsequently transferred to *S. clavuligerus* cells using the *Streptomyces* plasmid vector pIJ486. A *hom* mutant of *S. clavuligerus* (AK39) was formed through integration into the chromosome by double crossing over and the effects of *hom* disruption on cephamycin C yields were investigated. Disruption of *hom* gene resulted in a 1.7 to 2.0 fold increase in specific cephamycin C production in chemically defined medium (CDM).

**Keywords:** *Streptomyces clavuligerus*; homoserine dehydrogenase, cephamycin C, aspartate pathway, gene disruption.

## ÖZ

### ***STREPTOMYCES CLAVULIGERUS*'DA HOMOSERİN DEHİDROGENAZ GENİNİN HEDEFLENMİŞ BLOKASYONU VE BUNUN SEFAMİSİN C ÜRETİMİ ÜZERİNE ETKİLERİ**

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*Streptomyces* türleri, yüksek kapasitede protein ve sekonder metabolit üreticisidirler. Bu grubun önemli türlerinden biri olan *Streptomyces clavuligerus* sekonder metabolit olarak tıbbi açıdan oldukça değerli bir  $\beta$ -laktam antibiyotiği olan sefamisin C ve güçlü bir  $\beta$ -laktamaz inhibitörü olan klavulanik asit üretmektedir. *S. clavuligerus*'un aspartik asit metabolik yolu  $\beta$ -laktam sentezi için öncül metabolitler sağladığı için çok önemlidir. Bu metabolik yoldan L-lizin, L-metiyonin, L-izolösün ve L-treonin gibi 4 temel amino asit sentezlendiği gibi bir çok önemli öncül metabolit de oluşmaktadır.  $\beta$ -laktam sentezi için gerekli bir öncül olan

L- $\alpha$ -aminoadipik-asit ( $\alpha$ -AAA), aspartat metabolit yolundan sentezlenen L-lizinin katabolik bir ürünüdür. Aspartik asit yolunun L-lizin dalındaki karbon akışının, sefamisin C biyosentezi için hız sınırlayıcı olduğu bilinmektedir. Aspartat semialdehid (ASA)'den L-homoserin sentezlenmesi, aspartat metabolik yolunun L-treonin, L- metiyonin ve L-izolösin sentezinine giden çatalının ilk enzimatik reaksiyonudur ve homoserin dehidrogenaz (HSD, EC 1.1.1.3) enzimi tarafından katalizlenmektedir. *S. clavuligerus*'ta L-lizin ve  $\alpha$ -AAA sentezi için gerekli ASA miktarı, HSD'in hücredeki biyosentez seviyesi veya aktivitesinin regülasyonu ile kontrol edilir.

*S. clavuligerus* NRRL 3585'in homoserin dehidrogenaz geni (*hom*) laboratuvarımızda daha önce klonlanıp karakterize edilmişti. Şimdiki çalışmada, *S. clavuligerus*'un *hom* geni, içine kanamisin dirençlilik kaseti sokulmak suretiyle tahrip edilmiş ve inaktive edilen gen bir *Streptomyces* plazmid vektörü olan pIJ486 içerisinde *S. clavuligerus*'a aktarılmıştır. Kromozomal *hom* geni ve plazmid üzerindeki bloke edilmiş *hom* geni arasındaki homolog rekombinasyon sonucu oluşan *hom* okzotrof bir suş (AK39) seçilmiş ve *hom* geni blokasyonunun sefamisin C üretimine etkisi incelenmiştir. *hom* geninin blokasyonu sonucunda, kimyasal olarak tanımlanmış (CDM) besiyerinde sefamisin C spesifik üretiminde 1.7 ile 2.0 misli artış gözlenmiştir.

**Anahtar kelimeler:** *Streptomyces clavuligerus*; homoserin dehidrogenaz, sefamisin C, aspartat metabolik yolu, gen blokasyonu



*To My Family*

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## LIST OF SYMBOLS

$\alpha$ -AAA	: $\alpha$ -aminoadipic acid
ACV	: $\alpha$ -aminoadipyl-cysteinyl-valine
DAP	: Diaminopimelate
ASA	: Aspartate semialdehyde
AK	: Aspartokinase
bp	: Base pairs
<i>hom</i>	: Homoserine dehydrogenase gene
<i>kan</i>	: Kanamycin resistance gene
<i>hom::kan</i>	: Homoserine dehydrogenase disruption cassette
<i>tsr</i>	: Thiostrepton resistance gene
HSD	: Homoserine dehydrogenase enzyme
RT	: Room temperature
o/n	: Overnight
CDM	: Chemically defined medium

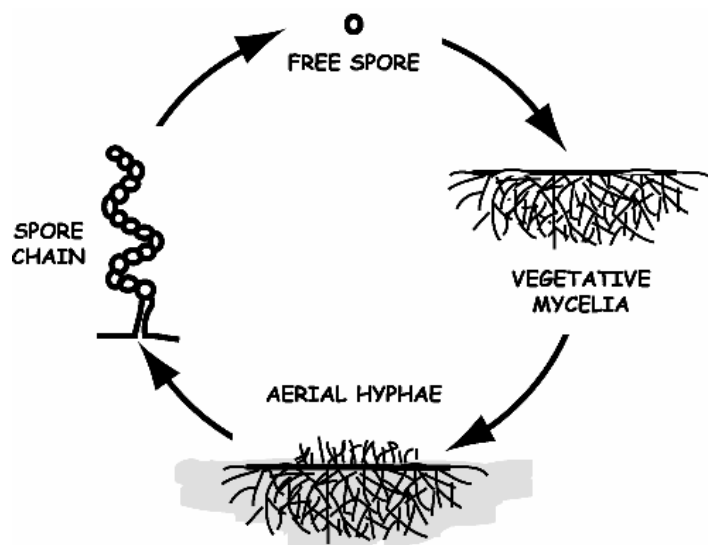
## CHAPTER 1

### INTRODUCTION

#### 1.1. The genus *Streptomyces*

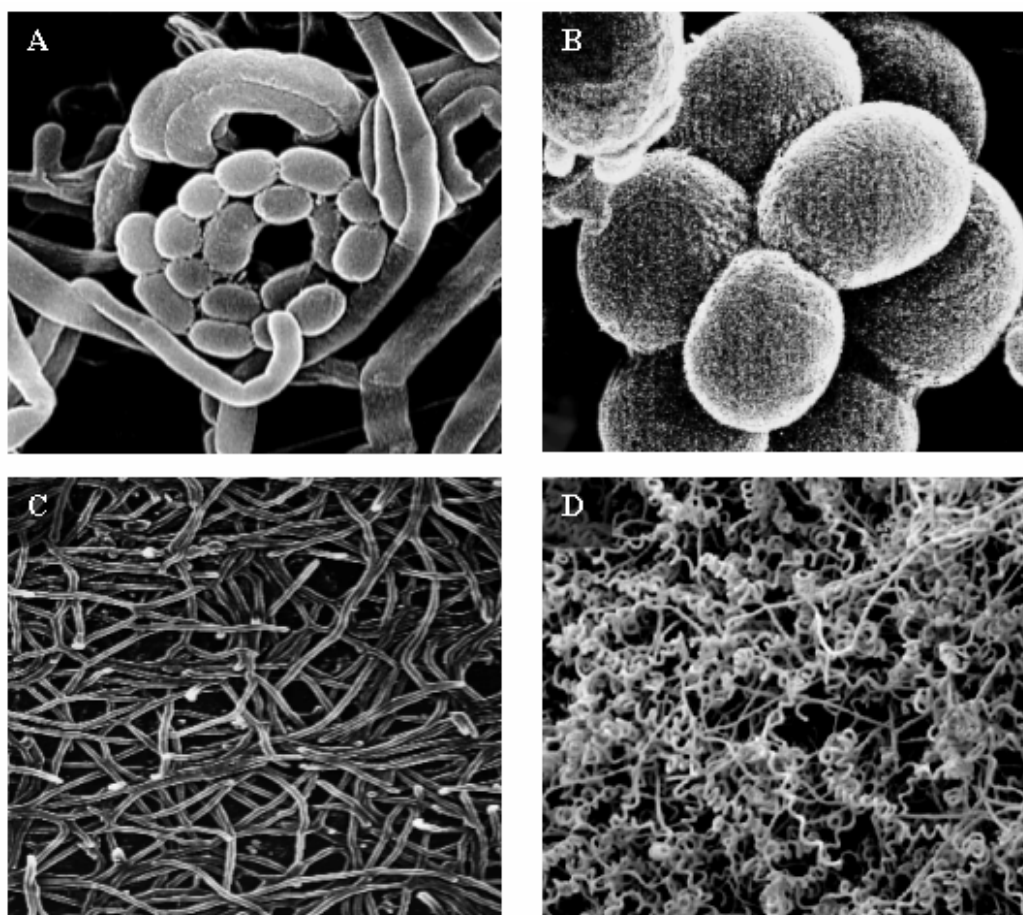
The members of the genus *Streptomyces* are gram positive, filamentous, spore forming soil bacteria belonging to the genera actinomycetes. As members of the high G/C branch of gram-positive bacteria, *Streptomyces* spp. possess one of the highest chromosomal G/C content, often quoted as 70-74 mole %. Large (~8 Mb) linear chromosomes and large (10-600 kb) linear plasmids are also striking features of streptomycetes (Keiser *et al.*, 2000). Another distinctive property of *Streptomyces* spp. is the presence of strong restriction modification systems, constituting a barrier to transfer of DNA from non streptomycetes origins to *Streptomyces* hosts, which makes them hardly transformable (Matsushima *et al.*, 1987). However, the most important characteristic of streptomycetes is their capacity to synthesize a vast repertoire of secondary metabolites, including many useful antibacterial and antifungal antibiotics, anticancer drugs, herbicides and immunosuppressive agents. Among all secondary metabolites with a known antibiotic activity, 55% are produced by *Streptomyces* spp. and the additional 11% by other actinomycetes (Weber *et al.*, 2003; Keiser *et al.*, 2000). With this knowledge in mind, one can appreciate the importance of streptomycetes for antibiotic industry.

Other noteworthy characteristic of *Streptomyces* is their remarkably complex developmental features in which they differ from most other bacteria (Kalakoutsii and Agre, 1976). Indeed, in terms of their complex life cycle, streptomycetes are superficially more reminiscent of filamentous fungi. They grow by tip extension to form a mycelium of branched hyphae (Flårdh, 2003). In a typical growth cycle, the spores bud into long filamentary hyphae which grow in and on the nutrient surface (Figure 1.1). The hyphae undergo branching and a dense mycelium (substrate mycelium) is gradually formed. This phase is usually referred as the vegetative growth phase. The vegetative phase is usually followed by a second aerial growth phase which can be accompanied by the generation of antibiotics. In this phase, the hyphae grow out of the mycelium, sometimes deforming into helical structures, and ultimately break up into spores which then start the vegetative growth cycle again (Goriely and Tabor, 2003) (Figure 1.2).



**Figure 1.1.** Life cycle of *Streptomyces* spp.

([http://biology.kenyon.edu/courses/biol114/Chap11/Chapter\\_11A.html](http://biology.kenyon.edu/courses/biol114/Chap11/Chapter_11A.html)).



**Figure 1.2.** *Streptomyces* cells in different developmental stages. (A) Scanning electron micrograph of spores and aerial mycelia of *S. coelicolor*. (B) Scanning electron micrograph of *S. coelicolor* spores. (C) Light microscope image of *S. coelicolor* young mycelia in liquid broth. (D) Scanning electron micrograph of curled aerial mycelium of *S. coelicolor* ([http://www.sanger.ac.uk/Projects/S\\_coelicolor/micro\\_images.shtml](http://www.sanger.ac.uk/Projects/S_coelicolor/micro_images.shtml)).

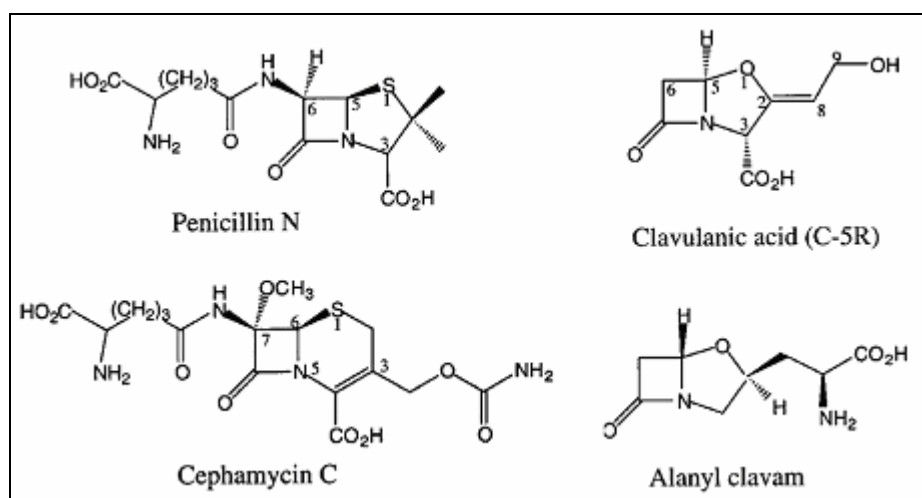
Secondary metabolite production in *Streptomyces* is usually growth phase-dependent. Production of secondary metabolites by streptomycetes generally coincides with the development of aerial hyphae in surface-grown cultures. In liquid-grown cultures, it is generally confined to stationary phase (Bibb, 2005).

Secondary metabolite production in streptomycetes is associated with low specific growth rates, and it is indeed the lowering of specific growth rate that signals a cascade of regulatory events resulting in chemical differentiation and morphological differentiation (Jonsbu *et al.*, 2002).

To sum up, the ability of *Streptomyces* spp. to produce clinically-useful antibiotics, their complex life cycles and distinctive genetic properties make them a very interesting subject to study.

### **1.2. *Streptomyces clavuligerus* and its secondary metabolites**

*Streptomyces clavuligerus* has been the subject of extensive research in the last three decades because of its ability to produce  $\beta$ -lactam metabolites with antibiotic, antifungal and  $\beta$ -lactamase-inhibitory activities (Thai *et al.*, 2001). *S. clavuligerus* produces two major groups of  $\beta$ -lactam compounds as secondary metabolites (Figure 1.3). The sulfur-containing  $\beta$ -lactam compounds have antibiotic properties and include isopenicillin N, desacetoxycephalosporin C and cephamycin C. The oxygen-containing  $\beta$ -lactam compounds, called as clavams include clavulanic acid as well as a number of related compounds. Clavulanic acid possesses a very weak antibiotic activity, but is a very potent  $\beta$ -lactamase inhibitor. Thus, clavulanic acid is used clinically extensively in combination with  $\beta$ -lactam antibiotics to combat infectious diseases caused by  $\beta$ -lactamase-producing pathogens. Other clavams such as alanylclavam have antifungal and antibacterial properties (Paradkar and Jensen, 1995). *S. clavuligerus* also produces a  $\beta$ -lactamase sensitive to clavulanic acid, a  $\beta$ -lactamase inhibitory protein (BLIP), and a BLIP-homologous protein (BLP) (Santamara *et al.*, 2002).



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

### 1.3. Importance of β-lactam antibiotics

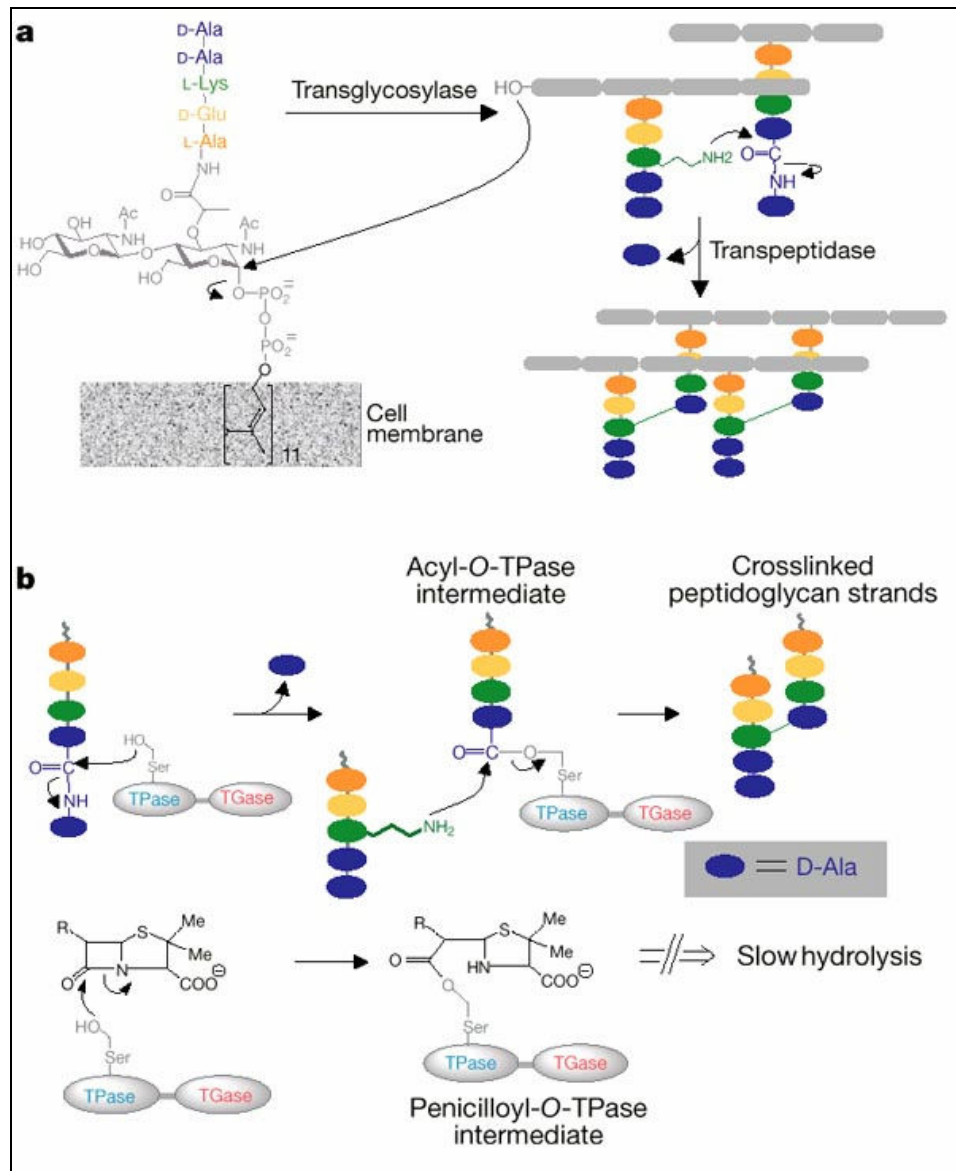
β-lactams constitute probably the most important class of antibiotics for several reasons. First of all, penicillin, the first antibiotic isolated and characterized, is a β-lactam. The discovery of antibiotics for clinical use started with the discovery of the efficacy of penicillin and is perhaps the most important discovery in the history of therapeutic medicine. Despite a growing number of antibiotics and the incidence of penicillin-resistant isolates, β-lactams are still the most frequently used antibiotics. The success of β-lactams in the treatment of infectious disease is due to their high specificity and low toxicity (Brakhage, 1998). 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 since 1940s and today penicillins are commodity-type products with annual production volumes exceeding 60,000 tons (Thykaer and Nielsen, 2003).

#### 1.4. Targets of $\beta$ -lactam antibiotics

As mentioned before, the  $\beta$ -lactam antibiotics are potent inhibitors of cell wall synthesis. Bacteria of all species rely on a heavily cross-linked peptidoglycan layer (cell wall) for the preservation of cell shape and rigidity. Peptidoglycan layer is a thin sheet composed of two sugar derivatives N-acetyl glucosamine and N-acetyl muramic acid, and a small group of amino acids consisting of L-alanine, D-glutamic acid, and either lysine or diaminopimelic acid (DAP). Adjacent peptide strands are connected in amide linkage by action of a family of transpeptidases, where as transglycosylases act on the glycan strands to extend the sugar chains by incorporation of new peptidoglycan units from *N*-acetylglucosamine –  $\beta$  – 1,4 – *N*-acetylmuramyl – pentapeptide – pyrophosphoryl – undecaprenol (Figure 1.4a). Bifunctional enzymes containing both transpeptidase and transglycosylase domains are the target sites for the killing of bacteria by the  $\beta$ -lactams (Walsh, 2000; Brock and Madigan, 1998).

The individual peptidoglycan units are produced inside the cell, but their final cross-linking is catalyzed outside the cytoplasmic membrane by a group of membrane anchored bacterial enzymes known as the cell-wall transpeptidases. In this cross-linking reaction, a peptide bond is formed between the penultimate D-alanine on one chain and the free amino end of a DAP or an L-lysine residue on the other chain. The linkage is formed with the penultimate D-alanine, causing the terminal D-alanine to be cleaved in the process. Transpeptidase enzymes utilize an active site serine and perform their catalytic cycle by way of an acylation/deacylation pathway. These enzymes are often termed penicillin binding proteins (PBPs) since  $\beta$ -lactam antibiotics irreversibly inactivate the bacterial transpeptidases. In the presence of the antibiotic, the transpeptidases form a lethal covalent penicilloyl–enzyme complex that serves to block the normal transpeptidation reaction. This results in weakly cross-linked peptidoglycan, which makes the growing bacteria highly susceptible to cell lysis and death (Figure 1.4b). In addition, antibiotic-PBP complex stimulates the





**Figure 1.4.** Cell wall biosynthesis and interruption of normal cross-linking by  $\beta$ -lactams. **a.** Transpeptidation and transglycosylation steps of normal cell wall biosynthesis **b.** Inhibition of transpeptidase activity by penicillins through formation of a slowly hydrolysing covalent acyl enzyme intermediate (Walsh, 2000).

release of autolysins that digest the existing cell wall. However, because peptidoglycan synthesis must be occurring for  $\beta$ -lactams to act,  $\beta$ -lactam-induced lysis occurs in only growing cells. In non-growing cells, the action of autolysins does not occur so that a break down of the cell wall peptidoglycan is prevented. These are the basis for how  $\beta$ -lactam antibiotics kill bacteria (Wilke *et al.*, 2005; Lee *et al.*, 2003; Brock and Madigan, 1998).

### **1.5. Cephamycin C biosynthesis in *Streptomyces***

Cephamycins are 7-methoxy-cephalosporins and called second-generation cephalosporins. Methoxylation of cephalosporins increases their inhibitory effect on transpeptidases involved in cell wall synthesis, reduces inactivation by  $\beta$ -lactamases and increases their activity against gram-negative and anaerobic pathogens, thus making the cephamycin C an important clinical antibiotic. Also promising is the fact that they have extremely low toxicity compared to other antibiotics. As representative of cephamycins, cephamycin C exhibits activity against many penicillin- and cephalosporin-resistant bacteria (Stapley *et al.*, 1979; Glazer and Nikaido, 1998).

Cephamycin C is produced in a nine step pathway by actinomycetes *Streptomyces clavuligerus* and *Nocardia lactamdurans* (Figure 1.5). Cephamycins are formed in a biosynthetic pathway which includes two steps also involved in penicillin formation (early steps), three steps common with cephalosporin C biosynthesis (intermediate steps) and specific steps for cephamycin C biosynthesis (late steps) (Liras, 1999).

The basic structure of the  $\beta$ -lactam nucleus is formed by condensation of the three precursor amino acids L- $\alpha$ -aminoadipic acid ( $\alpha$ -AAA), L-cysteine and L-valine by a mechanism designated as “non-ribosomal peptide synthesis”, which involves activation and condensation of the three component amino acids and epimerization of the L- to D-valine to form the tripeptide  $\delta$ - (L- $\alpha$ -aminoadipyl)-L-cysteinyl-D-valine (ACV).  $\alpha$ -AAA is a rare amino acid, hence it cannot be incorporated into

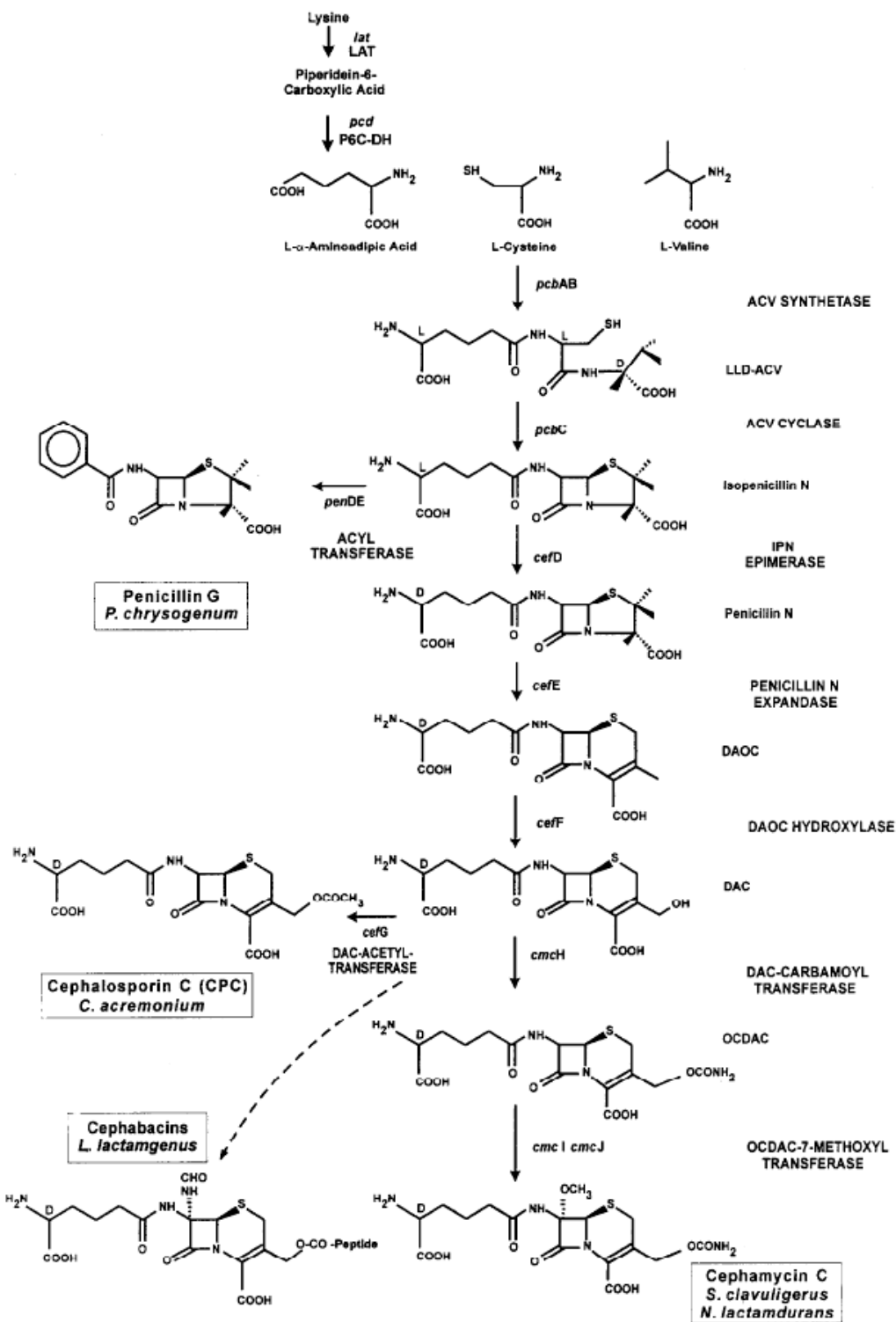


Figure 1.5. Cephamicin C biosynthetic pathway (Liras, 1999).

proteins by the standard ribosomal peptide synthesis (Martin, 1998). The multifunctional enzyme responsible for this step, ACV synthetase, is encoded by the *pcbAB* gene. ACV synthetase is one of the simplest known synthetases and therefore there is a great interest in the molecular mechanism of its activation and condensation. However, the molecular mechanisms of non-ribosomal ACV tripeptide synthesis are still obscure (Martin, 1998).

In the next step, the ACV tripeptide is cyclized by the ACV cyclase (also named isopenicillinN synthase) encoded by the *pcbC* gene, to give isopenicillin N (IPN), a  $\beta$ -lactam compound with a weak antibiotic activity (Liras, 1999). Then, IPN is epimerized to penicillin N by isopenicillin N epimerase (IPNE). Deacetoxycephalosporin C synthase (expandase) converts penicillin N to deacetoxycephalosporin C (DAOC) via an oxidative ring expansion that involves molecular oxygen and 2-oxoglutarate. DAOC is then hydroxylated to deacetylcephalosporin C (DAC) by another 2-oxoglutarate linked dioxygenase, deacetylcephalosporin C synthase (DACS). Finally for cephamycin C biosynthesis, the C-3 acetoxy group of DAC is replaced by a carbamyl group by DAC-carbaoyl transferase (Figure 1.5) (Thykaer and Nielsen, 2003).

Another important step of cephamycin C biosynthesis in *S. clavuligerus* is the synthesis of  $\alpha$ -AAA which is involved in the tripeptide ACV. In bacteria  $\alpha$ -AAA, is formed by deamination of lysine by the lysine-6-aminotransferase (LAT, encoded by *lat* gene) that converts lysine into piperidine-6-carboxylic acid (P6C). The activity of a second enzyme, P6C dehydrogenase (P6C-DH, coded by *pcd* gene), has been shown to be required for conversion of P6C into  $\alpha$ -AAA (Perez-Llarena *et al.*, 1998).

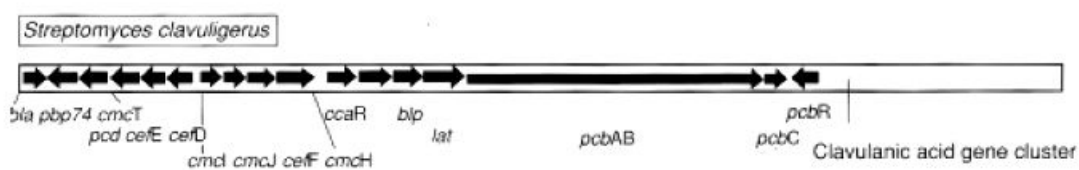
### **1.6. Cephamycin C gene cluster of *S. clavuligerus***

In general, the genes and enzymes for  $\beta$ -lactam biosynthesis are remarkably well conserved in prokaryotic and eukaryotic producers. Surprisingly, the percentages of

amino acid identity among the enzymes for  $\beta$ -lactam biosynthesis are higher than those for primary metabolism. This has been explained to be as the result of a stepwise horizontal transfer of  $\beta$ -lactam gene clusters from prokaryotic to eukaryotic producers (Landan *et al.*, 1990; Aharonowitz *et al.*, 1992; Brakhage, 2005).

The genes encoding enzymes involved in the synthesis of  $\beta$ -lactam antibiotics in *S. clavuligerus* have been cloned, many of the pathway enzymes have been purified and their kinetic properties were determined (Leskiw *et al.*, 1988; Madduri *et al.*, 1991; Jensen and Demain, 1995; de La Fuente *et al.*, 1997; Alexander and Jensen, 1998; Romero *et al.*, 1997, Khetan *et al.*, 1999; Santamarta *et al.*, 2002; Kovacevic *et al.*, 1991; Coque *et al.*, 1995a; Coque *et al.*, 1995b). It has been shown that all of the structural genes necessary for the biosynthesis of cephamycin C in *S. clavuligerus* are organized in a cluster together with regulatory and resistance genes (Figure 1.6) (Alexander and Jensen, 1998). Moreover, clavulanic acid gene cluster is located adjacent to cephamycin C gene cluster (Mosher *et al.*, 1999; Li *et al.*, 2000; Jensen *et al.*, 2000; Jensen *et al.*, 2004).

The genes in cephamycin C cluster of *S. clavuligerus* include *lat* and *pcd* which are involved in the formation of the  $\alpha$ -AAA precursor of the antibiotic, as well as the structural genes involved in the early steps of the pathway (*pcbAB* and *pcbC*) resulting in the formation of isopenicillin. *lat*, *pcbAB* and *pcbC* are expressed from polycistronic transcript under the promoter of *lat* (Alexeander *et al.*, 2000). The cluster also involves the genes required for the middle steps of the pathway (*cefD* and *cefE*, *cefF*), forming deacetylcephalosporin C, the late specific C-7 methoxylation (*cmcI* and *cmcJ*) and carbamoylation steps (*cmcH*) of cephamycin biosynthesis (Santamarta *et al.*, 2002).



**Figure 1.6.** Cephamycin C gene cluster of *S. clavuligerus*. (Liras *et al.*, 1998)

Besides the biosynthetic genes which encode the pathway enzymes of cephamycin C biosynthesis, genes for low affinity penicillin-binding proteins (*pbp74*, *pcbR*), a  $\beta$ -lactamase of poor activity (*bla*) involved in  $\beta$ -lactam resistance and a transmembrane protein (*cmcT*) that appears to be involved in cephamycin exportation, are clustered together in cephamycin C gene cluster (Pérez-Llarena *et al.*, 1997b; Paradkar *et al.*, 1996; Martin, 1998).

Finally, cephamycin C gene cluster of *S. clavuligerus* contains *blp* gene coding for a  $\beta$ -lactamase-inhibitory protein-like protein (BLP) and *ccaR* gene coding for a regulatory protein which will be further discussed in the next section (Alexander *et al.*, 1998).

### **1.7. Regulation of cephamycin C production in *S. clavuligerus***

The biosynthesis of a secondary metabolite can be regulated at least at four different levels. Highest in the hierarchy of regulation is the influence of global and specific regulators of secondary biosynthetic gene expression. These genetic elements, although poorly understood, are capable of influencing biosynthetic rates significantly in microorganisms that produce secondary metabolites. At the second level are the structural genes and their protein products that specify the biosynthetic pathway of a particular secondary metabolite. At this level, the activities of the enzymes constituting the pathway influence the rate of secondary metabolite production. At the interface between secondary and primary metabolism are the cofactors and precursors for secondary metabolite biosynthesis, whose supply rate represents a third level of cellular control. Finally, at the fourth level, the rate an individual cell can effectively transport the metabolites out is yet another controlling point on the production of secondary metabolites (Khetan *et al.*, 1999).

$\beta$ -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 *S. 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. However, when *S. clavuligerus* was grown on organic acids such as  $\alpha$ -ketoglutarate or succinate which are relatively poor carbon sources, specific production was higher 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).

Cephalosporin production was also found to be subjected to nitrogen source repression in *S. clavuligerus*. It has been known that the use of ammonium chloride as the sole nitrogen source or as an additive to asparagine decreases antibiotic production in *S. clavuligerus* (Aharonowitz, 1980). The decrease in  $\beta$ -lactam production was due to the repression of L- $\alpha$ -aminoacyl-L-cysteinyl-D-valine synthetase (ACVS), isopenicillin N synthase (cyclase), isopenicillin N epimerase and deacetoxycephalosporin C synthase (expandase) – four enzymes of the beta-lactam antibiotic pathway (Brana *et al.*, 1985; Brana *et al.*, 1986; Romero *et al.*, 1997). In addition, it was demonstrated that ammonium addition induced alanine dehydrogenase and increased intracellular alanine which also inhibits ACV synthetase, cyclase and expandase (Kasarenini *et al.*, 1994).

Antibiotic production in *S. clavuligerus* is also controlled by phosphate concentration. Aharonowitz and Demain (1977) showed that high concentration of inorganic phosphate inhibited cephalosporin production in *S. clavuligerus*. Jhang *et al.* (1989) explained that ACV synthetase, cyclase, epimerase and expandase were repressed by phosphate, with ACV synthetase being the main repression target. It was also demonstrated that high phosphate concentration inhibited isopenicillin N synthetase (Lubbe *et al.*, 1985).

Individual antibiotic biosynthesis pathways are also controlled by specific regulatory proteins that are frequently encoded by genes linked to the biosynthetic gene

clusters. It has been shown that *ccaR* regulatory gene (Cephamycin C and Clavulanic Acid Regulator) which is located within the cephamycin gene cluster of *S. clavuligerus*, codes for CcaR protein belonging to the SARP (Streptomyces Antibiotic Regulatory Proteins). Disruption of *ccaR* resulted in the inability to produce cephamycin C and clavulanic acid, suggesting that the *ccaR* product is an activator. Mutants disrupted in *ccaR* do not transcribe cephamycin or clavulanic acid structural genes and also lack several enzymes required for cephamycin C synthesis. Complementation of the disrupted mutant with *ccaR* restored production of both secondary metabolites while amplification of *ccaR* gene on a multicopy plasmid resulted in a two-to threefold increase in cephamycin and clavulanic acid production (Pérez-Llarena *et al.*, 1997a; Liras, 1999). On the other hand, what regulates the transcription of *ccaR* still needs investigation although there are reports describing autoregulation and regulation by a  $\gamma$ -butyrolactone-based quorum-sensing system (Santamarta *et al.*, 2002; Wang *et al.*, 2004; Santamarta *et al.*, 2005).

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 and cephamycin C production. Amplification of the *claR* gene in multicopy plasmids resulted in a threefold increase in clavulanic acid production and in a five- to sixfold increase of alanylclavam biosynthesis, whereas cephamycin production was significantly reduced both in defined and in complex media (Pérez-Redondo *et al.*, 1998). On the other hand, a gene replacement mutant disrupted in *claR* was unable to produce clavulanic acid (Paradkar *et al.*, 1998), while this type of disrupted mutants presented an increasing production of cephamycin C. However, the role of *claR* on cephamycin regulation has not been elucidated yet (Liras, 1999). Another finding was that the *claR* gene was not significantly expressed in mutants disrupted in the *ccaR* regulatory gene. These results indicate that clavulanic acid and cephamycin biosynthesis in *S. clavuligerus* is controlled by a cascade of regulatory proteins that include CcaR and ClaR (Pérez-Redondo *et al.*, 1998).



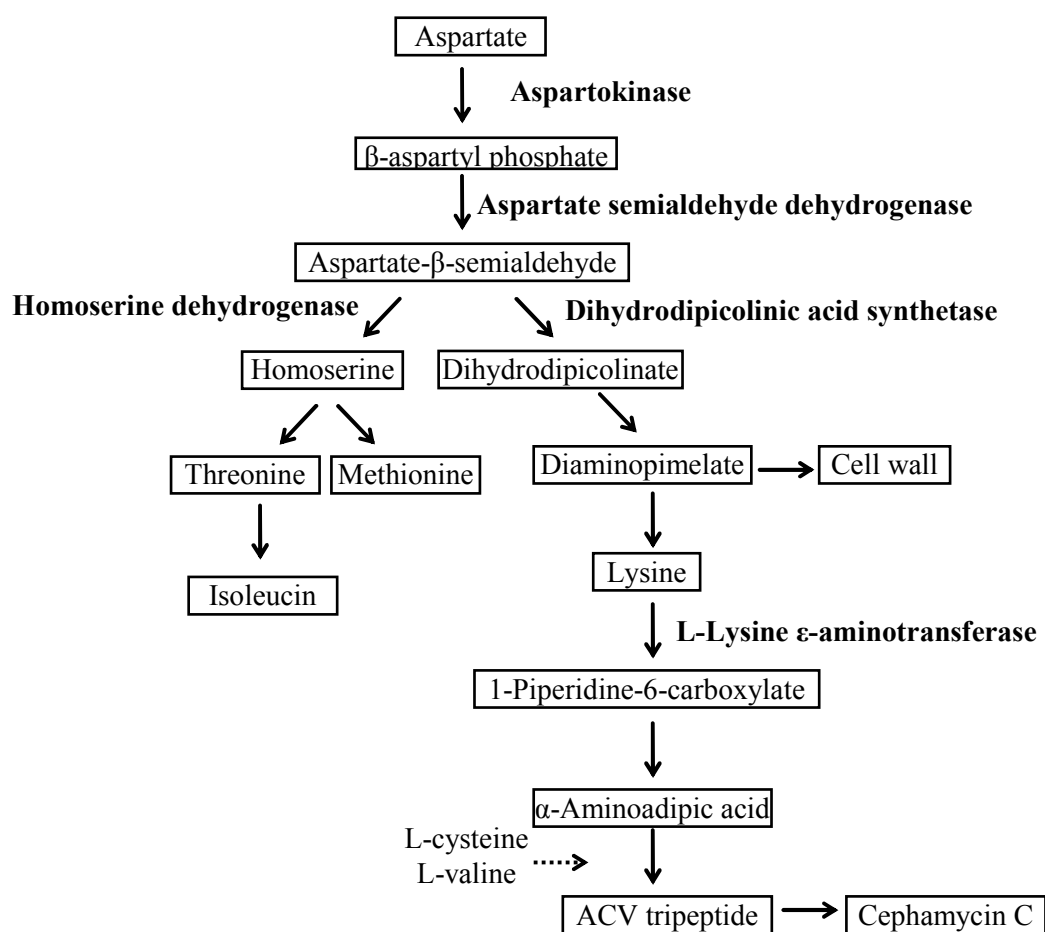
Antibiotic production in *Streptomyces* is generally growth-phase dependent. In liquid culture it begins as the culture enters stationary phase whereas in agar-grown cultures it starts with the onset of morphological differentiation. In addition, the occurrence of mutants deficient in both antibiotic production and the formation of aerial hyphae indicate at least some common elements of genetic control (Keiser *et al.*, 2000). Streptomycetes have a number of genes referred to as *bld* that function in the global regulation of morphological differentiation. Interestingly, some of the *bld* mutants are also defective in production of secondary metabolites. *bld* gene products are diverse and include a putative anti-anti-sigma factor. A *bldG* null mutant of *S. clavuligerus* was shown to be defective in both morphological differentiation and in the production of secondary metabolites, such as cephamycin C and clavulanic acid. This inability to produce cephamycin C and clavulanic acid was due to the loss of *ccaR* gene expression which is probably depended on *bldG* (Bignell *et al.*, 2005).

### **1.8. Aspartate pathway and cephamycin C production**

$\beta$ -lactams are very important class of antibiotics and improvement of  $\beta$ -lactam yields in the producer microorganisms is of great economical importance. For this reason, there have been many metabolic engineering attempts dealing with  $\beta$ -lactam biosynthetic genes and gene clusters (Thykaer and Nielsen, 2002). In this respect, as important as the secondary metabolite biosynthetic genes are the genes of primary metabolic pathways. The metabolic pathways of primary metabolism supply the precursors of secondary metabolism and hence, primary metabolism influences secondary metabolism. In a similar manner, factors controlling primary metabolism like induction, feedback inhibition/repression and catabolite repression will have effects on secondary metabolism (Drew and Demain, 1977).

The aspartate pathway of *S. clavuligerus* is an important primary metabolic pathway which provides substrates for  $\beta$ -lactam synthesis (Figure 1.7). This pathway uses L-aspartic acid as the precursor for the biosynthesis of the amino acids namely L-lysine, L-methionine, L-isoleucine, L-threonine and several important metabolic

intermediates including DAP, a key component required for cross-linking in bacterial cell-wall biosynthesis and  $\alpha$ -AAA, required for cephamycin C synthesis (Mendelovitz and Aharonowitz, 1982). As mentioned before, the initial step in the biosynthesis of  $\beta$ -lactams is the condensation of the three amino acids  $\alpha$ -AAA, L-cysteine and L-valine to form the tripeptide  $\alpha$ -aminoadipyl- cysteinyl-valine (ACV).



**Figure 1.7.** Biosynthetic pathway for the aspartate family amino acids and precursor flow to cephamycin C.

In fungi,  $\alpha$ -AAA is formed as an intermediate of the lysine pathway, but in actinomycetes it is synthesized from lysine branch of the aspartate pathway. Aspartate pathway of cephamycin C producer *Streptomyces* is the primary metabolic pathway of interest as  $\alpha$ -AAA, one of the precursors of cephamycin C, is synthesized thorough this pathway. However, there are only a limited number of studies dealing with the aspartate pathway of *S. clavuligerus* (Mendelovitz and Aharonowitz, 1982; Mendelovitz and Aharonowitz, 1983; Aharonowitz *et al.*, 1984; Malmberg *et al.*, 1993; Khetan *et al.*, 1999).

It has been shown that carbon flow through the lysine specific branch of the aspartic acid pathway is rate limiting in the formation of cephamycin C (Mendelovitz and Aharonowitz, 1982; Malmberg *et al.*, 1993; Khetan *et al.*, 1999). Malmberg *et al.* (1993) was able to elevate the cephamycin C yields two- to fourfold by insertion of an additional copy of L- lysine  $\epsilon$ - aminotransferase (*lat*) gene into the chromosome. Mendelovitz and Aharonowitz (1982) studied the effects of amino acids of the aspartic acid pathway on antibiotic production by *S. clavuligerus*. It was reported that DAP and L-lysine remarkably increased the specific cephamycin C production (1.86 and 1.69 fold increase, respectively). Since several branch points exist in the aspartate pathway, the flow of carbon from aspartate through lysine to the  $\alpha$ -aminoadipyl side chain of the cephalosporin molecules might be controlled by regulatory mechanisms operating at the initial and branching steps of pathway as well (Kern *et al.*, 1980; Mendelovitz and Aharonowitz, 1982).

*S. clavuligerus* aspartokinase (AK) catalyses a key step in the control of carbon flow towards  $\alpha$ -AAA. AK of *S. clavuligerus* was found to be subjected to concerted feed back inhibition by L-threonine and L-lysine which makes AK the first rate limiting step in the carbon flow from aspartate to  $\alpha$ -AAA (Mendelovitz and Aharonowitz, 1982). Mendelovitz and Aharonowitz (1983) demonstrated that aspartokinase deregulated mutants obtained by classical strain development methods (AEC-resistant strains), produced five times higher amount of cephamycin C than the wild type *S. clavuligerus*, accompanied with an increase in DAP accumulation

(Aharonowitz *et al.*, 1984). In our laboratory, Tunca *et al.* (2004) cloned and characterized as the first time the *ask-asd* gene cluster coding for aspartokinase and aspartate semialdehyde dehydrogenase from *S. clavuligerus* NRRL 3585. Also, in our laboratory, *ask* gene was introduced to *S. clavuligerus* in a multicopy plasmid (Taskin, 2005). This manipulation provided the multiple copies of the *ask* gene and resulted in two- to three fold increase in cephamycin C specific production in chemically defined medium (Taskin *et al.*, unpublished).

Formation of L-homoserine from aspartate semialdehyde (ASA) is the first step of the other branch of the pathway leading to L-threonine, L-isoleucine and L-methionine synthesis and catalyzed by homoserine dehydrogenase (HSD, EC 1.1.1.3). *S. clavuligerus* HSD was found to be strongly inhibited by L-threonine plus L-methionine (Mendelowitz *et al.*, 1982). The gene encoding homoserine dehydrogenase (*hom*) has been cloned and sequenced from diverse bacteria including *Corynebacterium glutamicum* (Peoples *et al.*, 1988), *Lactobacillus lactis* (Madsen *et al.*, 1996), *Bacillus* spp. (Parsot & Cohen, 1988; Malumbres *et al.*, 1995), *Pseudomonas aeruginosa* (Clepet *et al.*, 1992), *Methylobacillus glycogens* and *M. flagellatus* (Motoyama *et al.*, 1994; Marchenko *et al.*, 1999), *E. coli* and *S. typhimurium* (Cohen & Saint-Girons, 1987) and *Streptomyces* sp. NRRL 5331 (Fernandez *et al.*, 2002). The *hom* gene of cephamycin C producer *S. clavuligerus* was cloned and characterized in our laboratory (Yılmaz *et al.*, submitted) and its sequence was submitted to GenBank with the accession number AY802988.

## **1.9. Present study**

Regulation of the activity or biosynthesis of the HSD of *S. clavuligerus* might determine the availability of ASA for the biosynthesis of DAP, L-lysine, and  $\alpha$ -AAA. Such a regulation will also control the levels of L-threonine which together with L-lysine inhibits AK (Mendelowitz *et al.*, 1982). Hence, the absence of HSD activity in *S. clavuligerus* is expected to lead to an increase in cephamycin C

production levels in two ways. Firstly, all the carbon coming from aspartate would be directed to lysine rather than being shared between the two branches. This is quite important, because the carbon flow through the lysine-specific branch was shown to be rate limiting in cephamycin C biosynthesis (Mendelovitz and Aharonowitz, 1982; Malmberg *et al.*, 1993; Khetan *et al.*, 1999). Secondly, concerted feedback inhibition of AK by threonine and lysine would be prevented as no threonine could be produced in *S. clavuligerus* cells having no HSD activity. The aim of the present study was to disrupt the *hom* gene in *S. clavuligerus* NRRL 3585 and to investigate the effects of this disruption on cephamycin C yields. For this aim, a mutant allele (disruption cassette) was created, carried on a suitable vector and used to replace the chromosomal copy of the target *hom* gene via double crossing over.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. Bacterial strains, plasmids, media and culture conditions

Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* cultures were grown in either Luria broth liquid medium or on agar plates at 37 °C. *E. coli* was maintained on agar plates or kept in 20% glycerol at -80 °C. *S. clavuligerus* was maintained on sporulation medium. Spore suspensions of *Streptomyces* were prepared in 20% glycerol in TSB according to Keiser *et al.* (2000) and mycelium stocks of *S. clavuligerus* were also prepared in 25% glycerol in TSB. Both the spore suspensions and the mycelium stocks were stored at -80 °C. *Streptomyces* cultures were grown at 28 °C except for the protoplasts which were regenerated at 26 °C. Liquid cultures of *S. clavuligerus* were incubated on a rotary shaker (220 rpm) in baffled flasks.

For isolation of genomic DNA and for protoplast preparation, seed culture media containing trypticase soy broth (TSB) was inoculated with either spore or mycelium stocks of *S. clavuligerus* and was incubated for 48-60 h. 5 mL of this seed culture was inoculated into 50 mL of 2:3 (v/v) mixture of TSB and yeast extract - malt extract medium (YEME, Kieser *et al.*, 2000). To test auxotrophy in *S. clavuligerus*, *Streptomyces* minimal agar medium (MM) (Kieser *et al.*, 2000) was used as the solid medium. L-methionine and L-threonine (each 50 µg/mL) were added to the medium whenever required. For HSD and cephamycin C assay, 3 mL of *S. clavuligerus* mycelium stock was inoculated into 100 mL of chemically defined

medium (CDM) to prepare a seed culture (Malmberg *et al.*, 1993). CDM was supplemented with 150 µg/mL methionine and 50 µg/mL threonine for the growth of *S. clavuligerus* AK39. After 24 h incubation, 15 mL of seed culture was inoculated into 100 mL of fresh CDM to give an initial OD<sub>595</sub> of 0.03- 0.04 and grown for 120 h. The composition and preparation of culture media are listed in Appendix A.

To deal with plasmid containing cultures, ampicillin (100 µg/mL), kanamycin (25 µg/mL for *E. coli* and 200 µg/mL for *S. clavuligerus*) or thiostrepton (50 µg/mL for *S. clavuligerus*) was added into the medium. The maps of the plasmids used in the study are illustrated in Figure 2.1 and Figure 2.2.

## **2.2. Buffers and solutions**

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

## **2.3. Chemicals and enzymes**

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

**Table 2.1.** List of bacterial strains and plasmids used in this study

Strains and plasmids	Description	Source or reference
<b>Strains</b>		
<i>S. clavuligerus</i>		
NRRL 3585	Wild-type, cephamycin C and clavulanic acid producer	Prof. J. Piret, Northeastern University, USA
AK39	Disrupted in homoserine dehydrogenase gene, Kan <sup>R</sup>	This study
<i>S. lividans</i> 1326	Wild type	Prof. J. Piret, Northeastern University, USA
<i>E. coli</i>		
ESS	$\beta$ -lactam supersensitive <i>E. coli</i> strain	Prof. J. Piret, Northeastern University, USA
DH5 $\alpha$	F <sup>+</sup> $\phi$ 80dlacZ $\Delta$ M15 $\Delta$ (lacZYA-argF) U169 supE44 $\lambda$ <sup>-</sup> thi-1 gyrA recA1 relA1 endA1 hsdR17	<i>E. coli</i> Genetic stock center
ET 12567	F <sup>-</sup> dam 13::Tn9 dcm-6 hsdM hsdR lacYI	Prof. Keith Chater, John Innes Centre, UK
<b>Plasmids</b>		
pGEM-T	Amp <sup>R</sup> lacZ'	Promega
pK19	<i>E. coli</i> plasmid vector, Kan <sup>R</sup>	Pridmore R.D., 1987
piJ486	Streptomyces plasmid vector, PIJ101 replicon, Thio <sup>R</sup>	Prof. Keith Chater, John Innes Centre, UK
pGEM-TK	pGEM-T vector containing PCR amplified <i>kan</i> gene as <i>SacII</i> fragment, Amp <sup>R</sup> Kan <sup>R</sup>	This study
pEBH1	<i>BamHI/HindIII</i> fragment containing <i>hom</i> inserted into pQE-30 (Qiagen)	Assist. Prof. Ebru I.Yilmaz, Dicle University, Diyarbakir, Turkey
pEBHK	<i>SacII</i> fragment containing <i>kan</i> inserted into pEBH1	This study
pAEHK	<i>BamHI/HindIII</i> fragment containing <i>hom::kan</i> inserted into PIJ486	This study



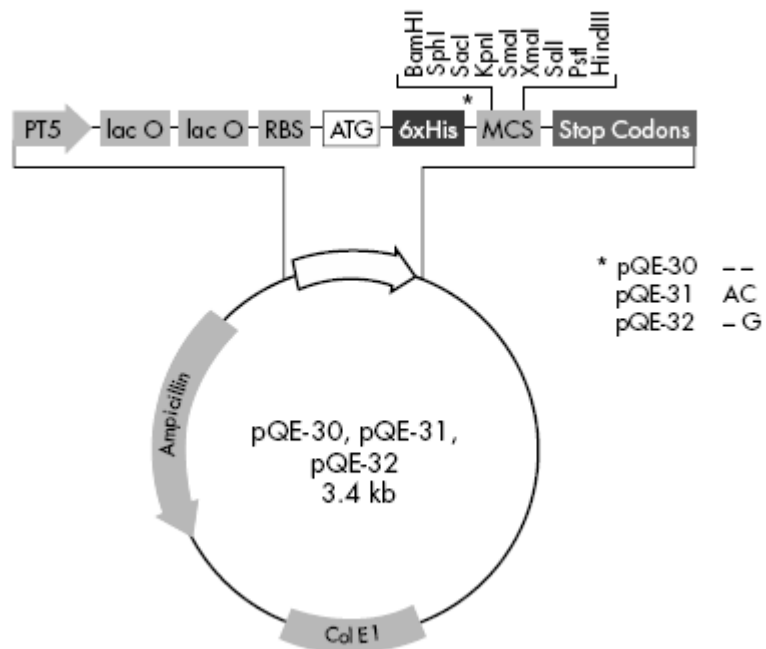
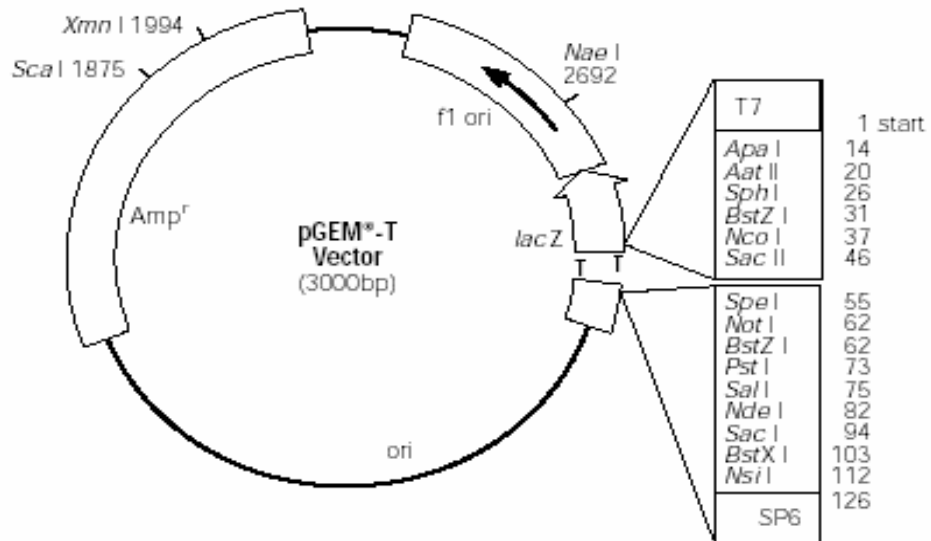


Figure 2.1. Maps of the pGEM-T and pQE-30 vectors.

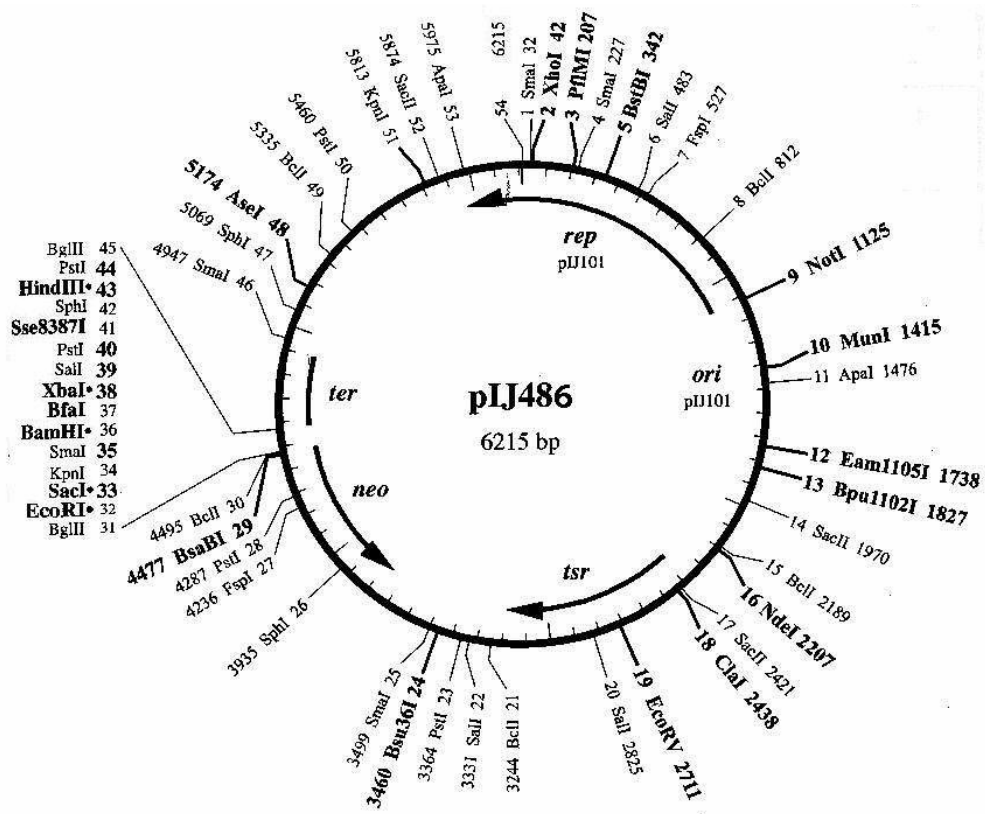
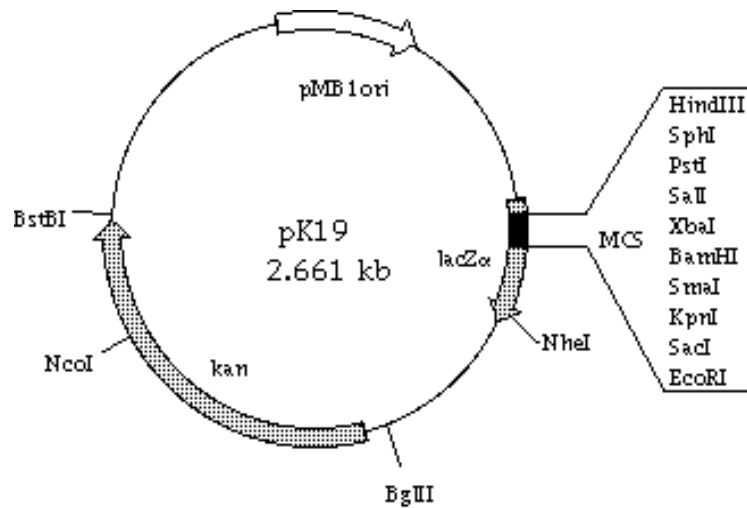


Figure 2.2. Maps of the *E. coli* plasmid pK19 and *Streptomyces* plasmid pIJ486.

#### **2.4. Preparation of *Streptomyces lividans* protoplasts**

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 h. The mycelium was harvested and washed twice with 10.3 % sucrose. The pellet was resuspended in 5 mL of P buffer (Appendix B) containing 2 mg/mL lysozyme. Following incubation at 30 °C for 30 min, 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 min. The pellets were resuspended in a final volume of 1 mL of P buffer and stored as 120 µL aliquots at –80 °C.

#### **2.5. 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 RT for 1 min. R2YE agar plates (Appendix A) dried for 2 h in a laminar flow hood were spread with 125 µL of the transformation suspension and incubated at 30 °C. *S. lividans* cells were incubated for 18 h, and then the plates were overlaid with soft nutrient agar (Appendix A) containing 50 µg/mL of thiostrepton. Incubation was continued at 30 °C for 2-4 days.

## **2.6 Preparation of *Streptomyces clavuligerus* protoplasts**

Protoplasts from *S. clavuligerus* were prepared according to Keiser *et al.* (2000) with some modifications made in our laboratory. *S. clavuligerus* seed culture was grown in TSB medium supplemented with 0.5% (w/v) maltose (Appendix A) at 28 °C on a rotary shaker (220 rpm) in baffled flasks for 48 - 60 h. 5 mL of this seed culture were inoculated into a 50 mL of 2:3 (v/v) mixture of trypticase soy broth: yeast extract - malt extract medium (TSB:YEME; Appendix A) supplemented with 0.3% (v/v) glycine and 3 mM MgCl<sub>2</sub> and incubated at 28 °C for 24 h. The mycelium was harvested by centrifugation of 25 mL culture at 3000g for 15 min. Harvested mycelium was washed twice with 10.3 % sucrose. At each wash, 10.3 % sucrose was added, vortexed, centrifuged and supernatant was discarded. The pellet was resuspended in 2 mL of PE buffer (Appendix B) containing 1 mg/mL lysozyme. Following incubation at 30 °C for 15 min, the cultures were examined microscopically to determine extent of protoplasting. If complete protoplasting was seen, the procedure was continued, if not additional incubation was performed at 30 °C until all the cells became protoplasts. Then, the protoplasts were diluted with 2.5 mL of PE buffer. Protoplast suspension was triturated 3 times with 1 mL pipette, before and after the dilution. Diluted protoplasts are filtered through non-absorbent sterile cotton plugs, washed with PE buffer and centrifuged three times at 2000 g for 10 min. Following the first 2 centrifugation, supernatant was discarded and the pellet was resuspended gently in a proper amount of PE buffer. Protoplasts were treated gently in each case, and never vortexed. After the third centrifugation, the pellet was resuspended in a final volume of 1 mL of PE buffer and stored as 50 µL aliquots at -80 °C.

## **2.7. Transformation of *Streptomyces clavuligerus* protoplasts**

*S. clavuligerus* transformations were performed via PEG mediated protoplast transformation according to Keiser *et al.* (2000) with some modifications.

Protoplasts stored at -80 °C were thawed on ice slowly and subjected to heat shock via incubation at 42 °C for 10 min (Bailey and Windstanley, 1986). 50 µL protoplast was transformed with plasmid DNA (2-10 µL; 0.05-1.5 µg) or ligation product, immediately mixed with 500 µL 50 % PEG 1000 in PE buffer, incubated at RT for 2-3 min, diluted with 1.5 mL of PE buffer and collected by centrifugation (at 2000 g for 10 min), pellet was resuspended gently in a smaller volume of PE buffer and plated on R2YE regeneration medium (Appendix A). Transformants were regenerated on R2YE medium at 26 °C (Garcia-Domiguez *et al.*, 1987) for 48 h and then each plate was overlaid with antibiotics containing 2.5 mL soft Nutrient Agar (SNA, 0.5 % agar) to give a final concentration of 8 µg/mL thiostrepton and/or 200 µg/mL kanamycin for selection. 48 h after the overlay, transformants appeared on the agar medium and single colonies were selected for further analysis.

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

*E. coli* competent cells were prepared according to the protocol described by Sambrook *et al.* (1989) with some 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 in an rotary shaker 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 and incubated for 2- 2.5 h at 37 °C with vigorous shaking around (300 cycles per min) in an orbital shaker to obtain an exponentially-growing culture. Then the culture was aseptically split into two sterile pre-chilled 40 mL screwcap centrifuge tubes and stored on ice for 10 min. Next, the cells were spun at 4000 rpm for 10 min 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<sub>2</sub> by vortexing. The cells were centrifuged at 3000 rpm for 10 min at 4 °C and supernatant was discarded. Finally, each pellet was resuspended very gently in 1 mL ice-cold 75 mM CaCl<sub>2</sub>. The competent cells were stored at – 80 °C as 100 µL aliquots.

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

Competent *E. coli* cells were transformed as described by Sambrook *et al.* (1989) with slight modifications. *E. coli* competent cells kept at -80 °C were slowly thawed on ice. Plasmid DNA 1-10 µL (1- 50 ng) or ligation product (in a maximum volume of 10 µL) was added to 100 µL of *E. coli* competent cells and mixed gently by pipetting. The tubes were left on ice for 30 min. Then, the tubes were placed in a 42 °C water bath and heat- pulsed for 90 sec. Next, the tubes were immediately put on ice for 2 min. 0.9 mL of LB was added to each tube and the cultures were incubated for 1.5 h at 37 °C with gentle shaking (100 rpm). Cells were centrifuged at low speed (3000 rpm) for 8 min and most of the supernatant was removed while 100-200 µL of the supernatant was left in the tube in which the pellet was resuspended. Finally, cells (100 µL) were spread onto selective LB agar plate and incubated o/n at 37 °C. The single colonies of transformants were selected and plasmids were isolated for further analysis.

## **2.10. Plasmid isolation from *Streptomyces* spp.**

Qiagen Plasmid Purification Kits (Qiagen Inc., Valencia, CA) were used for isolation of *Streptomyces* plasmid DNA, modifying the first steps of the manufacturer's instructions. 50 mL of TSB medium supplemented with 0.5% (w/v) maltose in 250 mL baffled flask was inoculated either with mycelium stocks or with one square cm of cell mass from an R2YE agar plate and incubated at 28 °C by continuous agitation on a rotary shaker at 220 rpm for 2 days. Then, 5 mL of this seed culture was used to inoculate the 50 mL YEME (Appendix A) medium supplemented with 0.5 % (v/v) glycine and 5 mM MgCl<sub>2</sub>. After incubation for 24-48 h, 25 mL of the culture was transferred to a sterile 40 mL centrifuge tube and centrifuged at 6000 rpm for 20 min. The pellet was washed once with 10.3% sucrose and was frozen at -20 °C o/n. Next day, the cells were resuspended in 1.5 mL of P1 buffer (from the kit) supplemented with 1 mg/mL lysozyme and by vortexed until cell clumps were no longer

visible. Resuspension was incubated at 37 °C for 15 min and 1.5 mL of P2 buffer was added. 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 on selective TSA (Appendix A). About 1 square cm of cell mass was scraped with a sterile flat toothpick and put into an Eppendorf tube containing 50 µL TSE solution (Appendix B) supplemented with 2 mg/mL lysozyme (Appendix B). After mixing the content of the tube, the mixture was incubated at 37 °C for 30 min. 3/5 volume of the lysis solution (Appendix B) was then added and immediately mixed by using vortex. The mixture was incubated at RT for 10 min to lyse cells, then at 70 °C for 10 min to denature DNA. The tube was then cooled 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 min at 13000 rpm. A sample of supernatant (10 µL) was loaded directly on a gel to screen plasmid sizes.

### **2.11. 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 miniprep method described by Hopwood *et al.* (1985). Each strain was grown as patches on selective medium, LB agar containing 100 µg/mL ampicillin and/or 25 µg/mL kanamycin. About 1 square cm of cell mass was scraped with a sterile toothpick and put into Eppendorf tube containing 100 µL cold TSE solution (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 min. 3/5 volume of lysis solution (Appendix B) was added to each tube and vortexed immediately. The mixture was incubated at RT for 10 min to lyse the cells and then at 70 °C for 10 min 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 min at 13 000 rpm to separate phases. 10 µL of supernatant was loaded directly on an agarose gel for electrophoresis.

## **2.12. Chromosomal DNA isolation from *S. clavuligerus***

*S. clavuligerus* seed culture was grown in TSB medium supplemented with 0.5% (w/v) maltose at 28 °C on a rotary shaker (220 rpm) in baffled flasks for 48-60 h. 5 mL of this seed culture was inoculated into 50 mL of 2:3 (v/v) mixture of trypticase soy broth: yeast extract - malt extract medium (TSB:YEME; Appendix A) supplemented with 0.3 % (v/v) glycine and 3 mM MgCl<sub>2</sub> and incubated at 28 °C for 24 h. 1.5 mL culture was harvested by centrifugation in 2 mL Eppendorf tubes. Harvested cells were washed twice with 10.3% sucrose solution. Pellet was resuspended in 500 µL TSE buffer (Appendix B) supplemented with 2 mg/mL lysozyme and 50 µg/mL RNase. After incubation at 37 °C for 30 min, 300 µL 2 % SDS was added and vortexed for 20 sec. 500 µL phenol:chloroform:isoamyl alcohol was finally added into the Eppendorf tubes and the mixture was vortexed until it became milky white. After centrifugation at 13000 rpm for 15 min, upper phase was taken to new tubes. If the upper phase was not clear, one more phenol:chloroform:isoamyl alcohol extraction was performed. Extraction with phenol:chloroform:isoamyl alcohol was followed by another extraction step with equal volume of chloroform:isoamylalcohol (24:1). Finally genomic DNA in the upper phase was precipitated by standard sodium acetate precipitation method which is described below: the upper phase was mixed with 0.1 volume of 3 M sodium acetate (pH: 5.2) and 2-3 volumes of cold absolute ethanol. After inverting the tubes



a few times, they were incubated at -20 °C for 30 min or more. At the end of incubation, tubes were centrifuged at maximum speed for 30 min at 4 °C and supernatant is discarded carefully. Pellet was washed with 70% ethanol and air dried. For the chromosomal DNA to be completely dissolved, a proper amount of distilled water was put into the tubes and incubated at 37 °C o/n.

## **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 generally used for DNA analysis at a concentration of 0.9%. The final concentration of ethidium bromide in the gels was 0.5 µg/mL. Gels were run for 1-1.5 h at 90 V. For Southern blot analysis 1.0% agarose gels were used and run at 25 V for 21-22 h. The electrophoresis buffer used was 1xTAE (Appendix B). The DNA bands were visualized on a UV transilluminator (UVP) and photographed by using Vilber Lourmat Gel Imaging System. Lambda/*Pst*I DNA size marker (Appendix C) was used to determine the molecular weights of DNA bands.

### **2.13.3. Extraction of DNA fragments from gels**

The desired fragments were extracted from the gel by using a Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA). The gel slice containing the DNA band was excised from the gel and weighed. Gel extraction was performed according to

the 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 pEBH1 and pIJ486 vectors were performed as follows: 1  $\mu$ L (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  $\mu$ L with H<sub>2</sub>O. Reaction mixtures were incubated at 4 °C or 16 °C, o/n. Ligation reaction of PCR products with pGEM-T vector was performed as follows: 1  $\mu$ L 10X ligase buffer, 1  $\mu$ L (55 ng/ $\mu$ L) pGEM-T vector, 500 ng insert DNA, 1  $\mu$ L T4 DNA ligase (3 unit/ $\mu$ L) were mixed and volume was completed to 10  $\mu$ L with H<sub>2</sub>O. Ligation was carried out as o/n incubation at 16 °C.

#### **2.14. Primer design**

The nucleotide sequences of the primers are given in Table 2.2. The underlined letters indicate *Bam*HI site in HomF primer, and *Hind*III site in HomR primer. In KanR and KanF primers underlined letters are *Sac*II sites. The designed primers were synthesized by the Iontek Company (İstanbul, Turkey). HomF and HomR primers were designed by Dr. Yilmaz, prior to this study for the cloning of the *hom* gene from *S. clavuligerus*. The same primers were used in the present study for the amplification of *hom* and *hom::kan* sequences. Sequence of *hom* gene and the positions of HomF and HomR primers are shown in Figure 2.3. KanF and KanR primers were designed in this study and used for the amplification of kanamycin resistance gene (*kan*) from *E. coli* plasmid vector pK19 (Pridmore, 1987). The sequence of kanamycin resistance gene including the promoter region and the location of primers are illustrated

in Figure 2.4. ThioR and ThioF are designed to amplify the thiostrepton resistance gene (*tsr*) of the plasmid vector pIJ486.

## 2.15. Polymerase chain reaction

PCR was performed in a total volume of 50  $\mu$ L. Reaction mixture components were as follows: 10X Fermentas PCR buffer without  $Mg^{+2}$ , 5  $\mu$ L; Fermentas  $MgCl_2$  solution, 6  $\mu$ L; primers (50 pmol of each), 1  $\mu$ L each; 10 mM dNTP mix, 2  $\mu$ L; DMSO, 2  $\mu$ L; 50 ng of template DNA (chromosomal DNA or plasmid DNA) and 1 Unit Taq Polymerase. PCR mixture was completed to 50  $\mu$ L with  $dH_2O$ . The PCR amplification condition was as follows: 95  $^{\circ}C$  (initial denaturation, 5 min) and 30 cycles of 95  $^{\circ}C$  (denaturation, 1 min), 63  $^{\circ}C$  (annealing, 1 min) and 72  $^{\circ}C$  (elongation, 2 min). At the end of the PCR, the PCR products were run in a 0.9% agarose gel, DNA bands are visualized, and PCR products were extracted from the gel, whenever necessary. Extracted PCR products were first cloned into the pGEM-T vector, prior to further cloning.

**Table 2.2.** Primers used for *hom*, *kan* and *tsr* genes' amplification.

Name of the primer	Sequence of the primer
<b>HomF</b>	5'- <u>AGGATCCATGATGCTGACGCGTCCG</u> -3'
<b>HomR</b>	5'- <u>TAAGCTTTTACTCCCCTTCAACACG</u> -3'
<b>KanF</b>	5'- <u>GCCGCGGGAACACGTAGAAAGCCAGT</u> -3'
<b>KanR</b>	5'- <u>CCC GCGGTCAGAAGA AACTCGTCAAGA</u> -3'
<b>ThioF</b>	5'-ATGACTGAGTTGGACACCAT-3'
<b>ThioR</b>	5'-TTATCGGTTGGCCGCGAGAT-3'

```

          HomF →
1 atgatgctga cgcgtccgct gaaggtggcg ctgctgggct gtggagtggg cggctcagag
61 gtggcgcgca tcatgacgac gcacggcgac gaccacgccg ccaggatcgg cggccccgtc
121 gaactggcgg ggggtggcgt gcgcaggccc tccaaggtac gggcgggcat cccgcaggag
181 ctcatcacca ccgacgccac cgcgctcgtc aaacgcggcg acatcgacgt cgtcatcgag
241 gtcateggcg gcatecgacc cgcgccacc ctcatcacca ccgccttcca gcacggcgcc
301 tccgtcgtct ccgccaacaa ggcgctgctg gccgaggacg gtgcgacgct ctaccgccc
361 gccgagcgcc acggcccgca cctctacttc gaggccgcgg tcgccggggc gatcccgtg
421 atcaggccgt tgcgcgagtc ctcgccgggg aacaaggtea accgggtgct cggcatcgtc
481 aacggcacca ccaacttcat cctggaccgg atggacagct ccggcgcccg ctacaccgag
541 gccctggacg agggcaccgc gctcgggtac gccgaggccg accccaccgc cgacgtcgag
601 ggcttcgacg ccgcccga aa ggccgccatc ctgcccggga tcgccttcca caccggggtc
661 cggctcgacg atgtgcaccg cgaggggctg accgaggtea ccgcccga catggctcg
721 gcccggcgga tgggctgcac cgtcaagctg ctgccatct gcgagcgcgc cgcgcagggc
781 cggtcggtea cggcccgggt gcaccccgcg atgatcccgc tcagccacc gcttgccctc
841 gtgcgagagg cgtacaacgc ggtcttcgct gagtcggagg ccgccgggca gctcatgttc
901 tacggccccg gcgcccggcg tccccacc gcgtccgcgc tcctcggcga cctcgtcgcc
961 gtgtgccgca acaagctcgc cgggtgcgaca gggccggggg agtcgcgta caccagctc
1021 cccgtgagcc ccagggcgga ggtggtcacg ccgtaccaca tcagtctcga cgtggccgac
1081 aagccgggtg tcctcggcca ggtcgcgacg gtcttcgccg agcacggggt gtcgatcgac
1141 accgtgcgcc agcaaggacg acaggactcc tccggcgagg cgtcgctcgt cgtcgccacc
1201 caccgcgcgc ccgacgccgc ccttcagggg accgtcgagg cgtcgcgctc gtcgcacacc
1261 gtgcgcggtg tcgccagcat catgctgtt gaaggggagt aa
          ← HomR

```

**Figure 2.3.** Nucleotide sequence of *S. clavuligerus hom* gene (GenBank AY802988). The sequences used in primer design are shown with an arrow.

```

          KanF →
1 gaacacgtag aaagccagtc cgcagaaacg gtgctgacc cggatgaatg tcagctactg
61 ggctatctgg acaagggaaa acgcaagcgc aaagagaaag caggtagctt gcagtgggct
121 tacatggcga tagctagact gggcggtttt atggacagca agcgaaccgg aattgccagc
181 tggggcgccc tctggttaagg ttgggaagcc ctgcaaagta aactggatgg ctttcttgcc
241 gccaaaggatc tgatggcgca ggggatcaag atctgatcaa gagacaggat gaggatcgtt
301 tcgcatgatt gaacaagatg gattgcacgc aggttctccg gccgcttggg tggagaggct
361 attcggctat gactggcgac aacagacaat cggctgctct gatgcccgcc tgttccggct
421 gtcagcgcag gggcgcccgg ttctttttgt caagaccgac ctgtcccgtg ccctgaatga
481 actccaagac gaggcagcgc ggtatcgtg gctggcccac acgggcgctc cttgcgcagc
541 tgtgctcgac gttgtcactg aagcgggaag ggactggctg ctattgggcg aagtgccggg
601 gcaggatctc ctgtcatctc accttgctcc tgccgagaaa gtatccatca tggctgatgc
661 aatgcggcgg ctgcatacgc ttgatccggc tacctgccc aatcgcacc aagcgaaca
721 tcgcatcgag cgagcacgta ctcgatgga agccggtctt gtcgatcagg atgatctgga
781 cgaagagcat caggggctcg cgcagcccga actgttcgcc aggctcaagg cgcggatgcc
841 cgacggcgag gatctcgtcg tgacccatgg cgatgcctgc ttgcccgaata tcatggtgga
901 aaatggcccg ttttctggat tcategactg tggccggctg ggtgtggcgg accgctatca
961 ggacatagcg ttggctaccg gtgatattgc tgaagagctt ggcggcgaat gggctgaccg
1021 cttcctcgtg ctttacggta tcgccgctcc cgattcgcag cgcatecctc tctatcgcct
1081 tcttgacgag ttcttctga
          ← KanR

```

**Figure 2.4.** Nucleotide sequence of the *kan* gene. ([http://seq.yeastgenome.org/vectordb/vector\\_descrip/COMPLETE/PK19.SEQ.html](http://seq.yeastgenome.org/vectordb/vector_descrip/COMPLETE/PK19.SEQ.html))

## **2.16. Southern blot hybridization**

### **2.16.1. Southern blotting**

Southern blotting was performed as described by Sambrook *et al.* (1989). DNA was run in 1% agarose gel at 25 Volts for 21-22 h. Following electrophoresis, the gel was first soaked in gel denaturation solution (Appendix B) with gentle agitation for 45 min and then neutralized in gel neutralization solution (Appendix B) with gentle agitation for 45 min. Then the gel was blotted for 20 h in 20 x SSC (Appendix B) using a positively charged nylon membrane (Ambion Brightstar<sup>TM</sup>-Plus). For DNA crosslinking, the membrane was exposed to UV light (300 nm) for 8 min.

### **2.16.2. Probe labeling and hybridization**

The probe DNA was labelled by using Gene Images AlkPhos Direct Labelling and Detection System (Amersham) according to the suppliers recommendations. Blots were put in hybridization tubes with DNA carrying sides facing the inside of the tube, and prehybridized with hybridization buffer (Appendix A) for at least 30 min at 50 °C. After prehybridization, denatured labelled probe was added to the prehybridization buffer and the membrane was hybridized o/n at 50 °C in hybridization oven with constant shaking. The membrane was washed twice with primary wash buffer (Appendix A) for 10 min at 50 °C and twice with secondary wash buffer (Appendix A) for 5 min at RT.

### **2.16.3. Signal generation and detection**

CDP-*Star* detection reagent was used for signal generation. After stringency washes, the blot was placed on a clean surface and CDP-*Star* Detection Reagent (30-40  $\mu\text{L}/\text{cm}^2$ ) was added directly on blots on the side carrying DNA. After 3-5 min incubation at RT, excess detection reagents were drained and the blot was wrapped in a Saran wrap. Then the blots were placed DNA side up in a film cassette. A sheet

of autoradiography film was placed on top of the blots under red light. Cassette was closed and the film (Kodak Scientific Imaging Film) was exposed for 2 h and developed.

### **2.17. Sample collection for growth determination, cephamycin C bioassay and HSD activity measurements**

3 mL of *S. clavuligerus* mycelium stock was inoculated into 500 mL triple-baffled flasks containing 100 mL of CDM (Appendix B). *hom* mutant of *S. clavuligerus* was supplemented with L-methione (150 µg/mL) and L-threonine (50 µg/mL). After 23-24 h of incubation at 28 °C with continuous shaking (220 rpm), optical density (OD<sub>595</sub>) of the suspension of broken mycelia generally reached to 0.2-0.3. 15 mL of this seed culture was inoculated into 500 mL baffled flasks containing 100 mL of CDM. Six such cultures were started for incubation with an initial OD<sub>595</sub> values of 0.030-0.035. Incubation was performed in an orbital incubator at 220 rpm for 120 h. Samples were taken at the 24, 48, 60, 72, 96 and 120 h of the fermentation. At each given specific hour of fermentation, one of the flasks was taken and samples were collected for optical density, dry cell weight and HSD assay measurements.

### **2.18. Determination of cultural growth**

Cultural growth was monitored by measurement of both OD at 595 nm and dry cell weight (DCW). OD measurements were done as indicated by Malmberg *et al.* (1993). A total of 0.5 mL of the culture was added to a tube containing 0.5 mL of 2.5 M HCl and 3 mL of distilled water. The mixture was homogenized by ultrasonification (Ultrasonic Processor, Cole Parmer) at 11 volt for 3 X 10 sec. The OD<sub>595</sub> of the resulting suspension was then measured. The cultures were diluted appropriately if the OD<sub>595</sub> was higher than 0.8. For DCW determination, 5 mL of culture was passed through Whatman cellulose nitrate membrane filters (0.2 µm pore size) by vacuum application. Filters were dried at 80 °C for 24 h and weighed.

Three independent measurements were done for both OD and DCW determination and their average value was used in further calculations.

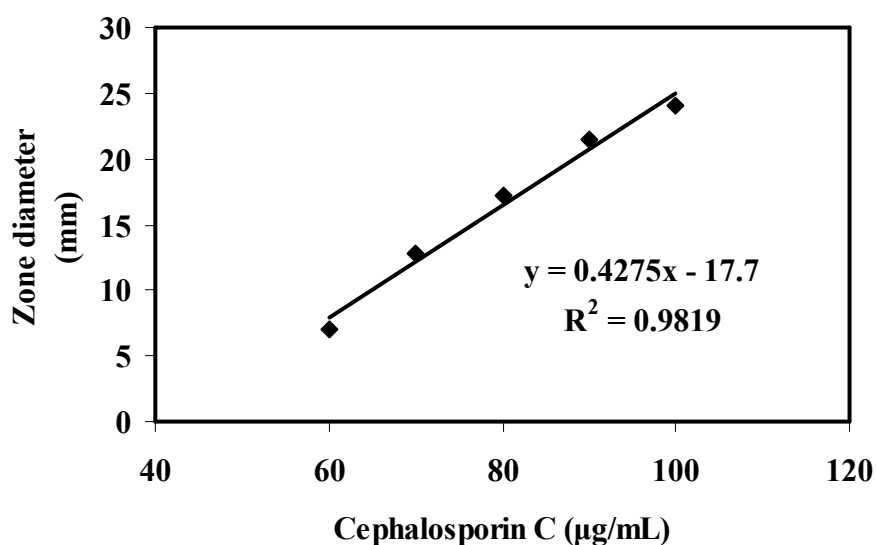
### **2.19. Cephamycin C bioassay**

Cephamycin C bioassay involved the use of the agar-diffusion method with *E. coli* ESS (a  $\beta$ -lactam-supersensitive mutant) as the indicator organism (Aharonowitz and Demain, 1978). For *E. coli* ESS glycerol stock preparation, a 2 mL of *E. coli* ESS o/n culture was centrifuged at 7000 g for 7-8 min and supernatant was discarded. Pellet was resuspended in 500  $\mu$ L, 50% glycerol and stored at -80 °C. 10 mL of SNA agar (NB + 0.5% (w/v) agar) (Appendix A) containing 100  $\mu$ L *E. coli* ESS glycerol stock 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  $\mu$ L of culture supernatant was impregnated into the disks, 10  $\mu$ L at a time. 5  $\mu$ L of acetone was next put on to the disks. After the soft agar was solidified, the disks were placed onto the agar surfaces and plates were incubated o/n at 37 °C. Zones of inhibition of growth were then measured after o/n growth. Cephamycin C production by both parental and *hom*-disrupted mutant *S. clavuligerus* cells was determined by using the standard curve constructed with cephalosporin C (CPC) as a standard.

### **2.20. Construction of cephamycin C bioassay calibration curve**

For the preparation of the cephamycin C bioassay calibration curve, cephalosporin C was used as a standard. The curve was constructed using the agar-diffusion method with *E. coli* ESS as the indicator organism (Aharonowitz and Demain, 1978). 10 mL of soft NB agar containing 100  $\mu$ L of *E. coli* ESS glycerol stock was poured into a bioassay plate containing 15 mL NB agar. Oxoid bioassay disks (England) were placed on the surface of a sterile, empty petri plate cover. Then 20  $\mu$ L of different concentrations 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 o/n at 37 °C. One unit of  $\beta$ -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 o/n growth. An inhibition zone versus CPC concentration graph was plotted as a calibration curve which is presented in Figure 2.5.



**Figure 2.5.** Cephamycin C bioassay calibration curve.

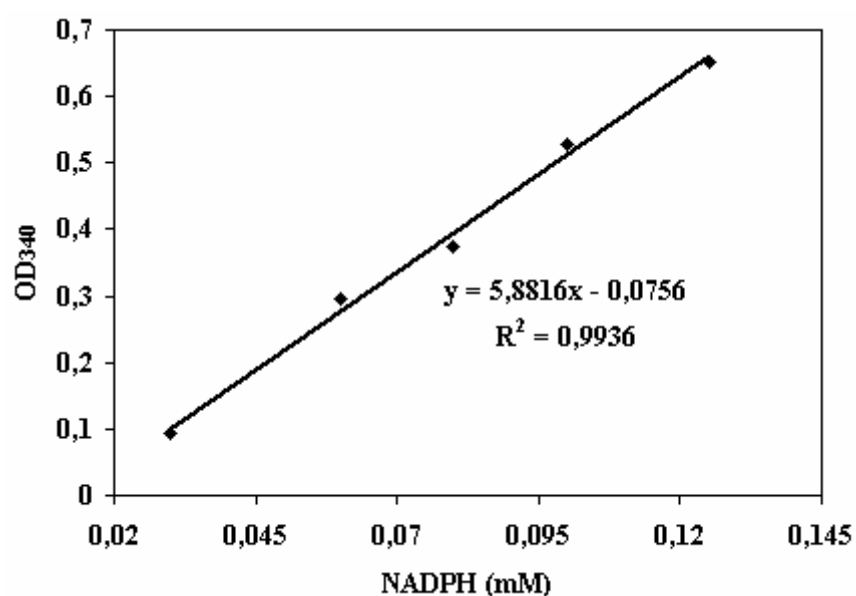
### 2.21. Measurement of homoserine dehydrogenase (HSD) activity

*S. clavuligerus* cells were grown in CDM. Samples were taken at 24, 48, 60, 72, 96, and 120 h for the measurement of enzyme activity. For each measurement, a 50 mL cell culture was centrifuged at 10,000 rpm for 15 min at 4 °C. Cell pellets were washed twice with cold phosphate buffer (50 mM, pH 7.5) and stored at -80 °C until use. For performing enzyme assay, the cells prepared for HSD assay were thawed slowly on ice and crude extracts were prepared as follows: Pellets were resuspended in sonication lysis buffer (Appendix A) which were then lysed by ultrasonication (Ultrasonic Processor, Cole Parmer). Sonication was conducted for 3 X 1 min with



pulses of 10 sec at 11 volts. After each 1 min sonication, the cells were rested for an additional 1 min. All the sonication procedure was performed on ice. Crude extracts were centrifuged at 10.000 rpm for 15 min at 4 °C. The pellets were discarded and the supernatants were used as crude extracts in HSD reaction mixture.

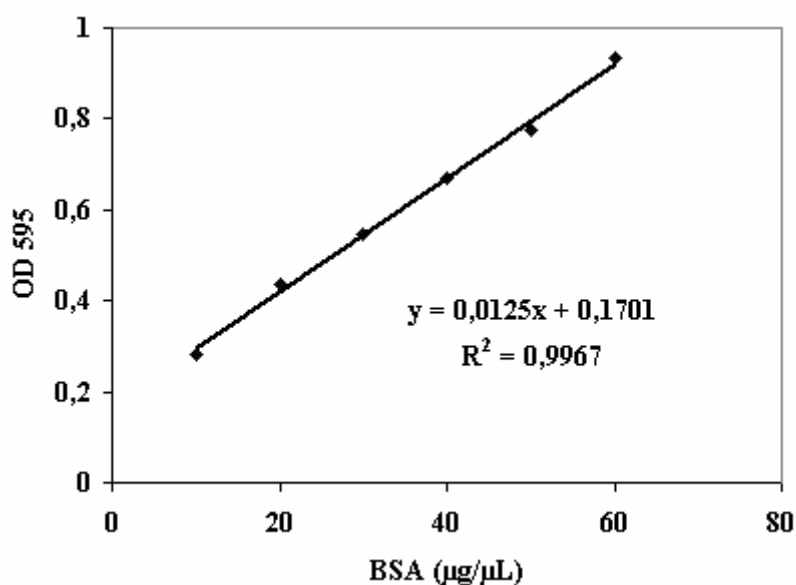
HSD activity was measured in the reverse reaction by determining the initial rate of increase of absorbance at 340nm. Standard assay mixture contained; 0.2 mL of 500 mM Tris-HCl buffer containing 5 mM EDTA, pH 8.4; 0.1 mL 100 mM L-homoserine, pH 7.0; 0.1 mL 4 mM NADP solution, 0.5 mL distilled water and 0.1 mL of crude extract. OD<sub>340</sub> was measured for 15 min and the change in first 6 min was used in calculations. The specific activity of HSD was expressed as micromoles of NADPH formed per min per mg of protein using the standard curve shown in Figure 2.6.



**Figure 2.6.** Calibration curve for the determination of NADPH concentration.

## 2.22. Determination of protein concentration

Protein concentration was measured by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard. 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 Whatman No. 1 filter paper. Bovine serum albumin (BSA) was used as the standard for preparation of protein calibration curve. Volumes of 10, 20, 30, 40, 50 and 60  $\mu\text{L}$  of 1 mg/mL BSA were added to tubes and volumes were adjusted to 500  $\mu\text{L}$  with water. 500  $\mu\text{L}$  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 protein concentration was prepared and the amounts of proteins were determined from the slope of the curve (Figure 2.7).



**Figure 2.7.** Calibration curve for the protein concentration determination.

## CHAPTER 3

### RESULTS AND DISCUSSION

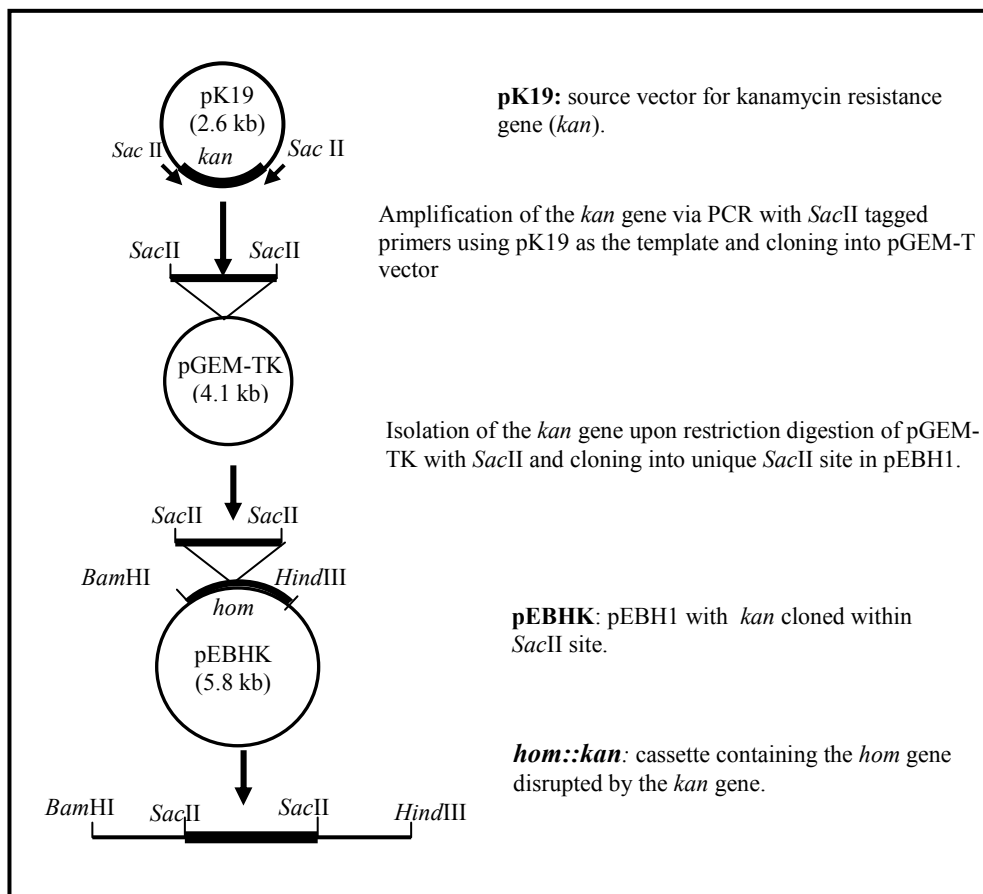
#### 3.1. Construction of homoserine dehydrogenase gene disruption cassette

Homoserine dehydrogenase gene disruption cassette was prepared by inserting a kanamycin resistance (*kan*) gene into the homoserine dehydrogenase gene (*hom*). Figure 3.1 summarizes the construction of the cassette.

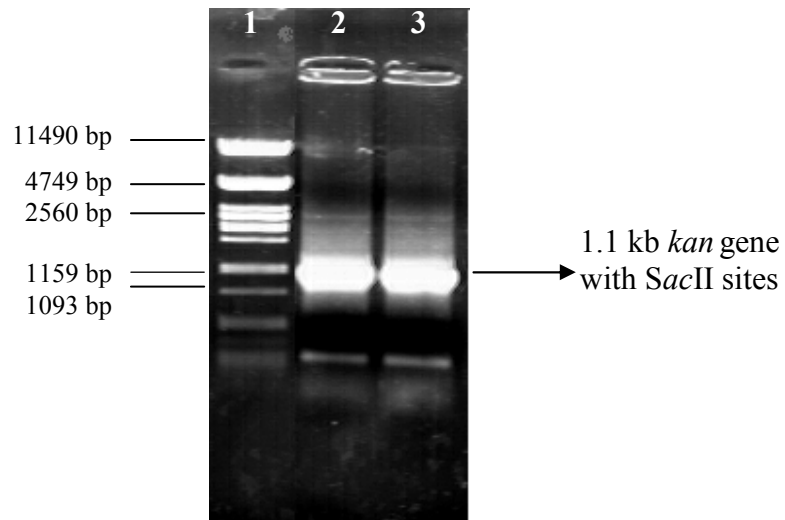
*hom* gene of *S. clavuligerus* was previously cloned in our laboratory and the *E. coli* plasmid (pQE-30) containing the *hom* gene flanking with *Bam*HI (5' end) and *Hind*III (3' end) restriction sites was named pEBH1. As the source of *kan* gene, *E. coli* plasmid vector pK19 (Figure 2.2) was used. Regarding the sequence of *kan* gene in pK19 plasmid, the primers tagged with *Sac*II restriction sites on 5' ends (KanF, KanR) were designed (Table 2.2). The forward primer was designed to be complementary to a region approximately 300 bp upstream of the start codon of the *kan* gene so that amplified fragment would also contain the promoter region of the gene (Figure 2.4). *kan* gene was amplified by PCR with KanF and KanR primers using pK19 as the template. Amplification resulted in 1.1 kb *kan* gene fragment (Figure 3.2).

Amplified *kan* gene fragment was extracted from the gel, ligated into pGEM-T vector and the resultant recombinant plasmid (pGEM-TK) was introduced into *E. coli* DH5- $\alpha$ . Plasmids were isolated from transformed *E. coli* cells and digested with

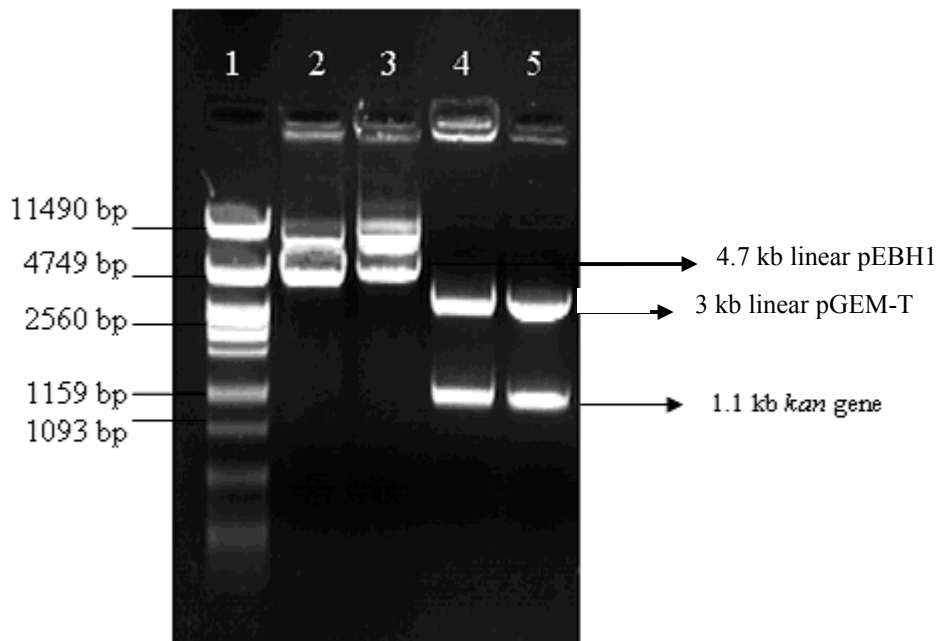
*Sac*II to obtain the *kan* gene (Figure 3.3). Meanwhile, pEBH1, the plasmid containing the *hom* gene, was also digested with the same enzyme. In pEBH1, the unique *Sac*II restriction site is located at the nucleotide 404 within the *hom* gene.



**Figure 3.1.** Construction of the *hom* disruption cassette.

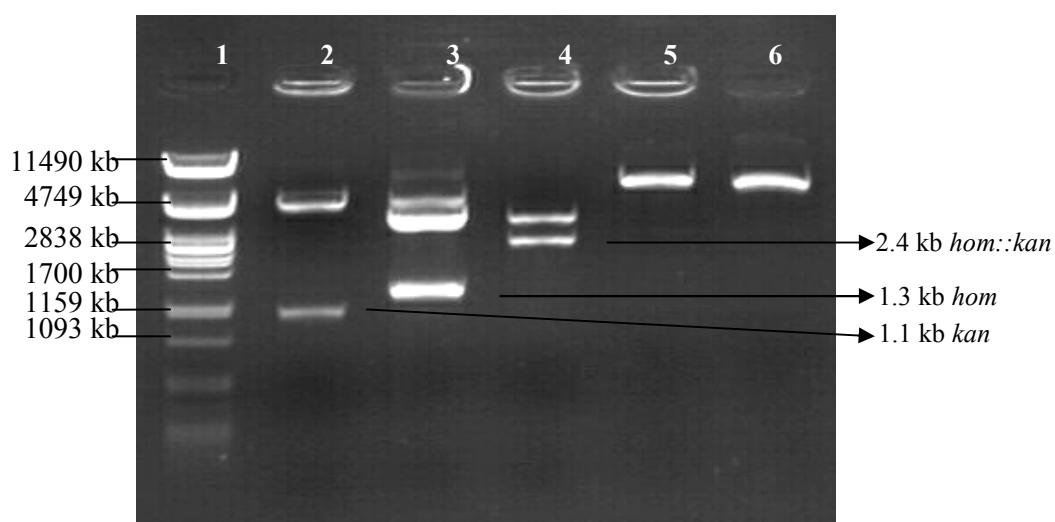


**Figure 3.2.** PCR amplification of the *kan* gene using pK19 as the template. 1) Lambda/*Pst*I size marker, 2,3) PCR product with KanF and KanR primers.



**Figure 3.3.** Restriction of pEBH1 and pGEM-TK with *Sac*II. 1) Lambda/*Pst*I size marker, 2,3) pEBH1 digested with *Sac*II, 4,5) pGEM-TK digested with *Sac*II.

Next, 1.1 kb *kan* gene and 4.7 kb linearized pEBH1 (Figure 3.3) were extracted from the gel and ligated. Ligation product was used to transform competent cells of *E.coli* ET12567. Transformants were selected on selective medium containing ampicillin and kanamycin. Plasmids were isolated from the transformants and both single digested with *Sac*II and *Bam*HI and double digested with *Bam*HI-*Hind*III to confirm that they contain the *hom* gene disrupted by the *kan* gene (Figure 3.4). The resultant recombinant plasmid was designated pEBHK. 2.4 kb *hom::kan* fragment was extracted from the gel and stored for further use.



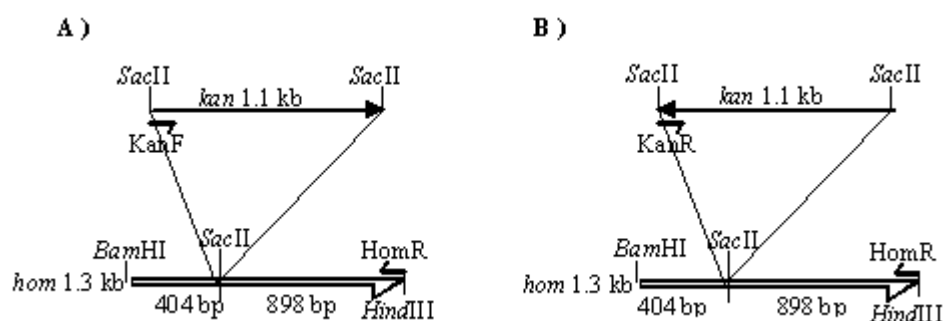
**Figure 3.4.** Digestion of pEBH1 and pEBHK with *Sac*II, *Bam*HI and *Hind*III.

1) Lambda/*Pst*I size marker, 2) Digestion of pEBHK with *Sac*II, 3) Digestion of pEBH1 with *Bam*HI and *Hind*III, 4) Digestion of pEBHK with *Bam*HI and *Hind*III, 5) Digestion of pEBHK with *Bam*HI, 6) Digestion of pEBH1 with *Bam*HI.

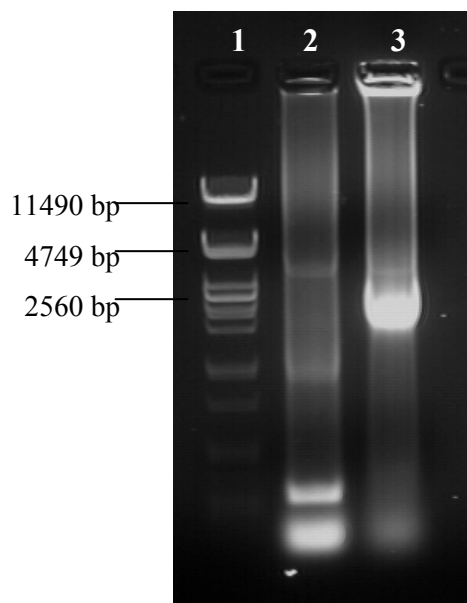
The transfer of pEBHK into the methylation-deficient host *E. coli* ET12567, was an important step in order to avoid streptomycetes' restriction barriers. Many streptomycetes possess restriction systems that can drastically reduce transformation

frequencies. One way to eliminate this is the usage a methylation-deficient *E. coli* as an intermediate host (MacNeil et al., 1992).

The transcriptional direction of *kan* gene within the *hom* gene was also determined. To reveal the correct direction, two PCRs were designed using pEBH1 as the template and two different primer pairs, HomR-KanF and HomR-KanR, respectively. With these primers, amplification would be only in one of the PCR tubes. As illustrated in the Figure 3.5, if *hom* and *kan* were in the same direction, amplification would occur only with HomR-KanF primer pair. On the other hand, if the *kan* gene was inserted in the reverse direction with respect to the *hom* gene, only HomR-KanR primer pair would give rise to amplification. An amplicon of 1998 bp was expected in either case. When PCRs were performed, amplification was seen only with the HomR-KanR primer pair (Figure 3.6). Thus, it was concluded that the the *kan* gene was in reverse orientation with respect to the *hom* gene.



**Figure 3.5.** Diagram showing two possible PCR amplifications when A) the *kan* gene is in the same direction with the *hom* gene and B) the directions of the *hom* and *kan* genes are different.



**Figure 3.6.** PCR with 2) HomR-KanF and 3) HomR-KanR primer pairs using pEBHK as the template. 1) Lambda/*Pst*I size marker.

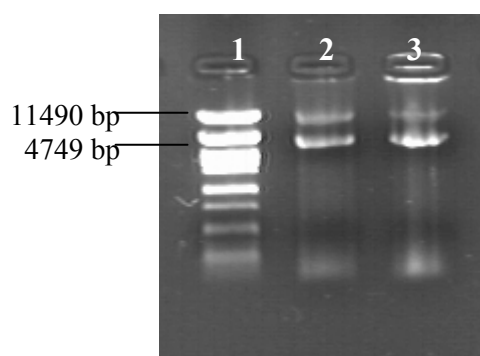
### 3.2. Transformation of *S. clavuligerus*

According to the existing literature reports (Matsushima *et al.*, 1987; Keiser *et al.*, 2000) and our previous experience, *S. clavuligerus* is hardly transformable because of restriction endonuclease barriers. Therefore, an efficient transformation procedure was to be adopted by optimizing the available protocols which are based on the transformation of protoplasts. The *Streptomyces* plasmid vector we used in these attempts was pIJ486 (Figure 2.2) which has thiostrepton resistance gene (*tsr*) as a selective marker. pIJ486 is a multicopy, stable plasmid when there is thiostrepton in growth medium. On the other hand, in the absence of thiostrepton, this vector is unstable and can be easily cured from the cells after a few rounds of sporulation. A closely-related species, *S. lividans*, was much easier to transform, therefore the protoplasts of this organism were prepared and transformed with pIJ486 in order to



amplify this vector. The transformants of *S. lividans* were selected with thiostrepton, the plasmid DNA was isolated from them (Figure 3.7) and used to develop an optimized transformation protocol for *S. clavuligerus*.

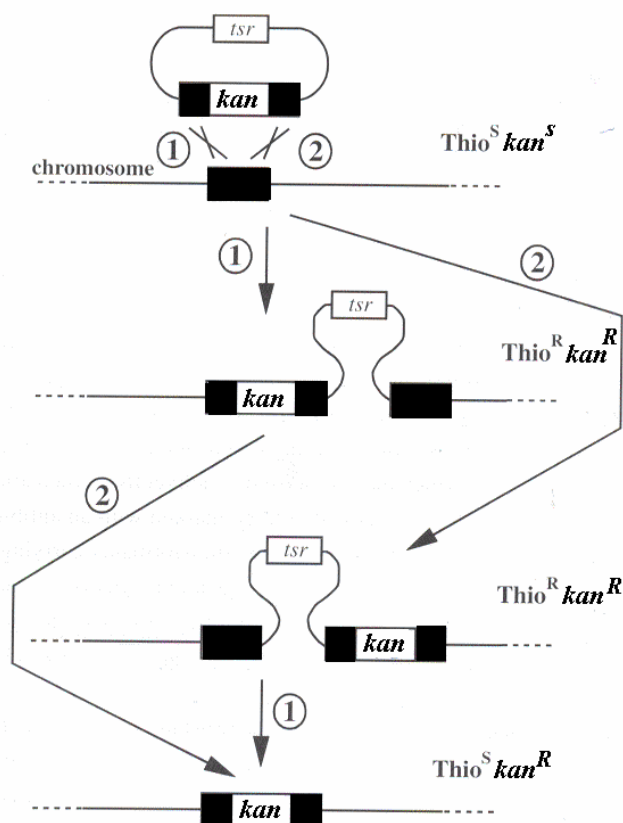
PEG-mediated protoplast transformation (Keiser *et al.*, 2000; Garcia-Dominguez *et al.*, 1987; Rodicio and Chater, 1982) which is a widely used *Streptomyces* transformation method was taken as a base and the parameters most likely affecting the transformation of *S. clavuligerus* were outlined as follows: (i) Protoplast buffer which is used both in protoplast preparation and transformation, (ii) age of the culture used for protoplast preparation, (iii) heat-treatment of protoplasts prior to transformation, (iv) PEG concentration, (v) protoplast regeneration medium, (vi) incubation temperature for protoplast regeneration and (vii) the final thiostrepton concentration for selection. After testing 3 to 4 variables for each parameter independently, transformation frequencies were greatly improved under the following conditions: PE buffer as the protoplast buffer, protoplast preparation from 24 h-old *S. clavuligerus* culture still in exponential growth phase, heat treatment of protoplasts at 42°C for 10 min prior to transformation, PEG concentration of 50% during transformation, R2YE medium as the protoplast regeneration medium, 26 °C as the protoplast regeneration temperature and 8 µg/mL final thiostrepton concentration for the selection of the transformants.



**Figure 3.7.** pIJ486 vector isolated from *S. lividans* (2,3) and Lambda/*Pst*I size marker (1).

### 3.3. Delivery of *hom* disruption cassette

Insertional inactivation via double crossing over is a widely used technique for the inactivation of *Streptomyces* genes (Jensen *et al.*, 2004; Li *et al.*, 2000; Mosher *et*



**Figure 3.8.** Insertional inactivation via double crossing over. The two crossovers may occur simultaneously (1) or successively (2). (Modified from Keiser *et al.*, 2000)

*et al.*, 1999; Jensen *et al.*, 2000; Paradkar *et al.*, 1995; Paradkar *et al.*, 2001). In this method, a selectable marker such as the kanamycin resistance gene employed in the present study is put into a cloned copy of the target gene. The mutant allele (disruption cassette) is thus created, carried on a suitable vector, and then used to replace the chromosomal copy of the target gene via double crossovers, one on

either site of the selectable marker (Figure 3.8), resulting in a mutation that cannot revert (Keiser et al., 2000). Although the basic principle of gene replacement via double crossover is unique, we tried different types of vectors for introduction of the *hom* disruption cassette into the *S. clavuligerus* cells.

### **3.3.1. The use of pEBHK as a suicide vector for the delivery of *hom::kan* cassette**

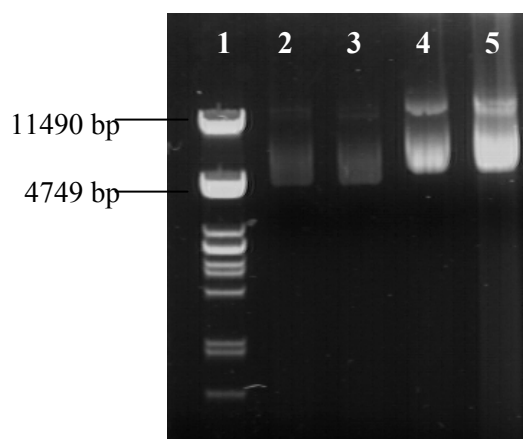
The plasmid vector pEBHK containing the *hom::kan* cassette as a *Bam*HI-*Hind*III fragment was the first delivery vector used in this study. Being an *E. coli* vector, this plasmid cannot replicate in *Streptomyces*, hence it would act as a suicide plasmid in *S. clavuligerus*. Only in the condition of homolog recombination between the *hom* gene on the chromosome and on the vector, the *S. clavuligerus* cells would be kanamycin-resistant. Thus, the use of this suicide vector would provide an easy selection for the mutants in spite of the fact that the transformation frequency of the cells with this kind of a vector of non-*Streptomyces* origin must be low (Keiser et al., 2000). However, no transformants could be obtained when this vector was employed and kanamycin selection was made.

It is known that single-stranded DNA gives transformation frequencies 10- to 100-fold higher than the corresponding double-stranded molecule (Hillemann et al., 1991). Hence, the suicide vector pEBHK was denatured with alkaline treatment (Keiser et al., 2000) and the resulting single-stranded pEBHK was used to transform *S. clavuligerus* protoplasts. Still, no transformants were obtained with this manipulation.

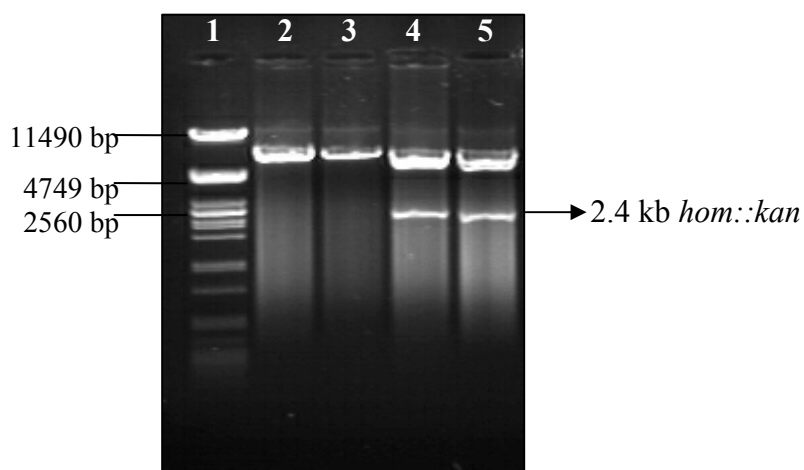
### **3.3.2. The use of pIJ486 for *hom::kan* delivery**

Another candidate delivery vector was the *Streptomyces* plasmid pIJ486 (Figure 2.2). The *hom:kan* fragment obtained by the digestion of pEBHK with *Bam*HI and *Hind*III (Figure 3.4) was ligated to *Bam*HI-*Hind*III digested pIJ486. *S. clavuligerus*

protoplasts were transformed with the ligation product. The transformants selected with kanamycin were further screened for thiostrepton sensitivity after a few rounds of sporulation to allow plasmid curing.

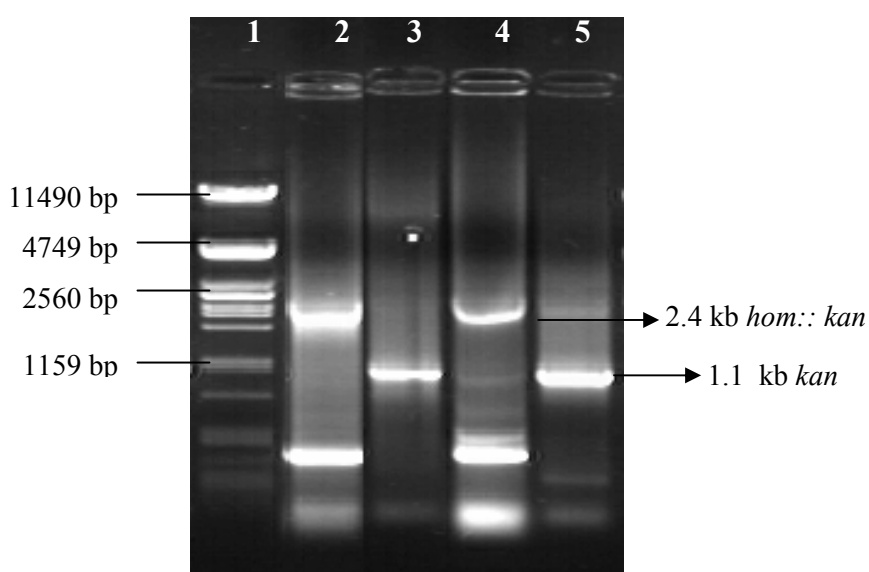


**Figure 3.9.** pIJ486 (2,3) and pAEHK (4,5) plasmids isolated from the cells of *S. clavuligerus*. Lambda/PstI (1) was used as a size marker.



**Figure 3.10.** Digestion of pIJ486 and pAEHK plasmids from *S. clavuligerus*. 1) Lambda/PstI size marker, 2,3) pIJ486 digested with *Bam*HI-*Hind*III, 4,5) pAEHK digested with *Bam*HI-*Hind*III.

A fraction of the regenerated protoplasts was selected with both thiostrepton and kanamycin for the amplification of the recombinant plasmid named pAEHK. Figure 3.9 shows the plasmid pAEHK. Verification of recombination was made by double digestion of pAEHK with *Bam*HI-*Hind*III (Figure 3.10) and by PCR with *hom* and *kan* primers using this recombinant plasmid as a template (Figure 3.11).



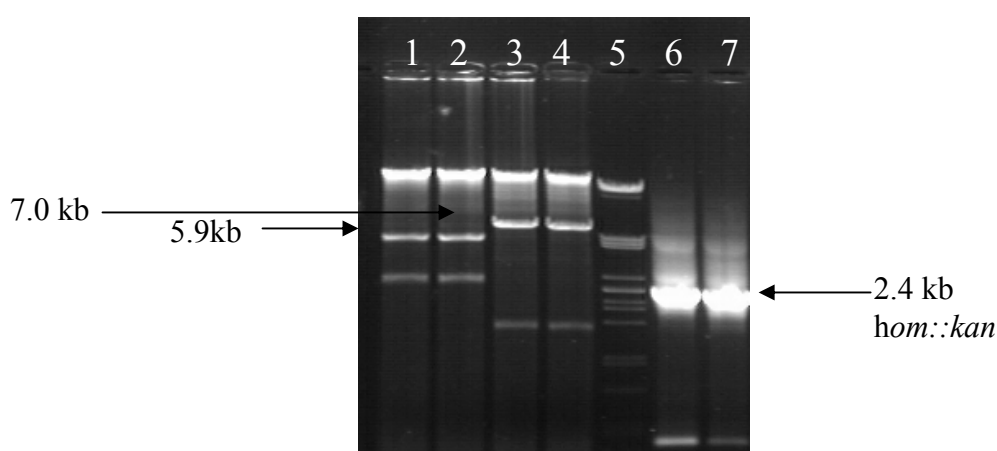
**Figure 3.11.** PCR with *hom* and *kan* primers using pAEHK from *S. clavuligerus* as a template. 1) Lambda/*Pst*I size marker, 2,4) PCR with *hom* primers, 3,5) PCR with *kan* primers.

### 3.3.3. Use of linearized pAEHK vector for *hom::kan* delivery

The linearized DNA is known to increase the percentage of the primary double crossovers. In order to maintain chromosomal integrity, double crossovers do occur more frequently than single crossover. However, it is also known that upon

transformation with linearized DNA, transformation efficiencies are greatly reduced (Keiser et al., 2000).

To test this strategy, pAEHK vector was linearized and used for transformation. In order to obtain linear vector, pAEHK was double digested with *EcoRV-XhoI* and *EcoRV-NotI*, respectively, to leave behind an intact *hom::kan* cassette in either case (Figure 3.12).



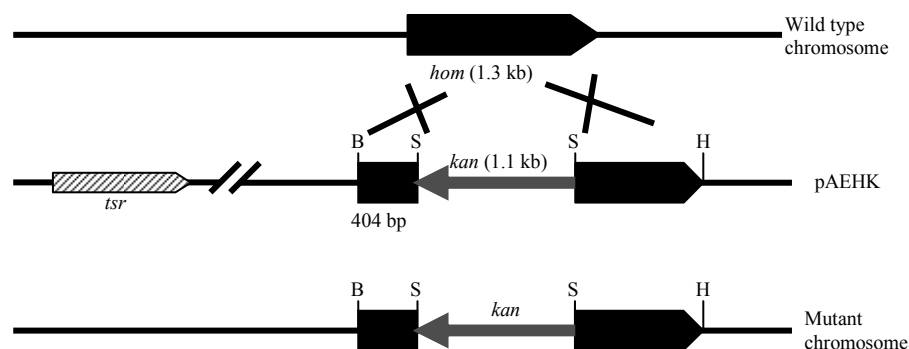
**Figure 3.12.** Linearization of pEBHK vector for the delivery of the disruption cassette into *S. clavuligerus*. 2) *EcoRV-XhoI* digested pEBHK from *S. clavuligerus*, 3, 4) *EcoRV-NotI* digested pEBHK from *S. clavuligerus*, 5) Lambda/*PstI* size marker, 6, 7) PCR with pAEHK template with *hom* primers.

5.9 kb fragment obtained from *EcoRV-XhoI* digestion and the 7.0 kb fragment obtained from *EcoRV-NotI* digestion were extracted from the gel and used for the transformation of *S. clavuligerus* protoplasts. The *hom::kan* fragment obtained by the PCR from pAEHK template was (Figure 3.12) also used to transform protoplasts. However, none of these linearized DNA yielded any transformants. The molecules were also denatured and the resultant single-stranded linear DNA

fragments were used for the transformation of *S. clavuligerus*. However, no transformants could be obtained.

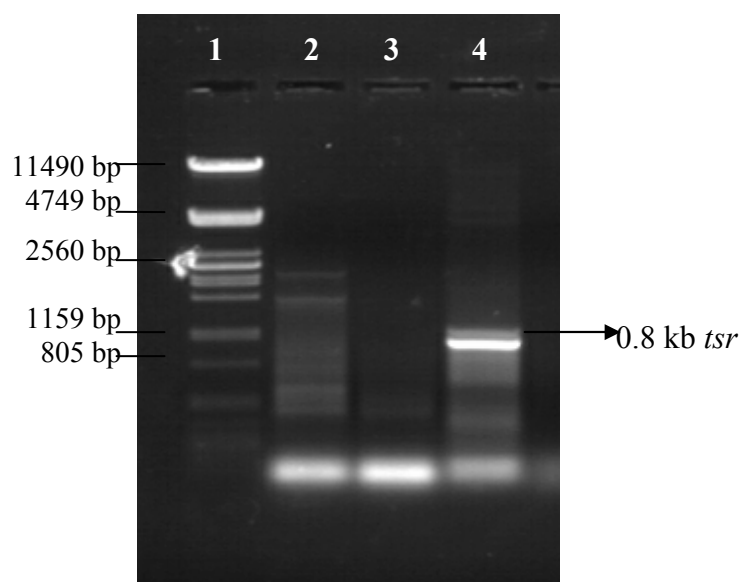
### 3.4. Selection of the putative *hom*-disrupted mutants of *S. clavuligerus*

The cells transformed with pAEHK and selected for kanamycin resistance (described in the section 3.3.2) were replica plated on *Streptomyces* sporulation medium for plasmid curing. Plasmid curing is important for selection of the double crossovers. Only after all plasmids are cured, one can conclude that the antibiotic resistant colonies (in our case, kanamycin resistant) are the ones in which double crossovers occurred. The phenotype of putative mutants was expected to be kanamycin resistant ( $\text{kan}^R$ ) and thiostrepton sensitive ( $\text{thio}^S$ ) as illustrated in Figure 3.8 and Figure 3.13. After the first round of sporulation, colonies were replica plated onto fresh sporulation medium and a second round of sporulation was performed. Replica plating onto fresh sporulation media was continued to make sure that plasmids were cured in the majority of the colonies. After the fifth round of sporulation, colonies were replica plated onto TSA medium containing kanamycin and another TSA medium containing thiostrepton. Finally, one colony was found to be kanamycin resistant and thiostrepton sensitive. This colony was chosen as a putative *hom* mutant *S. clavuligerus* and named AK39.



**Figure 3.13.** Diagram of the *hom* gene disruption via double crossing over. Restriction enzyme sites are shown as B, *Bam*HI; S, *Sac*II; H, *Hind*III.

The loss of plasmid from *S. clavuligerus* AK39 was first verified by plasmid analysis. PCR was next performed with the primers ThioR and ThioF which were designed for the amplification of the thiostrepton resistance gene (*tsr*). There was no amplification in the reactions performed with genomic DNA of the wild type and recombinant *S. clavuligerus* used as two different templates (Figure 3.14). If there were any amplification with the recombinant DNA, it would indicate the integration via single cross over of whole plasmid into the genomic DNA or the presence of plasmid DNA in the cell.



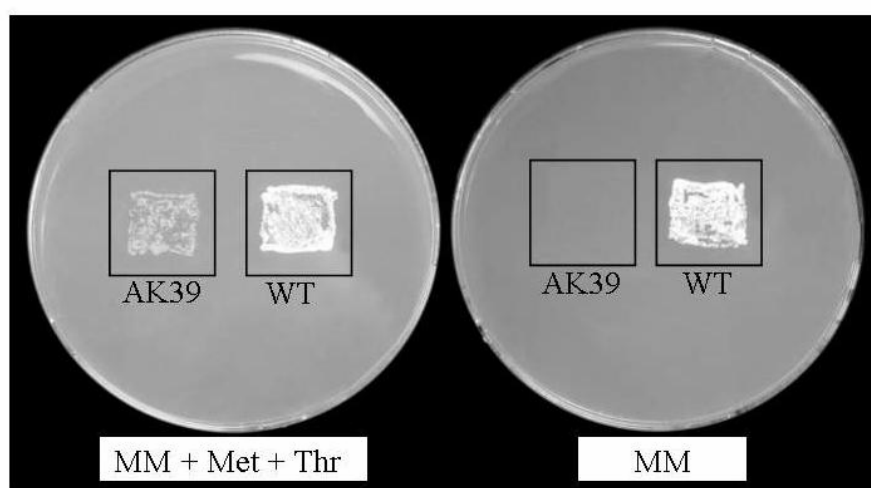
**Figure 3.14.** PCR with ThioR and ThioF primers. 1) Lambda/*Pst*I size marker, 2) PCR product using the wild *S. clavuligerus* genomic DNA, 3) PCR using the mutant *S. clavuligerus* genomic DNA 4) PCR product with pAEHK as the template (positive control).



### 3.5. Verification of the targeted *hom* mutation in *S. clavuligerus*

#### 3.5.1. Test for auxotrophy

Putative *hom* mutant *S. clavuligerus* cells were streaked onto minimal medium (MM) to test for auxotrophy. The mutant could not grow on MM, while it could grow well on MM supplemented with L- methionine and L-threonine (Figure 3.15). This provided strong evidence that the *hom* gene coding for HSD was successfully disrupted with *hom::kan* cassette resulting in a L-methionine and L-threonine auxotrophy and kanamycin resistance.

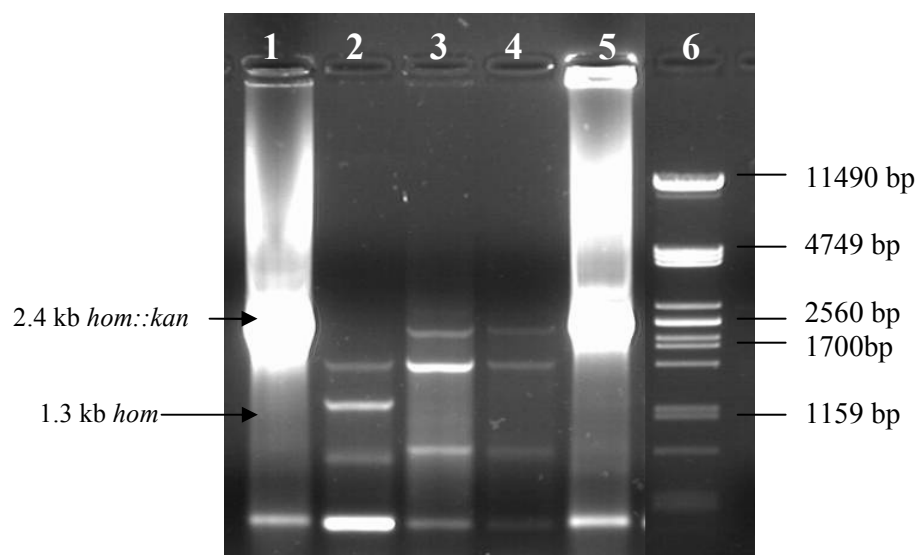


**Figure 3.15.** *hom*-disrupted mutant AK39 exhibiting L-methionine and L-threonine auxotrophy. Identical volumes of the exponentially growing wild and mutant strains were streaked onto MM plates and incubated at 28 °C for 72 h.

#### 3.5.2. Verification with PCR

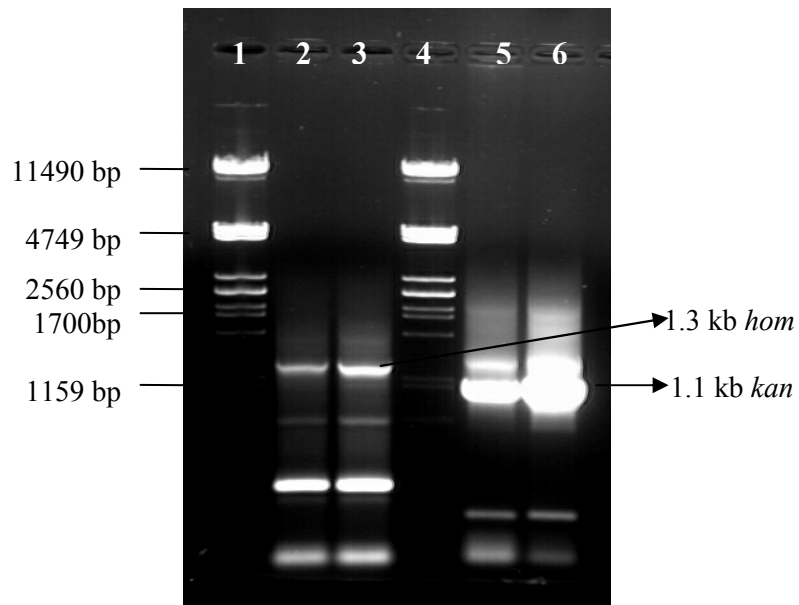
The disruption of the *hom* gene in *S. clavuligerus* AK39 was proved by PCR with the chromosomal DNA from the wild and mutant strains by using *hom* primers. The PCR product of the reaction with the chromosomal DNA of the wild strain gave an expected 1.3 kb band corresponding to the *hom* gene. However, instead of the

amplification of 1.3 kb *hom* gene, 2.4 kb *hom::kan* fragment was amplified in the PCR with the chromosomal DNA of the mutant (Figure 3.16), verifying the disruption of the *hom* gene in *S. clavuligerus* AK39.



**Figure 3.16.** PCR for the verification of *hom* disruption (I). 1,5) PCR with the *hom* primers using pAEHK as a template (positive control), 2) PCR with the *hom* primers using the chromosome of the wild *S. clavuligerus*, 3,4) PCR with the *hom* primers using the chromosome of the mutant *S. clavuligerus*, 6) Lambda/*Pst*I size marker.

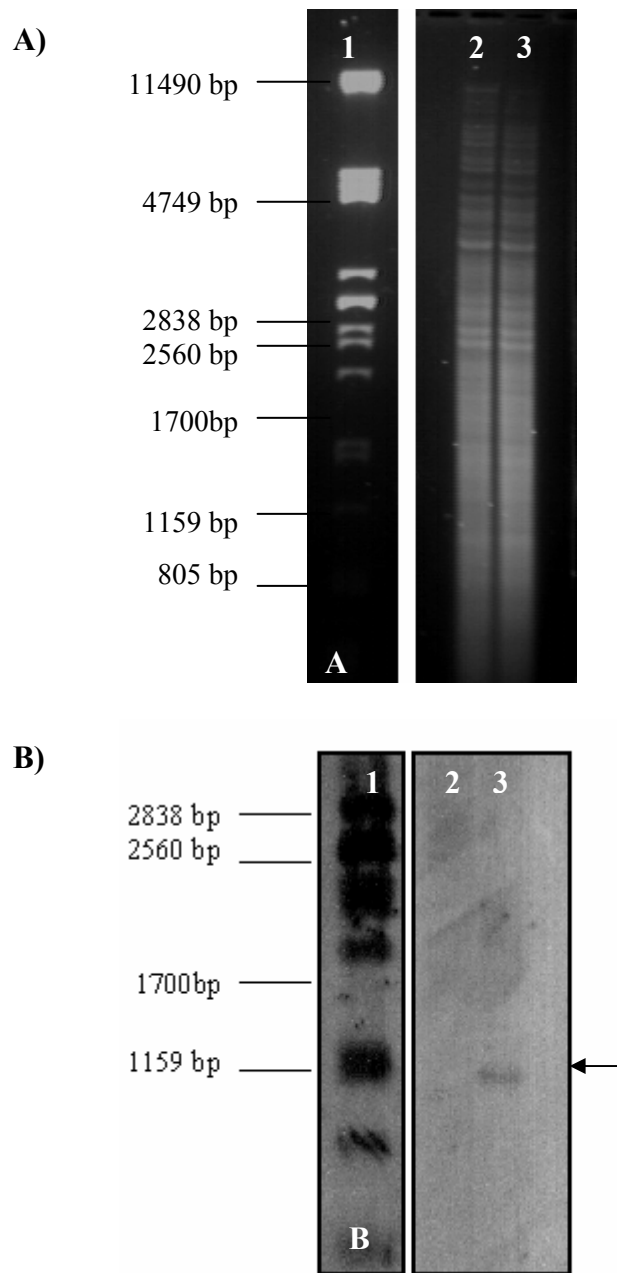
Next, the 2.4 kb PCR product obtained from the mutant *S. clavuligerus* chromosomal DNA was extracted from the gel and used as a template in a second PCR, this time with *kan* primers. 1.1 kb *kan* gene was successfully amplified (Figure 3.17), providing further confirmation for *hom* disruption with the cassette in AK39. Similarly, 1.3 kb PCR product obtained with the *hom* primers using the chromosome of wild *S. clavuligerus* was isolated from the gel and used as a template in a second PCR again with the *hom* primers. This PCR was made just for another control yielded the expected 1.3 kb *hom* (Figure 3.17).



**Figure 3.17.** PCR for the verification of *hom* disruption (II). 1,4) Lambda/PstI size marker, 2,3) PCR with the *hom* primers using the 1.3 kb amplicon as a template, 5,6) PCR with the *kan* primers using the 2.4 kb amplicon as a template.

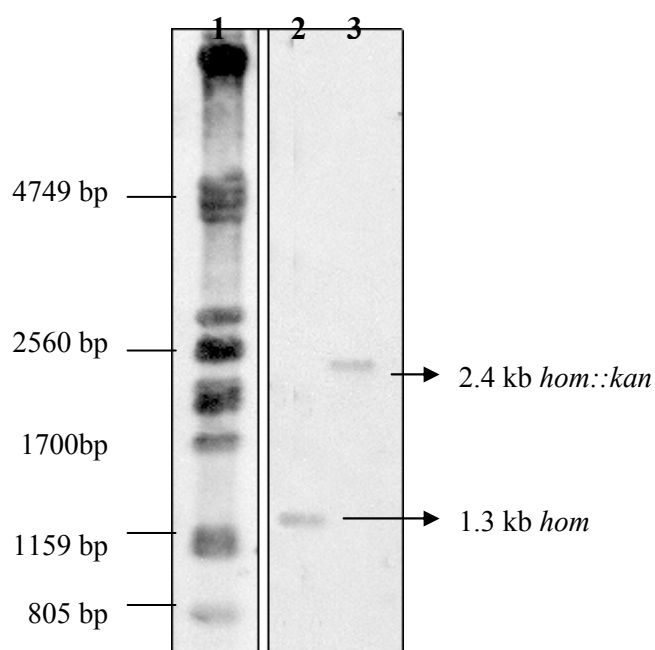
### 3.5.3. Verification with Southern blot hybridization

Disruption of the *hom* gene was also verified by performing Southern blot hybridization. Chromosomal DNAs from the wild and mutant *S. clavuligerus* were digested with *Sac*II, run on a gel (Figure 3.18.A), blotted and hybridized with a labeled *kan* gene fragment as the probe. As expected, *Sac*II digestion released the intact kanamycin resistance gene from the chromosome of the *hom* mutant strain which gave signal when hybridized to the *kan* probe (Figure 3.18.B).



**Figure 3.18.** Southern blot hybridization analysis using labeled *kan* gene as a probe. A) *Sac*II-digested and blotted chromosomal DNA from the 2) wild strain and 3) mutant, 1) Lambda/*Pst*I size marker. B) Detection of hybridization with 1) Lambda/*Pst*I size marker hybridized with labeled Lambda/*Pst*I fragments, 2) *Sac*II-digested chromosomal DNA from the wild strain hybridized with labeled *kan* gene as a probe, 3) *Sac*II-digested chromosomal DNA from the mutant strain hybridized with labeled *kan* gene as a probe.

In addition, 1.3 kb and 2.4 kb PCR products obtained with the *hom* primers from the mutant and wild type *S. clavuligerus* chromosomal DNA, respectively (Figure 3.16) were extracted from the gel and loaded to an agarose gel for Southern blotting followed by hybridization performed with a *hom* gene probe (Figure 3.19). The results showed that PCR-amplified fragments contained the *hom* gene and thus the amplifications were not non-specific.



**Figure 3.19.** Southern blot hybridization analysis using labeled *hom* gene as a probe. 1) Lambda/*Pst*I size marker hybridized with labeled Lambda/*Pst*I fragments, 2) 1.3 kb PCR product obtained with the *hom* primers from the wild type *S. clavuligerus* chromosomal DNA, 3) 2.4 kb PCR product obtained with the *hom* primers from the mutant *S. clavuligerus* chromosomal DNA.

### 3.5.4. Verification with HSD assay

Disruption of *hom* gene was also proved at enzyme level. Wild and mutant *S. clavuligerus* strains were grown in CDM. Samples were taken at 48, 60, 72, 96, and 120 h and HSD activities were determined. As expected, no HSD activity was detected from the mutant *S. clavuligerus* (Table 3.1).

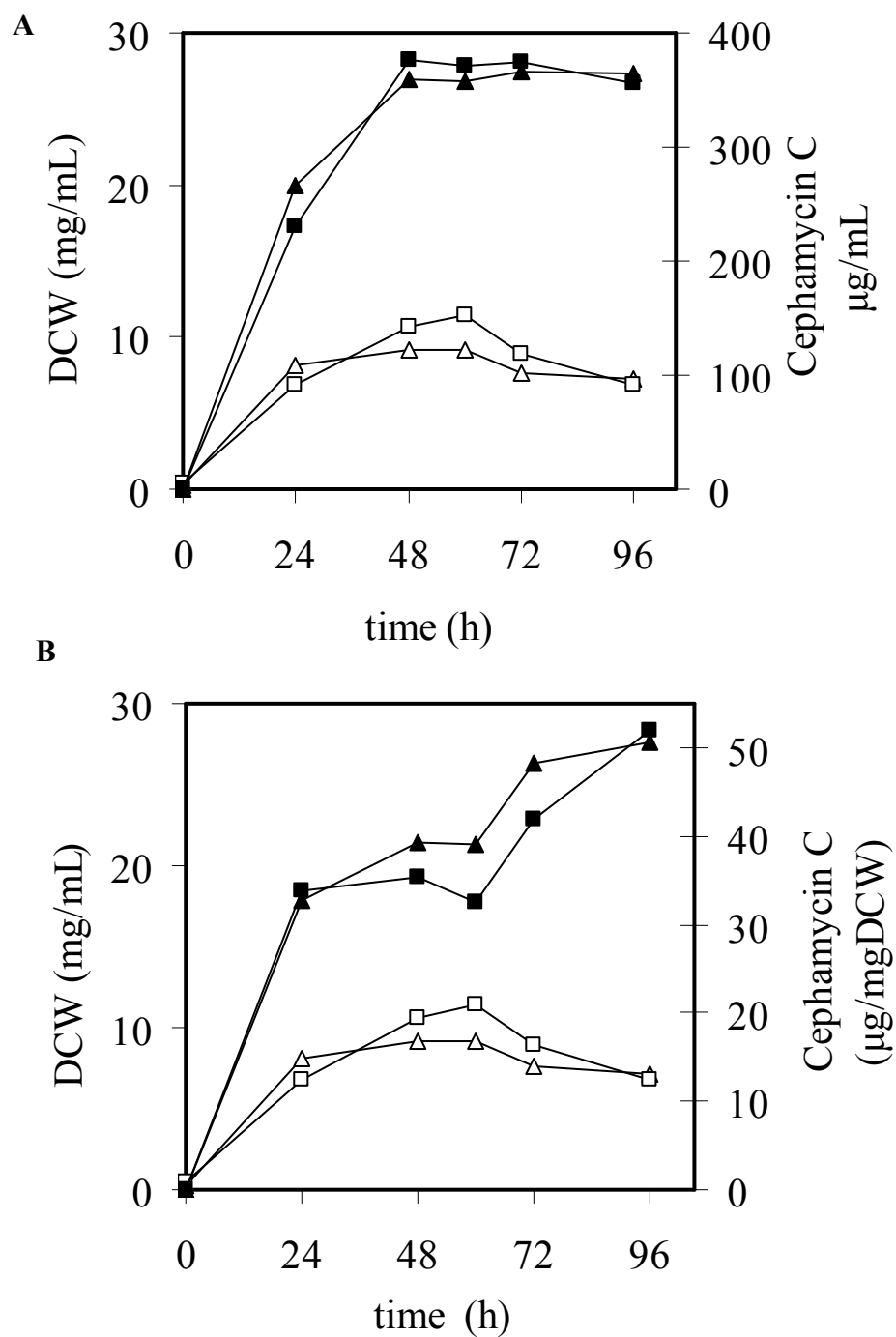
**Table 3.1.** HSD activities from wild and mutant strains of *S. clavuligerus*

Cultivation time (h)	Specific HSD activity ( $\mu\text{mol}/\text{min}/\text{mg}$ protein)	
	Wild type	Mutant
48	0.02	0
60	0.02	0
72	0.05	0
96	0.07	0
120	0.04	0

### 3.6. Growth and cephamycin C production patterns of the wild and *hom*-disrupted mutant strains of *S. clavuligerus*

The effect of *hom* gene disruption on growth and cephamycin C production was determined by growing the wild type and mutant *S. clavuligerus* in CDM and TSBS for 96 h and taking the aliquots from the cultures at time intervals. Cephamycin C levels in cultures were tabulated as both volumetric and specific production. Specific production of cephamycin C was reported as  $\mu\text{g}$  cephamycin C produced by mg dry cell weight (DCW) of bacteria and hence, it reflects the antibiotic production capacities of the cells much better than volumetric production.

*hom* gene disruption in AK39 resulted in a much poorer growth in CDM supplemented with L-methionine and L-threonine while its growth was even better than that of the wild strain in TSBS rich in amino acids (Figure 3.20 and Figure 3.21). In TSBS, volumetric production of cephamycin C was slightly higher in the

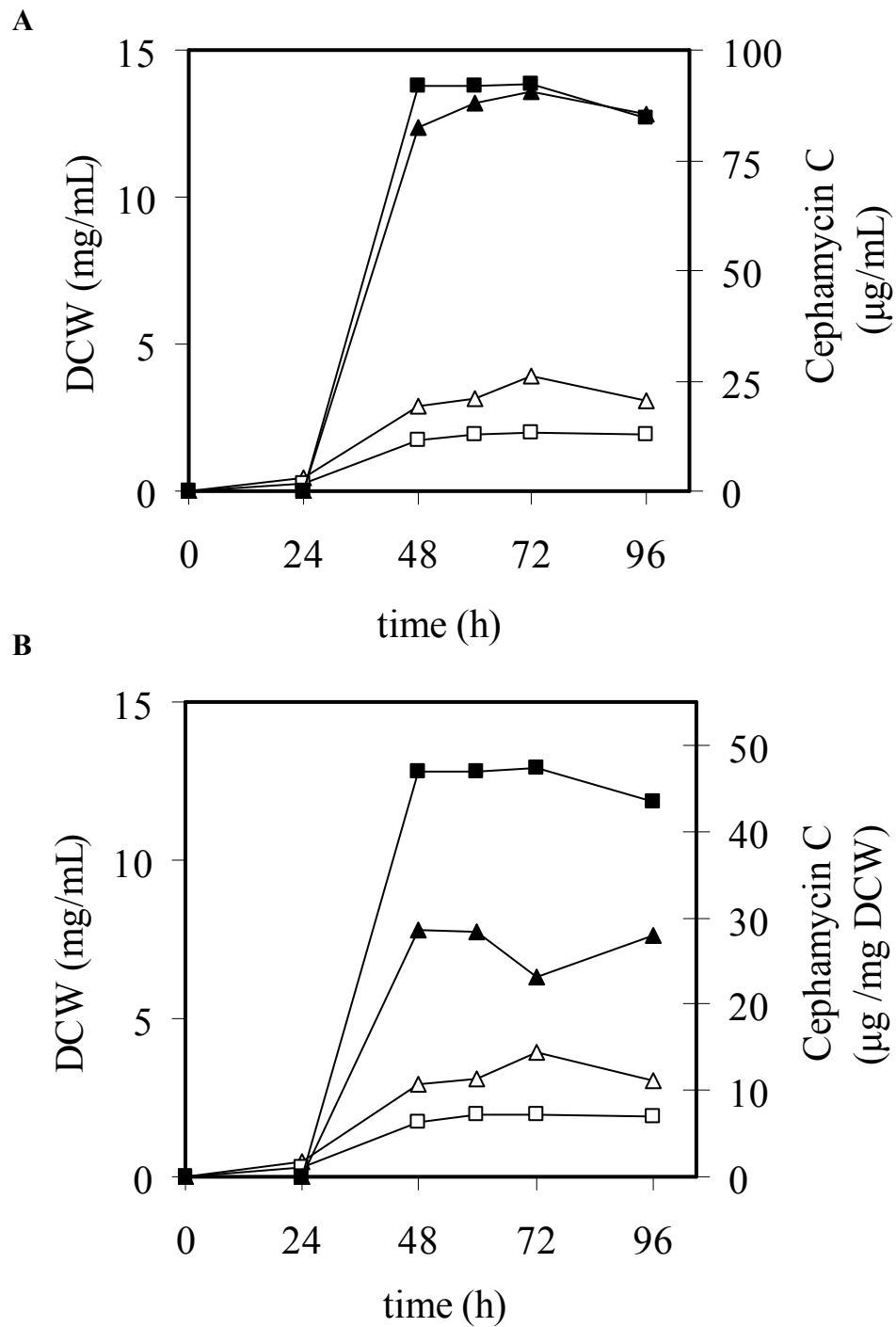


**Figure 3.20.** (A) Volumetric and (B) specific cephamycin C production in TSBS medium. Open symbols represent DCW and closed symbols represent cephamycin C production. Squares show the mutant *S. clavuligerus* and triangles symbolize the wild strain.

mutant *S. clavuligerus* than the wild type (Figure 3.20.A) while the specific cephamycin C production by the wild strain was about 10-20% higher than that of the mutant at 48, 60 and 72 h of cultivation (Figure 3.20.B). Therefore, there was not a remarkable difference in cephamycin C levels of the wild and mutant strains in rich TSBS medium. Indeed, rich medium is not suitable to reveal the effects of *hom* disruption on cephamycin C production, because it contains amino acids in high amounts. When the wild type is provided with plenty of L-methionine and L-threonine externally, as occurs in TSBS, it is not even expected to use the pathway producing L-methionine and L-threonine.

The effect of *hom* disruption on antibiotic biosynthesis was observed in CDM where AK39 produced 1.7 to 2 fold higher specific yields of cephamycin C (Figure 3.21.B). It is to be noted that the entire experiment was repeated three times with similar results. Mendelovitz and Aharonowitz (1982) studied the effects of amino acids of the aspartic acid pathway on cephamycin C production by *S. clavuligerus* NRRL 3585. It was reported that DAP and L-lysine exerted precursor effect, increasing specific cephamycin C production by 1.9 and 1.7 fold, respectively. In the same study, the specific activity of dihydrodipicolinic acid synthetase was found to be higher than that of HSD which suggested conversion of ASA to dihydrodipicolinate in a greater efficiency. This might have accounted for the lack of a massive increase of several folds in cephamycin C yields upon *hom* disruption. Otherwise, regarding the dual effect of *hom* disruption, one being an increase in the carbon flow towards L-lysine-specific branch and the other being the elimination of concerted feedback inhibition of the key enzyme AK by L-lysine and L-threonine, an increase of maximum two fold in cephamycin C production was not as much as anticipated.



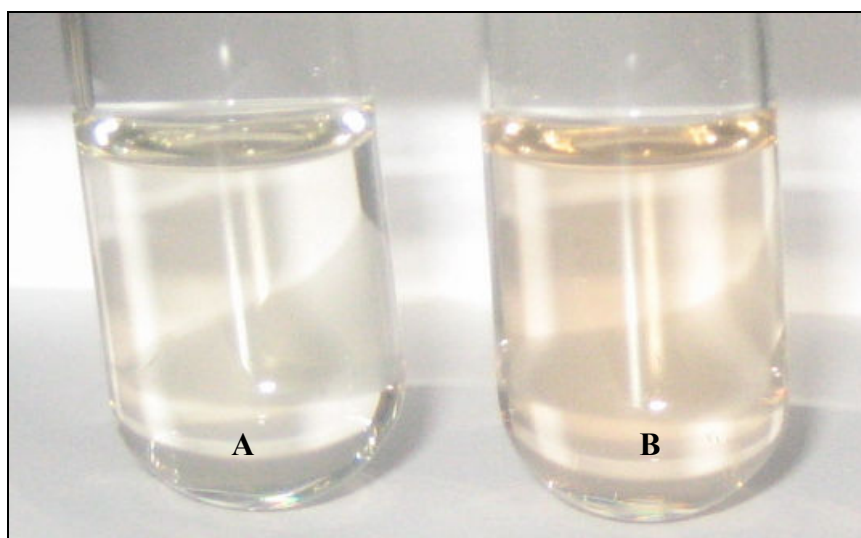


**Figure 3.21.** (A) Volumetric and (B) specific cephamycin C production in CDM. Open symbols represent DCW and closed symbols represent cephamycin C production. Square symbols show the mutant *S. clavuligerus* and triangles symbolize the wild strain.

Another limitation for our mutant could be the insufficiency of L-cysteine and L-valine, other precursor amino acids of cephamycin C, in CDM. As mentioned before, biosynthesis of the cephamycin C begins with non-ribosomal condensation of L- $\alpha$ -AAA, L-cysteine and L-valine to form the tripeptide  $\alpha$ -aminoadipyl-cysteinyl-valine (ACV). Thus, even when there occurs an increased production of L- $\alpha$ -AAA, it may not be converted to ACV efficiently. This possibility can be tested by externally adding these two amino acids to the medium when culturing the *hom*-disrupted mutant. Finally, we should also consider the linkage between the primary and secondary metabolism in that even when the precursor synthesis in primary metabolic pathways is increased, these precursors might be degraded to prevent entry of excess precursors into stationary phase and secondary metabolism. Our future work related with the present study will include the determination of the free amino acid pools in both the wild and *hom* mutant *S. clavuligerus* to understand the impact of *hom* gene disruption on pathway flux distributions.

Another interesting aspect of *hom* gene disruption was its pleiotropic effects on *S. clavuligerus*. The mutant AK39 obtained in this study was defective in sporulation on MM supplemented with L-methionine and L-threonine, but not on sporulation agar. This finding was in accord with that of Gehring *et al.* (2004) who reported that an L-methionine auxotroph of *S. coelicolor* had developmental defects which also increased secondary metabolite production. A functional *metH* (methionine synthase gene) was required for conversion of aerial hyphae into chains of spores in this mutant. In our study, the disruption of the *hom* gene also resulted in a morphological change. When observed under the light microscope, the mycelia of the mutant cells were more densely packed, but formed smaller clumps than the wild strain. This morphological difference is most likely related with its defect in sporulation which also includes morphological differentiation. It must be noted as well that DAP, the cell wall component in bacteria, is synthesized from the L-lysine branch of the aspartate pathway. When the *hom* gene is blocked, all the carbon flow will be directed to the lysine branch of the aspartate pathway which might increase the intracellular DAP concentration and its deposition into the cell wall. The possible

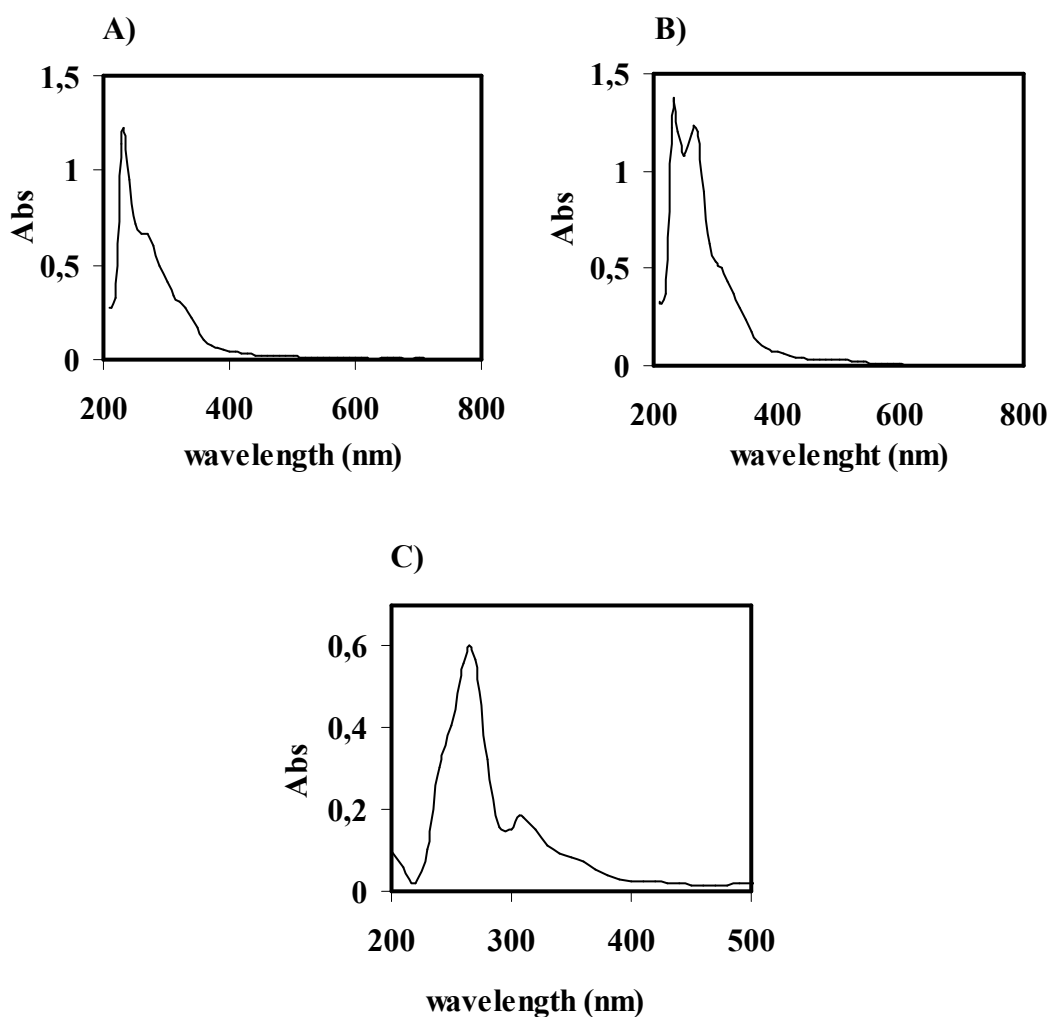
differences in developmental stages of wild type and *hom* mutant *S. clavuligerus* are worthy of clarifying and elucidation of pleiotropic effects of its auxotrophy will provide integrative insights into the regulation of morphological and biochemical differentiation.



**Figure 3.22.** Supernatants of 60 h cultures of the wild (A) and *hom* mutant (B) strains when grown in CDM.

When AK39 was grown in CDM, the color of the culture fluid turned into faint red after 48 h (Figure 3.22). When a sample from the culture was centrifuged, the color remained in the supernatant suggesting that a colored substance is secreted to the medium. Such a color difference was not detected in the wild strain of *S. clavuligerus*. The absorption spectra between 200-800 nm of 60 h culture supernatants of the wild and mutant strains grown in CDM revealed the existence of a major peak at 265 nm in the mutant which was absent from the wild strain (Figure 3.23). Red pigment accumulation by the blocked mutant was reminiscent of characteristic red pigment formation in certain adenine auxotrophs of *Schizosacharomyces pombe* due to the accumulation of two intermediates

(phosphoribosylaminoimidazole and phosphoribosyl aminoimidazole carboxylate) of purine biosynthetic pathway which was relieved by intracellular homocysteine accumulation due to a methionine synthase mutation (Fujita, 2006). Yet, the structure of our colored substance and its relation to *hom* mutation remains to be elucidated.



**Figure 3.23.** Absorption spectra of the 60 h culture supernatants grown in CDM. A) Absorption spectra of  $\frac{1}{4}$  diluted (A) wild and (B) mutant culture supernatant. (C) Differences in the absorption spectra of the culture supernatants of the wild and mutant strains when the absorption of the mutant culture supernatant was measured against that of the wild.

There has been only a limited number of studies focusing on improvement of cephamycin C yields by manipulating aspartate pathway in *S. clavuligerus*. Malmberg *et al.* (1993) was able to elevate cephamycin C yields 2 to 5 fold by inserting an additional copy of *lat* gene into the chromosome of *S. clavuligerus* NRRL 3585. In another study, AK-deregulated mutants (AEC-resistant strains) produced 2 to 7 times more cephamycin C than the wild type (Mendelovitz & Aharonowitz, 1983). When AK is deregulated, it can not be inhibited by the concerted action of L-lysine and L-threonine. However, these AK-deregulated mutants were obtained by random mutagenesis, therefore the reversion of the *ask* mutation can always take place. In our case, since *S. clavuligerus* AK39 was obtained by insertional inactivation of *hom* gene, the mutation is quite stable and cannot be reverted. Our *hom* mutant is indeed similar to AK-deregulated mutant in that AK enzyme cannot be inhibited by L-lysine and L-threonine since *hom*-disrupted cells are not able to produce L-threonine. Furthermore, the concentration of L-threonine externally added into CDM (50 mg per liter) is very much lower than that can inhibit AK (Mendelovitz and Aharonowitz, 1982; Tunca *et al.*, 2004). As L-threonine levels do not increase in our mutant *S. clavuligerus*, concerted feedback inhibition of AK is not possible even when L-lysine level is elevated.

Metabolic engineering has become a rational alternative to classical strain improvement in optimization of production of antibiotics, including  $\beta$ -lactams (Thykaer and Nielsen, 2003). The information obtained by metabolic engineering approaches will reveal the complex regulatory mechanisms involved in the biosynthesis of secondary metabolites. These approaches involve directed genetic modifications of the strains to increase the antibiotic production. In most of the studies, extra copies of the antibiotic biosynthetic genes are introduced into the cells or existing genes are modified to improve the strain properties. Modification of regulatory genes also enables to improve antibiotic production. Very recently, we accomplished the expression of *ask* gene in a multi-copy plasmid in *S. clavuligerus* with 2 to 3 fold increase in specific cephamycin C yields (unpublished) and the studies are under way to express multiple copies of *ask* gene in AK39. The present

study determined the beneficial effect of closing L-threonine branch of aspartate biosynthetic pathway and as stated earlier, further work will focus on determination of the impact of *hom* gene disruption on pathway flux distributions and optimization of the nutritional composition of CDM for higher cephamycin C yields from the disrupted mutant.

## CHAPTER 4

### CONCLUSION

- A homoserine dehydrogenase disruption cassette was prepared by inserting the kanamycin resistance gene into the homoserine dehydrogenase gene. Disruption cassette was introduced into *S. clavuligerus* cells on a *Streptomyces* plasmid, pIJ486 for its homologous recombination. A putative *hom*-disrupted strain displaying L-threonine and L-methionine auxotrophy was isolated and designated *S. clavuligerus* AK39.
- *hom* disruption in this putative mutant was verified via PCR, Southern blot hybridization analysis and homoserine dehydrogenase enzyme assays.
- Growth patterns and cephamycin C levels of wild type and mutant strains of *S. clavuligerus* were compared in both TSBS and CDM. *hom* gene disruption in AK39 resulted in a much poorer growth in CDM supplemented with L-methionine and L-threonine while its growth was even better than that of the wild strain in TSBS rich in amino acids. While there was no remarkable difference in specific cephamycin C production levels of mutant and wild strains in rich medium, the effect of *hom* disruption on antibiotic biosynthesis was observed in CDM where AK39 produced 1.7 to 2 fold higher specific yields of cephamycin C.
- *hom* gene disruption had some pleiotropic effects in *S. clavuligerus*. The mutant was defective in sporulation on MM supplemented with L-methionine

and L-threonine. The morphology of the *hom*-disrupted mutant was also different than that of the wild strain. Moreover, when the mutant was grown in CDM, it secreted a faint red pigment into the culture medium after 48 h growth. Existence of a major peak at 265 nm in the mutant culture supernatant which was totally absent from the wild culture supernatant was demonstrated. The differences in developmental stages of the wild and mutant *S. clavuligerus* remain to be clarified and elucidation of pleiotropic effects of its auxotrophy will provide integrative insights into the regulation of morphological and biochemical differentiation in this organism.

- The metabolic engineering approach which involved turning off L-threonine branch of aspartate biosynthetic pathway had beneficial effect with a two fold increase in cephamycin C production in *S. clavuligerus*. However, regarding the dual positive effect of *hom* disruption, i.e. increment in carbon flow towards L-lysine-specific branch and elimination of concerted feedback inhibition of the key enzyme AK by L-lysine and L-threonine, a massive increase in cephamycin C yields had been anticipated. Our future work related with the present study will include the determination of the free amino acid pools in both the wild and *hom* mutant strains and further determination of the impact of *hom* gene disruption on pathway flux distributions.



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## APPENDIX A

### COMPOSITION AND PREPARATION OF CULTURE MEDIA

#### 1. Media

##### 1.1. Liquid Media

###### Luria Broth (LB)

	<u><b>g.L<sup>-1</sup></b></u>
Luria Broth	25
Sterilized at 121 °C for 15 min	

###### Nutrient Broth (NB)

	<u><b>g.L<sup>-1</sup></b></u>
Nutrient Broth	8
Sterilized at 121 °C for 15 min	

###### Tryptone Soy Broth (TSB)

	<u><b>g.L<sup>-1</sup></b></u>
Tryptic Soy Broth	30
Sterilized at 121 °C for 15 min	

### **Tryptone Soy Broth With Sucrose (TSBS)**

	<b><u>g.L<sup>-1</sup></u></b>
Tryptic Soy Broth	30
Sucrose	100
Sterilized at 121 °C for 15 min	

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

	<b><u>g.L<sup>-1</sup></u></b>
Yeast Extract	3
Malt Extract	3
Bacto-peptone	5
Glucose	10
Sucrose	340
Sterilized at 121 °C for 15 min	

After sterilization following sterile components were added to the medium:

MgCl <sub>2</sub> .6H <sub>2</sub> O (2.5M)	2 mL/L
Glycine (20%)	25 mL/L

## **1.2. Solid Media**

### **LB Agar**

	<b><u>g.L<sup>-1</sup></u></b>
Luria Broth	25
Agar	15
Sterilized at 121 °C for 15 min	

**NB Agar**

	<b><u>g.L<sup>-1</sup></u></b>
Nutrient Broth	8
Agar	15
Sterilized at 121 °C for 15 min	

**Soft NB Agar**

	<b><u>g.L<sup>-1</sup></u></b>
Nutrient Broth	8
Agar	5
Sterilized at 121 °C for 15 min	

**TSA**

	<b><u>g.L<sup>-1</sup></u></b>
Tryptic Soy Broth	30
Agar	15
Sterilized at 121 °C for 15 min	

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

	<b><u>g.L<sup>-1</sup></u></b>
Sucrose	103
K <sub>2</sub> SO <sub>4</sub>	0.25
MgCl <sub>2</sub> .6H <sub>2</sub> O	10.12
Glucose	10
Yeast Extract	5
Casaminoacids	0.1
Distilled water added to	800 mL
Sterilized at 121 °C for 15 min	

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

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

KH <sub>2</sub> PO <sub>4</sub> (0.5 %)	2 mL
CaCl <sub>2</sub> .2H <sub>2</sub> O (3.68 %)	16 mL
L-Proline (20 %)	3 mL
TES Buffer (5.73 %, pH 7.2)	20 mL
NaOH (1 N)	1 mL
* Trace elements solution	0.4 mL

\*Trace elements solution

	<u>mg .L<sup>-1</sup></u>
ZnCl <sub>2</sub>	40
FeCl <sub>3</sub> .6H <sub>2</sub> O	200
CuCl <sub>2</sub> .2H <sub>2</sub> O	10
MnCl <sub>2</sub> .4H <sub>2</sub> O	10
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> .10H <sub>2</sub> O	10
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> .4H <sub>2</sub> O	10

### Sporulation Medium

	<u>g.L<sup>-1</sup></u>
Yeast extract	4
Malt extract	10

pH is adjusted to 7.3 with 10N KOH and agar is added.

	<u>g.L<sup>-1</sup></u>
Agar	20

Sterilized at 121 °C for 15 min

After sterilization following sterile components were added to the medium:

	<u>mL.L<sup>-1</sup></u>
Glucose (50%)	8
CoCl 6H <sub>2</sub> O(100mg/mL)	0.2

**Minimal Agar Medium,MM (Keiser *et al.*, 2000; modified )**

	<u>g.L<sup>-1</sup></u>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.14
K <sub>2</sub> HPO <sub>4</sub>	0.5
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.01

pH is adjusted to 7-7.2 with KOH, the volume is completed to 965 mL and agar is added.

	<u>g.L<sup>-1</sup></u>
Agar	20

Sterilized at 121 °C for 15 min

After sterilization following sterile components were added to the medium:

	<u>mL.L<sup>-1</sup></u>
Glycerol (50 %)	20
L-Methionine (4 mg/mL) (added if necessary)	12.5
L-Threonine (40 mg/mL) (added if necessary)	1.25

**CDM (Malmberg *et al.*, 1993; modified)**

	<u>g.L<sup>-1</sup></u>
K <sub>2</sub> HPO <sub>4</sub>	3.5 g
MOPS	20.9 g

pH is adjusted to 6.9 and volume is completed to 780 mL

Sterilized at 121 °C for 15 min

After sterilization following sterile components were added to the medium:

	<u><b>mL.L<sup>-1</sup></b></u>
L-Asparagine (10g/L)	200
MgSO <sub>4</sub> ·7 H <sub>2</sub> O (0.615 g/mL)	2
Glycerol (50 %)	20
* trace salt solution	1

\* trace salt solution

	<u><b>g.100mL<sup>-1</sup></b></u>
MnCl <sub>2</sub> ·4 H <sub>2</sub> O	0.1
FeSO <sub>4</sub> ·7 H <sub>2</sub> O	0.1
ZnSO <sub>4</sub> ·7 H <sub>2</sub> O	0.1
CaCl <sub>2</sub>	0.1

## APPENDIX B

### BUFFERS AND SOLUTIONS

#### P Buffer

	<u>g. L<sup>-1</sup></u>
Sucrose	103
K <sub>2</sub> SO <sub>4</sub>	0.25
MgCl <sub>2</sub> .6H <sub>2</sub> O	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 °C for 15 min. Before use following sterile components were added into 80 mL buffer in order:

KH <sub>2</sub> PO <sub>4</sub> (0.5%)	1 mL
CaCl <sub>2</sub> .2H <sub>2</sub> O (3.68)	10 mL
TES Buffer ( 5.73%, pH 7.2)	10 mL

\*Trace Elements Solution

mg.L<sup>-1</sup>



ZnCl <sub>2</sub>	40
FeCl <sub>3</sub> .6H <sub>2</sub> O	200
CuCl <sub>2</sub> .2H <sub>2</sub> O	10
MnCl <sub>2</sub> .4H <sub>2</sub> O	10
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> .10H <sub>2</sub> O	10
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> .4H <sub>2</sub> O	10

**PE Buffer**

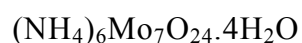
	<b><u>g.L<sup>-1</sup></u></b>
Sucrose	171
K <sub>2</sub> SO <sub>4</sub>	0.1

Completed to 784 mL with distilled water. Sterilized at 121 °C for 15 min. Before use following sterile components were added:

*Trace Elements Solution	1 mL
TES (5.72%, pH 7.2)	25 mL
190 mL CaCl <sub>2</sub> .2H <sub>2</sub> O	190 mL

\* Trace Elements Solution

	<b><u>mg.L<sup>-1</sup></u></b>
ZnCl <sub>2</sub>	40
FeCl <sub>3</sub> .6H <sub>2</sub> O	200
CuCl <sub>2</sub> .2H <sub>2</sub> O	10
MnCl <sub>2</sub> .4H <sub>2</sub> O	10
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> .10H <sub>2</sub> O	10



10

**TSE Buffer**

Sucrose	0.3 M
Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	25 mM
Supplemented with:	
Lysozyme	2 mg/mL
RNase	50 $\mu\text{g}/\text{mL}$

**Lysis Solution**

0.3 M NaOH

2% SDS

**Phenol-Chloroform Solution (water-saturated)**

Phenol	500 g
Chloroform	500 mL
Distilled water	400 mL

The solution was stored at RT, protected from light.

**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

**Gel Soak I (denaturation)**

NaOH	0.5 N
NaCl	1 M

**Gel Soak II (neutralization)**

Tris HCl (pH 7.2)	1 M
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NaCl	1M
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**20 X SSC (standart saline citrate)**

NaCl	3 M
Nacitrate	0.3 M

**Hybridization buffer (AlkPhos direct)**

Followings were dissolved in Hyb buffer provided by Amersham AlkPhos direct labelling and detection kit :

NaCl	0.25 M
blocking reagent	4% (w/v)

**Primary Wash Buffer**

Urea	60 g
SDS	0.5g
0.5M NaH <sub>2</sub> PO <sub>4</sub> (pH: 7.0)	50mL
NaCl	4.35 g
1 M MgCl <sub>2</sub>	0.5 mL
Blocking reagent	1 g

**Secondary Wash Buffer (20X)**

Tris HCl (pH: 10.00)	1M
NaCl	2M

**50 mM Phosphate Buffer**

	<b><u>g.L<sup>-1</sup></u></b>
KH <sub>2</sub> PO <sub>4</sub>	6,8
KOH	1,9

pH adjustment was done to with

\*50 mM KH<sub>2</sub>PO<sub>4</sub> solution (without added KOH) to lower the pH

\*0.1 M KOH, added dropwise, to raise the pH

#### **Sonication Lysis Buffer**

Tris HCl (pH: 8.00)	50mM
NaCl	100mM
EDTA	1mM
PMSF	1mM

#### **Assay Mixture**

Tris HCl (pH: 8.4), 500mM	200 $\mu$ L
NADP, 4mM	100 $\mu$ L
L-homoserine ( pH:7), 100mM	100 $\mu$ L
Crude extract	400 $\mu$ L

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

X-Gal	20 mg
Dimethylformamide	1 mL

The solution was stored at  $-20^{\circ}$  C protected from light.

#### **IPTG (Isopropyl- $\beta$ -D-thiogalactoside)**

IPTG	100 mg
Distilled water	1 mL

The solution was filter sterilized and stored at  $-20^{\circ}$  C.

## APPENDIX C

### CHEMICALS AND THEIR SUPPLIERS

#### Chemicals

Agar	Merck
Agarose	Prona
Ampicillin	Sigma
L-Asparagine	Sigma
Bacto-peptone	Difco
BSA (Bovine Serum Albumin)	Sigma
CaCl <sub>2</sub> .2H <sub>2</sub> O	Merck
Casaminoacids	Difco
Cephalosporin C (CPC)	Sigma
Chloroform	Merck
CoCl .6H <sub>2</sub> O	Sigma
Coomassie Brilliant Blue G-250	Merck
CuCl <sub>2</sub> .2H <sub>2</sub> O	Sigma
Dimethylformamide	Merck
EDTA	AppliChem
Ethanol	Botafarma
Ethidium bromide	Sigma
FeCl <sub>3</sub> .6H <sub>2</sub> O	Sigma
Glacial Acetic Acid	Merck
Glucose	Merck

Glycerol	Merck
Glycine	Merck
HCl	Fluka
L-homoserine	Sigma
IPTG	MBI Fermentas
Isopropanol	Merck
Kanamycin	Sigma
K <sub>2</sub> SO <sub>4</sub>	Merck
KCl	Merck
K <sub>2</sub> HPO <sub>4</sub>	Meck
KH <sub>2</sub> PO <sub>4</sub>	Merck
KOH	Merck
Luria Broth	Q-Biogene
Malt Extract	LabM
L- Methionine	Sigma
MOPS	Sigma
MgCl <sub>2</sub> .6H <sub>2</sub> O	Merck
MnCl <sub>2</sub> .4H <sub>2</sub> O	Merck
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> .10H <sub>2</sub> O	Sigma
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> .4H <sub>2</sub> O	Sigma
NADP	Sigma
NaCl	Merck
Nacitrate	Merck
NaH <sub>2</sub> PO <sub>4</sub>	Merck
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Merck
NaOH	Merck
Nutrient Broth	Merck
PEG-1000	Aplichem
Phenol	Merck
PMSF (Phenylmethylsulfonylfluoride)	Sigma
L-Proline	Sigma

SDS	Merck
Sucrose	Merck
TES Buffer	AppliChem
Thiostrepton	Sigma
L-Threonine	Sigma
Tris Base	Merck
Tris-HCl	Merck
Tryptic Soy Broth	Oxoid
X-Gal	MBI Fermentas
Yeast Extract	Difco
ZnCl <sub>2</sub>	Sigma

### **Enzymes**

Lysozyme	Q-Biogene
Rnase	MBI Fermentas
T4 DNA Ligase	MBI Fermentas
<i>Bam</i> HI	MBI Fermentas
<i>Sac</i> II	MBI Fermentas
<i>Eco</i> RV	MBI Fermentas
<i>Xho</i> I	MBI Fermentas
<i>Hind</i> III	New England
<i>Not</i> I	Fermentas
Yellow/Tango Buffer	MBI Fermentas
NE2 Buffer	New England
BSA	New England
<i>Taq</i> DNA polymerase	MIB Fermentas

### **Size Markers**

<i>Pst</i> I digested /Lambda DNA	MBI Fermentas
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## **Kits**

DNA Extraction Kit

Qiagen

Plasmid isolation Kit

Qiagen

p-GEM T Vector

Promega

AlkPhos direct labelling and detection Kit

Amersham