CLONING AND CHARACTERIZATION OF *STREPTOMYCES CLAVULIGERUS* MESO-DIAMINOPIMELATE DECARBOXYLASE (*LYS*A) GENE

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ABSTRACT

CLONING AND CHARACTERIZATION OF *STREPTOMYCES CLAVULIGERUS* MESO-DIAMINOPIMELATE DECARBOXYLASE (*LYS*A) GENE

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In *Streptomyces clavuligerus*, the route to the biosynthesis of α-aminoadipic acid (α-AAA) represents an important primary metabolic pathway providing carbon flux to the synthetases of antibiotic formation. This carbon flow comes through the lysine-specific branch of the aspartate pathway and is rate limiting in the formation of cephamycin C, a second generation cephalosporin produced by this organism. In this study, the *lys*A gene which encodes for an important key enzyme of aspartate pathway; meso-diaminopimelic acid (DAP) decarboxylase (E.C.4.1.1.20)

catalyzing the conversion of diaminopimelate to lysine was cloned and characterized for the first time from *S. clavuligerus* NRRL 3585. The attempts to clone the gene by constructing libraries of *S. clavuligerus* genomic DNA and screening of the libraries either by homologous probing or complementation approach gave no positive results. Then, PCR-based cloning was taken as the approach and the gene was amplified with PCR using the primers derived from the conserved sequences of *lys*A genes in two fragments (620 and 983 bp) which had overlapping regions. Fragments were then cloned and nucleotide sequencing revealed a complete open reading frame (ORF) encoding a protein of 463 aa (M_r 49, 907). The GC content of the gene was identified as 70.98 %. The gene sequence showed 83 % identity to the sequence of *S. coelicolor lys*A gene and 81 % identity to *S. avermitilis lys*A gene. By comparing the amino acid sequence of this protein to those available in database, the sites of the enzyme important for catalysis were identified.

Keywords: *Streptomyces clavuligerus*; Gene cloning; DAP decarboxylase; Cephamycin C, antibiotic biosynthesis.

ÖZ

STREPTOMYCES CLAVULIGERUS MEZO-DİAMİNOPİMELİK ASİT DEKARBOKSİLAZ (LYSA) GENİNİN KLONLANMASI VE KARAKTERİZASYONU

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Streptomyces clavuligerus'da α-aminoadipik asit (α-AAA) biyosentezi, antibiyotik üretiminde görev yapan sentetazlar için karbon akışını sağlayan önemli bir primer metabolik yoldur. Bu karbon akışı, aspartat metabolik yolunun Lizin-spesifik dalından gelmekte olup, organizmanın ürettiği ve ikincil kuşak sefalosporin olarak da bilinen sefamisin C biyosentezinde hız sınırlayıcıdır. Bu çalışmada, *Streptomyces clavuligerus* NRRL 3585'in aspartik asit amino asit yolunun anahtar enzimlerinden birisi olan, mezo-diaminopimelik asit (DAP) dekarboksilaz (E.C.4.1.1.20)'ı kodlayan *lys*A geni ilk kez klonlanmış ve karakterize edilmiştir. *lys*A genini *S. clavuligerus* genomik DNAsının kütüphanelerini oluşturup bu kütüphaneleri homolog problama veya komplementasyon yaklaşımı ile tarama

yöntemiyle klonlama çalışmaları olumlu sonuç vermemiştir.Bunun üzerine PCR'ı temel alan klonlama yöntemine başvurulmuş ve *lys*A geninin korunmuş bölgelerine karşılık gelen primer çiftleri kullanılarak genin birbiriyle çakışan bölgelerine sahip iki farklı parçası (620 ve 983 bp) PCR ile çoğaltılmıştır. Nükleotid dizi analizi sonuçları eksiksiz bir open reading frame'in (ORF) varlığını göstermiştir. Bu ORF, 463 amino asitlik (M_r 49, 907) bir proteini kodlamaktadır. *lys*A geninin GC içeriği % 70.98 olarak belirlenmiştir. Gen sekansı *S. coelicolor lys*A gen sekansı ile % 83, *S. avermitilis lys*A gen sekansı ile % 81 aynılık göstermiştir. Bu proteinin amino asit dizisi veri tabanında mevcut diğer DAP dekarboksilaz enzimlerinin amino asit dizileriyle karşılaştırılmak suretiyle enzimin katalitik aktivitesi için önemli olan bölgeleri saptanmıştır.

Anahtar kelimeler: *Streptomyces clavuligerus*; Gen klonlaması; DAP dekarboksilaz; Sefamisin C, antibiyotik biyosentezi.

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LIST OF SYMBOLS

α-AAA	: α-aminoadipic acid
DAP	: diaminopimelate
U	: unit
aa(s)	: amino acid(s)
bp	: base pairs
kb	: kilobase
orf(s)	: open reading frame(s)
MCS	: multiple cloning site

CHAPTER 1

INTRODUCTION

1.1. Antibiotic producing *Streptomycetes*

Streptomyces is a genus represented by a large number of species and varieties. This genus belongs to the order *Actinomycetes*. Phylogenetically, the actinomycetes form a subdivision of gram-positive bacteria, with most representatives having mole percent GC ratios in 60s and 70s (Brock and Madigan, 1988). Actinomycetes are rich sources of a variety of bioactive products and these organisms have been famous as producers of secondary metabolites including antibacterial and antifungal antibiotics, anticancer drugs, natural herbicides and immunusuppressive agents (Paradkar *et al*., 2001; Challis and Hopwood, 2003). As antibiotics are typical secondary metabolites, they do not seem to play a central role in growth and catabolism of the organism and they tend to be produced after cell population ceases growth (Glazer and Nikaido, 1998). Within the actinomycetes, the members of the genus *Streptomyces* account for 70-80% of secondary metabolites (Challis and Hopwood, 2003).

Antibiotic production in *Strepomyces* species is regulated by a variety of physiological and nutritional conditions and is co-ordinated with morphological development of the organism that includes formation of aerial mycelia and sporulation; a process which is triggered by nutrient depletion (Glazer and

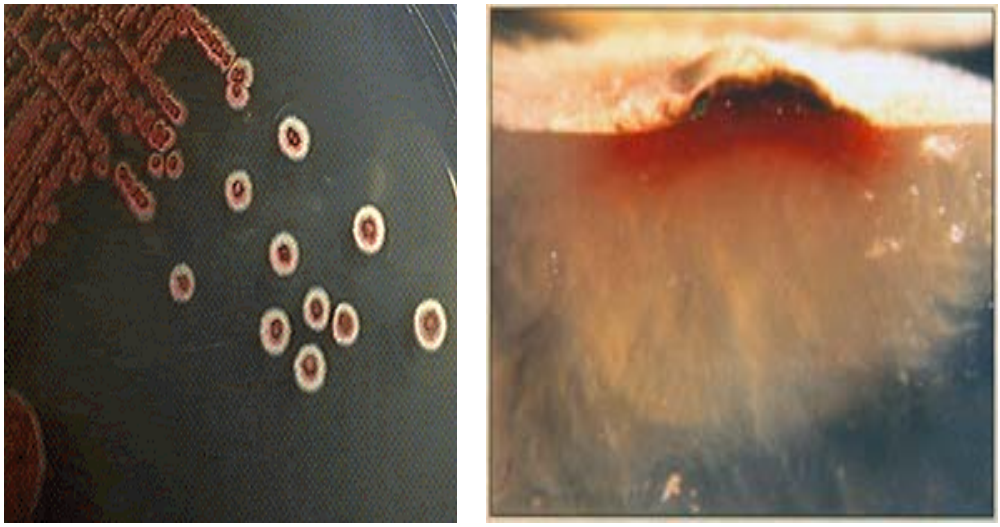
Nikaido, 1998; Paradkar *et al*., 1998). *Streptomyces* species differ conspicuously from most other bacteria in their growth and developmental biology. They are superficially more reminiscent of filamentous fungi, growing by tip extension to form a mycelium of branched hyphae. During the development of *Streptomyces* colonies, the specialized spore-bearing hyphae emerge through the air-water interface to give a fluffy layer of aerial mycelium. Once aerial hyphae have formed, their differentiation into spores called conidia involves the syncronous formation of several tens of sporulation septa (Flärdh, 2003). The conidia and sporophores are often pigmented and contribute a characteristic color to the mature colony (Brock and Madigan, 1988) (Figure 1.1). Concerning the biotechnological importance of streptomycetes, the mycelial growth habit strongly affects their behaviour and productivity in large-scale fermentations. A mechanistic and physiological understanding of tip growth, hyphal branching and mycelium fragmentation and a detailed knowledge about the genetic components involved in antibiotic production should improve their value as producers of antibiotics and other natural products, and should permit construction of strains that can overproduce these commercially important substances (Paradkar *et al*., 1998; Flärdh, 2003).

1.2. *Streptomyces clavuligerus* and β-lactam antibiotics it produces

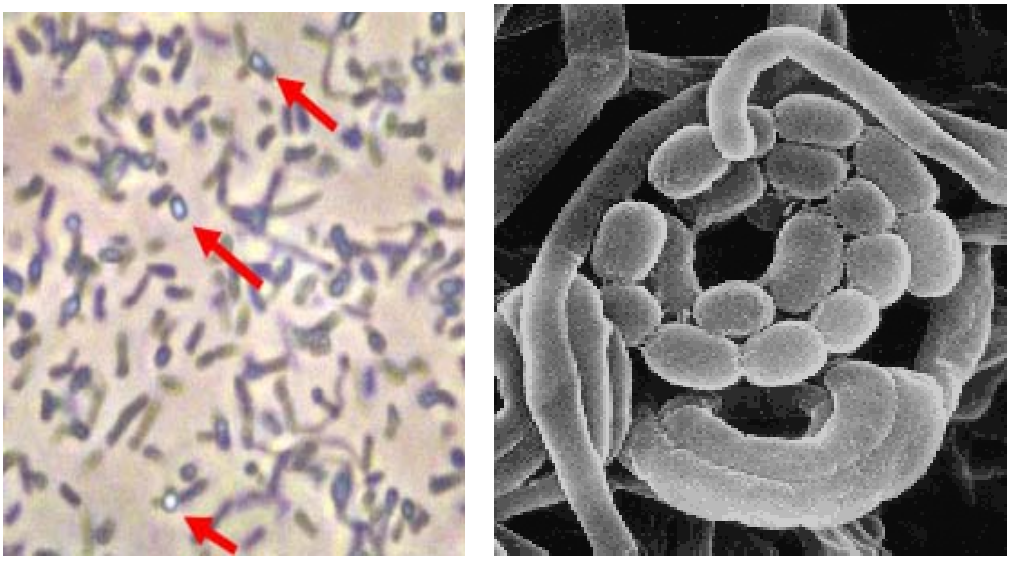
Streptomyces clavuligerus is an actinomycete well known for its ability to produce a variety of β-lactam antibiotics such as isopenicillin N, desacetoxycephalosporin C and cephamycin C (Figure 1.2). The same organism produces another group of structurally related β-lactam compounds, the clavams. The best known of these compounds, clavulanic acid possesses weak antibiotic activity, but is a very potent β-lactamase inhibitor (Paradkar *et al*., 2001).

1.2.1. Importance and market of β-lactam antibiotics

The discovery and development of β-lactam antibiotics are among the most powerful and successful achievements of modern science and technology (Demain



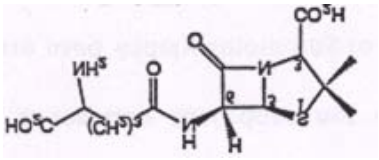
(a) (b)



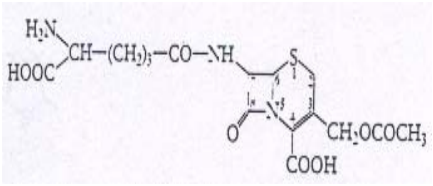
(c) (d)

Figure 1.1. Morphological differentiation of *Streptomyces.* (a) Streptomyces colonies on agar (b) Cross-section of a Streptomyces colony (c) Streptomyces spores (d) electron micrograph of sporulating aerial hyphae of *Streptomyces* (Brock and Madigan, 1988).

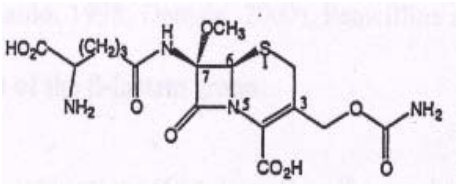
and Elander, 1999). β-lactams are probably the most important class of antibiotics for several reasons. For one thing, penicillin, the first antibiotic characterized, is a β-lactam (Glazer and Nikaido, 1998). β-lactams have been used extensively for treatment of various bacterial infections for more than half a century and today penicillins are commodity-type products with annual production volumes exceeding 60,000 tons, amounting to 60% of the 30 billion dollar world-wide market (Demain, 2000; Thykaer and Nielsen, 2003). Second, the inherent toxicity of β-lactams against higher animals is extremely low, yet they are often very effective at killing infecting bacteria. This is because they inhibit peptidoglycan synthesis, a reaction that does not exist in the eukaryotic world (Glazer and Nikaido, 1998).



Penicillin N



Cephalosporin C



Cephamycin C

Figure 1.2. β-lactam antibiotics produced by *S. clavuligerus*

1.2.2. Action mechanism of β-lactam antibiotics

The β-lactam antibiotics are potent inhibitors of cell wall synthesis. Peptidoglycan is synthesised from two amino sugars (N-acetyl muramic acid [MurNAc] and N-acetyl glucosamine [GlcNAc]) and ten amino acids. This structure gives peptidoglycan exceptional stability, mechanical strength and rigidity. The long single peptidoglycan chains are joined together with crosslinks by a transpeptidase

which is also capable of binding to penicillin or other antibiotics with the β-lactam ring, thus transpeptidase is also known as penicillin binding protein (PBP). In the first step of transpeptidation reaction, the D-Ala-D-Ala peptide bond of a donor peptide is cleaved by a PBP enzyme and a covalent bond between the carboxyl group of the penultimate D-Ala and the hydroxyl group of a serine in the active center of the enzyme is formed, giving rise to a mureinyl enzyme intermediate. In a second reaction step, the mureinyl moiety is transferred to a free epsilon amino group of a diaminopimelic acid residue in another peptide moiety (acceptor peptide) (Figure 1.3). In the reaction of a β-lactam antibiotic with the PBP enzyme, the β-lactam ring is cleaved and a penicilloyl enzyme intermediate is formed. In contrast to the mureinyl enzyme intermediate, the penicilloyl enzyme intermediate is rather inert, and thus the enzyme is blocked by a covalent bond to the antibiotic, a kind of suicide substrate for PBPs (Brock and Madigan, 1988; Höltje, 1998).

1.2.3. Biosynthesis of cephamycin C

In the first steps of cephamycin C biosynthesis, lysine is converted to α-aminoadipic acid (α-AAA) through a two step conversion. The deamination of lysine by lysine-6-aminotransferase (LAT) to form 1-piperidine-6-carboxylate marks the first step of cephamycin C biosynthesis in *S.clavuligerus*. LAT uses α-ketoglutarate as a cosubstrate and pyridoxyl phosphate as a cofactor (Kern et al., 1980). The second step is mediated by piperideine-6-carboxylate dehydrogenase (PCD) which converts 1-piperidine-6-carboxylate to α-aminoadipic acid (α-AAA) and requires NAD as a cofactor (de La Fuente *et al*., 1997). The α-AAA then undergoes a condensation reaction with two amino acids, cysteine and valine. The basic structure of the β-lactam nucleus is formed from three amino acids L-α-aminoadipic acid, L-cysteine and L-valine, which are activated with ATP and linked together to form the tripeptide δ-(L-α-aminoadipyl-L-cysteinyl-D-valine-ACV) in a reaction catalyzed by a multifunctional ACV synthetase. The ACV tripeptide is cyclized by the ACV cyclase (also named isopenicillin N synthase-IPNS) to give isopenicillin N, a β-lactam compound with weak antibiotic activity (Liras, 1999; Khetan *et al*. 1999). The next three steps of the pathway are identical

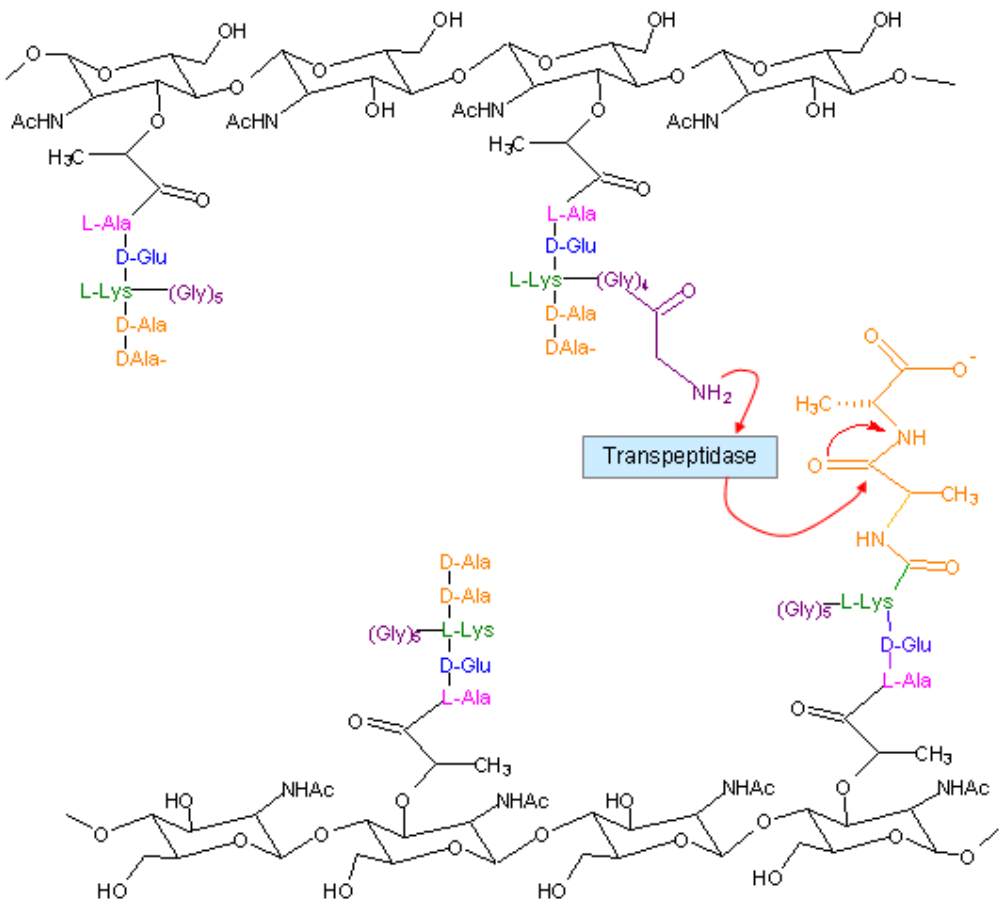


Figure 1.3. The transpeptidation reaction (Höltje, 1998)

to those existing in *Cephalosporium acremonium*, the cephalosporin C producer, but are not present in penicillin forming *Penicillium chrysogenum*. The L-α-aminoadipyl lateral chain of isopenicillin N is isomerized to the D configuration by the isopenicillin N isomerase to give penicillin N. The five-membered ring of the penicillin N is then expanded to a six-membered dihydrothiazinic ring by the penicillin N expandase (also named deacetoxycephalosporin C synthase). Finally, the deacetoxycephalosporin C is hydroxylated at C-3' by the deacetoxycephalosporin C hydroxylase, to form deacetylcephalosporin C, the immediate precursor of cephalosporin C (Liras, 1999). In cephamycin C producing actinomycetes, further reactions are involved in the synthesis of C-7 methoxyl group and in the attachment of the carbamoyl group at C-3'. Little information is

available about the so-called late genes, the product of which convert deacetoxycephalosporin C into cephamycin C. Deacetylcephalosporin C is converted into O-carbamoyldeacetylcephalosporin C by an O-carbamoyltransferase that transfers a carbamoyl group from carbamoylphosphate to the 3'-hydroxyl group of deacetylcephalosporin C (Figure 1.4). The methoxyl group at C-7 in the cephamycins derives from molecular oxygen and methionine by the apparent action of oxygenase and a methyltransferase (Coque *et al*., 1995).

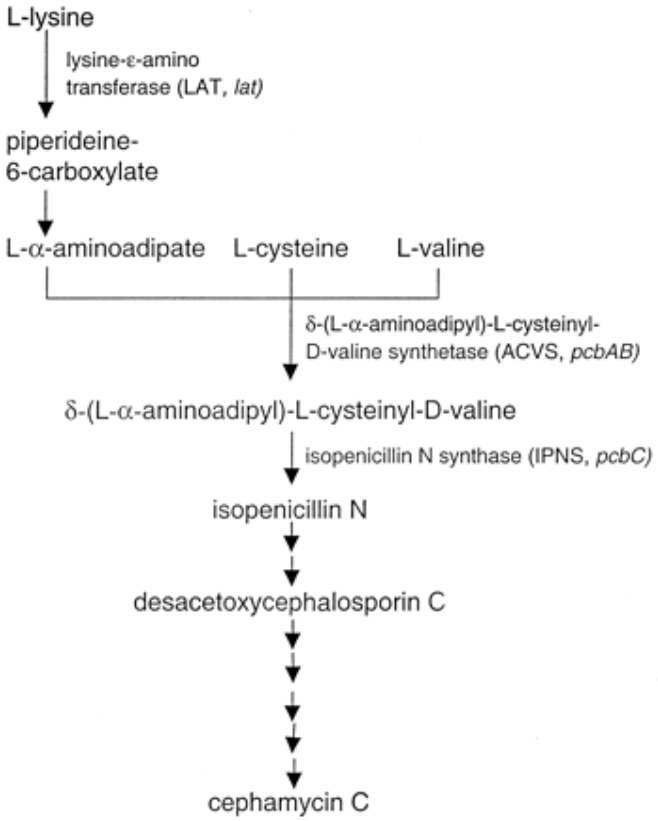


Figure 1.4. Cephamycin C biosynthesis

A search through the culture filtrates of *Streptomyces* species yielded cephamycins, with a methoxy group at the 7-α position of the nucleus. Methoxylation of cephalosporins was reported to increase their inhibitory effect on transpeptidases involved in bacterial cell wall synthesis and to reduce inactivation by β-lactamases, which makes the cephamycins clinically more important (Glazer

and Nikaido, 1998; Kim et. al., 2000). Hence, introduction of methoxy groups to certain cephalosporins in cell-free extracts of *S. clavuligerus* has increased the antibiotic activity in this organism (O'Sullivan *et al*., 1980).

1.3. Physiology of antibiotic production

It is difficult to produce antibiotics continuously during the prolonged cultivation of microbial cells. In most cases, continuous fermentation is not an option because these cultures do not produce antibiotics unless they are entering the stationary phase. Also avoiding contamination in continuous cultures is difficult as they are open systems. Antibiotic production have been performed in batch systems successfully (Glazer and Nikaido, 1998; Devi and Sridhar, 2000).

For carrying out fermentations, it is important to know the growth pattern of the producing organism. For this purpose, a study was made with *S. clavuligerus* by real-time biomass monitoring to determine biomass concentration. In complex medium cultivation, a lag phase of about 10 hours was detected. The exponential growth phase lasted till ca. 25 hours and was followed by a long transition period, till ca. 75 hours (Neves *et al*., 2000). Repeated batch operations with *S. clavuligerus* revealed that 96 hours is the optimum time for antibiotic production (Devi and Sridhar, 2000).

Many antibiotic producing organisms are less productive in the presence of excess carbon source, especially of compounds that are rapidly degraded such as glucose (Brock and Madigan, 1988). This is quite reminiscent of the catabolite repression well-characterized in *E. coli*. Ecological advantage of this event could be explained in such a way that antibiotic-producing soil organisms need to produce antibiotics and kill off their competitiors only in a nutritionally poor environment, and catabolite repression is useful for this arrangement. Aharonowitz and Demain (1978) first suggested that cephalosporin production in *S. clavuligerus* is under some type of carbon catabolite control. In order to overcome catabolite repression, carbon sources must be added to the medium in carefully adjusted, small

increments. Alternatively, mutants in which antibiotic production is no longer repressed by the presence of excess carbon source may be isolated (Glazer and Nikaido, 1998).

In many cases, the presence of excess nitrogen compounds in the fermentation medium decreases antibiotic production severely (Aharonowitz and Demain, 1979). Ecological advantage of this regulation is probably similar to that of catabolite repression. Excess phosphate has also been shown to repress some genes of antibiotic synthesis (Glazer and Nikaido, 1998).

1.4. Genetics of antibiotic production in *Streptomycetes*

Recent advances in actinomycete molecular biology and knowledge of antibiotic biosynthetic pathways started a new era in improvement of antibiotic production by these organisms. In conjunction with the publication of two complete *Streptomyces* genome sequences, *S. coelicolor* by Bentley *et al.* (2002) and *S. avermitilis* by Ikeda *et al*. (2003), the studies on these complex processes have certainly become more rewarding (Madduri *et al*., 1989; Liras, 1999; Flärdh, 2003).

It has been postulated that nucleoid migration occurs in *Streptomyces* such that the chromosomes move in relation to the cell envelope to populate the extending tips and lateral branches. In addition to poleward migration, newly replicated chromosomes segregate from each other (Flärdh, 2003). A recently discovered peculiarity of *Streptomyces* genome is that the chromosome may sometimes exist as linear DNA with telomeric sequences (Glazer and Nikaido, 1998). The linear *Streptomyces* chromosomes are covalently attached to terminal proteins (Tpg) at the 5' ends which are believed to prime the last Okazaki fragments, thereby providing a solution to the end replication problem of linear chromosomes (Volff and Altenbuchner, 2000).

A striking feature of 8.7 megabase *Streptomyces coelicolor* genome is its notional division into a core region of 4.8 megabase and left and right arms of 1.5 and 2.3 megabases, respectively. Classes of genes that encode unconditionally essential functions such as the machinery of DNA replication, transcription and translation are found in the core, whereas examples of conditionally adaptive functions occurred predominantly in the arms. The genome sequence revealed 23 clusters of genes which were predicted to encode biosynthetic enzymes for a wide range of secondary metabolites. An even larger number of secondary metabolic gene clusters was found in the recently sequenced *S. avermitilis* genome, again many of them located in the arm regions (Challis and Hopwood, 2003). In the genome of *Streptomyces* spp, very large-scale deletions occur at high frequency and these deletions frequently involve the antibiotic production genes. This frequent loss of antibiotic biosynthesis genes resembles the loss of a plasmid which causes the simultaneous loss of all the genes located on it (Glazer and Nikaido, 1998).

In general, the genes and enzymes for β-lactam biosynthesis are remarkably well conserved in prokaryotic and eukaryotic producers which is surprising if β-lactam antibiotics confer no advantage to the cells. Surprisingly, the percentages of amino acid identity among the enzymes for β-lactam antibiotic biosynthesis are higher than those for primary metabolism. This has been explained as the result of a stepwise horizontal transfer of fragments of the β-lactam clusters from prokaryotic to eukaryotic producers (Landan *et al*., 1990; Aharonowitz *et al*.,1992).

The genetic organization of the cephamycin C gene clusters is well known in actinomycetes (Liras, 1999). All of the structural genes necessary for the biosynthesis of cephamycin C in *S. clavuligerus* are organized into a cluster together with the regulatory and resistance genes. The genes encoding three of the early enzymes in the biosynthetic pathway; lysine-6-aminotransferase (LAT), ACV synthetase and isopenicillin N synthase (IPNS) are designated *lat*, *pcb*AB and *pcb*C, respectively. The uniform transcriptional orientation and short intergenic regions separating *lat*, *pcb*AB and *pcb*C in *S. clavuligerus* suggested that these genes might be organized in an operon for coordinate expression from a

polycistronic transcript (Alexander *et al*., 2000). The genes for LAT and piperideine-6-carboxylate dehydrogenase (PCD) are clustered with the genes for the rest of the cephamycin biosynthesis enzymes in *S. clavuligerus* (Madduri *et al*., 1991). The genes encoding for the enzymes of the intermediate steps; *cef*D gene encoding isopenicillin N isomerase, *cef*E gene encoding penicillin N expandase and cefF gene encoding deacetoxycephalosporin C hydroxylase are linked together in all cephamycin producers. Also the genes for the last steps of cephamycin C pathway (*cmc*I-*cmc*J encoding ocdac-7-methoxyl-transferase and *cmc*H encoding deacetylcephalosporin C O-carbamoyl transferase) are linked (Liras, 1999).

Antibiotic-producing species possess suicide-avoiding mechanisms. For example, low affinity penicillin-binding proteins (PBPs) have been described as a mechanism of resistance to β-lactam antibiotics in gram-positive bacteria. *Streptomyces* species contain 3 to 8 PBPs (Ogawara and Horikawa, 1980). The presence of genes encoding PBPs in the cluster of β-lactam biosynthetic genes was first reported in cephamycin C producing *N. lactamdurans* (Coque *et al*., 1993). It has been determined that resistance to β-lactam antibiotics is a result of the produciton of penicillin-binding proteins having low affinity for β-lactams in *S. clavuligerus* (Paradkar *et al*., 1998).

The traditional genetic approach to improve the yield from antibiotic-producing organism depends entirely on random mutagenesis and screening of overproducers. However, recent advances in actinomycete molecular biology and knowledge of antibiotic biosynthesis pathways and the gene clusters that encode them offer genetic engineering and cloning as alternative approach to the improvement of strains in a targeted manner. Engineered strains of *Streptomyces* species have been constructed in which gene dosage has been increased by introduction of additional copies of genes on high-copy number plasmids. Gene cloning offers some practical benefits such as the direct manipulation of biosynthetic pathways and construction of novel compounds besides overproducing of antibiotic-synthesizing enzymes and increasing the yield of

fermentation via gene dosage effect (Glazer and Nikaido, 1998; Paradkar *et al*., 2001).

Many enzymes of cephamycin C payhway have been cloned and characterized to date. The enzyme reducing P6C to α-AAA P6C-dehydrogenase has been purified from *S. clavuligerus* to homogeneity (de la Fuente *et al*.,1997). The *pcb*AB gene of *N. lactamdurans*, encoding the ACV synthetase has been cloned and sequenced (Coque *et al*.,1991). The same gene has been partially sequenced by several investigators in *S. clavuligerus* (Doran *et al*., 1990; Tobin *et al*., 1991; Yu et al., 1994). The ACV cyclase encoded by *pcb*C gene have been purified from *S. clavuligerus* and *N. lactamdurans* (Jensen *et al*., 1986; Castro *et al*., 1988). Nine *pcb*C genes have been studied from actinomycetes, fungi and gram negative bacteria (Aharonowitz *et al*., 1992). Genes encoding isopenicillin N epimerases (*cef*D) have been cloned from actinomycetes and gram negative bacteria and expressed in *S. lividans* and *E. coli* (Kovacevic *et al*.,1990; Coque *et al*.,1993; Kimura *et al*., 1996).

Combinations of β-lactam antibiotics and β-lactamase inhibitors are well known to be effective against β-lactam resistant bacteria when compared to the use of β-lactam antibiotics alone. This is because of the synergistic action of these metabolites. The fact that no known actinomycetes produce clavulanic acid alone, but there are actinomycetes that produce just cephamycin C suggests that the production of clavulanic acid evolved in a cephamycin C producer as a response to the acquisition of β-lactamase-mediated resistance in bacteria inhabiting the same environmental niche and thus posing biological competition (Challis and Hopwood, 2003). Thus, the clavulanic acid gene cluster is directly adjacent to the cephamycin cluster in the chromosomes of *S. clavuligerus*, *S. jumonijensis* and *S. katsurahamanus* This superclustering would be expected for gene clusters that direct the production of metabolites acting synergistically to benefit the producing organism (Ward and Hodgson, 1993).

Although there are no biosynthetic enzymes shared by the cephamycin C and clavulanic acid pathways, the two biosynthetic pathways are coregulated by a single transcription activation protein, CcaR. *cca*R gene codes an OmpR-like transcriptional regulator and is located within the cephamycin cluster. Disruption of *cca*R by targeted double recombination resulted in the loss of the ability to synthesize cephamycin C and clavulanic acid. Amplification of *cca*R in *S. clavuligerus* resulted in a two to three fold increase in the production of cephamycin and clavulanic acid (Perez-Llerna *et al*., 1997).

Another regulatory gene, *cla*R has been described in *S. clavuligerus* (Paradkar *et al*., 1998; Perez-Redondo et al., 1998). It is located in the clavulanic acid cluster and has a significant degree of homology to many transcriptional activators of the *lys*R family. Disruption of *cla*R yields a mutant producing higher levels of cephamycin C, but unable to produce clavulanic acid. This may be a mechanism to balance formation of the β-lactam cephamycin and the β-lactamase inhibitor clavulanic acid according to the cell's needs (Perez-Redondo *et al*., 1998).

The *cmc*T gene encoding a transmembrane protein is also located within the cephamycin biosynthetic gene cluster, with its primary function being cephamycin transport from the cell (Alexander and Jensen, 1998). *cmc*T genes have analogies to ABC transporter genes which are known to confer resistance to the antibiotic produced (Fernandez *et al*., 1996).

1.5. The aspartate pathway

Secondary metabolites have to be synthesized from primary metabolites, thus the efficient production of antibiotics requires a steady flow of their precursors (Glazer and Nikaido, 1998). As mentioned earlier, the basic structure of β-lactam nucleus is formed form three amino acids L-α-aminoadipic acid (α-AAA), L-cysteine and L-valine. The metabolic origin of the AAA moiety of β-lactam antibiotic produced by fungi differs from that of streptomycetes: In fungi, AAA is an intermediate of the lysine biosynthetic pathway. Because lysine is the end

product of a biosynthetic pathway, a high level of lysine in the medium shuts off biosynthesis by inhibiting the first enzyme of the pathway (feedback inhibition). Thus, the presence of excess lysine strongly inhibits penicillin production in *P. chrysogenum* fermentations. In streptomycetes, AAA originates as a catabolic product of lysine produced from the diaminopimelic acid (DAP)-lysine branch of the aspartate pathway. Therefore, the involvement of lysine metabolism in controlling β-lactam antibiotic synthesis in fungi and streptomycetes becomes of special interest from both a comparative biochemical as well as the practical point of view (Mendelovitz and Aharonowitz, 1983; Glazer and Nikaido, 1998).

In most bacteria, amino acids of the aspartate family, such as lysine, methionine and threonine are produced from aspartate by the aspartate pathway by a series of enzymatic steps (Figure 1.5). One of the intermediates of this pathway, diaminopimelic acid (DAP) has an important metabolic function on its own right as a constituent of the bacterial cell wall peptidoglycan (Zhang *et al*., 1999).

In streptomycetes, where aspartate is a precursor not only of the related protein amino acids but sometimes also of secondary metabolites, there is evidence that regulatory mechanisms acting on core reactions in the aspartate pathway control the flow of metabolic intermediates to such products as the β-lactam antibiotics which are derived from lysine via L-α-aminoadipic acid (Le *et al*.,1996).

In *S. clavuligerus*, aspartate pathway provides the α-aminoadipate side chain of cephamycin C and provides the methyl at the C-7 position of the antibiotic, involves cysteine as an intermediate and shares common enzymes with the valine biosynthetic pathway; both cysteine and valine are moieties are part of the cephamycin molecule. When such a link exists between primary metabolism and antibiotic metabolism, it is not surprising that mechanisms controlling the supply of the required substrates for antibiotic biosynthesis affect the ability of the cell to produce antibiotics (Aharonowitz *et al*., 1984).

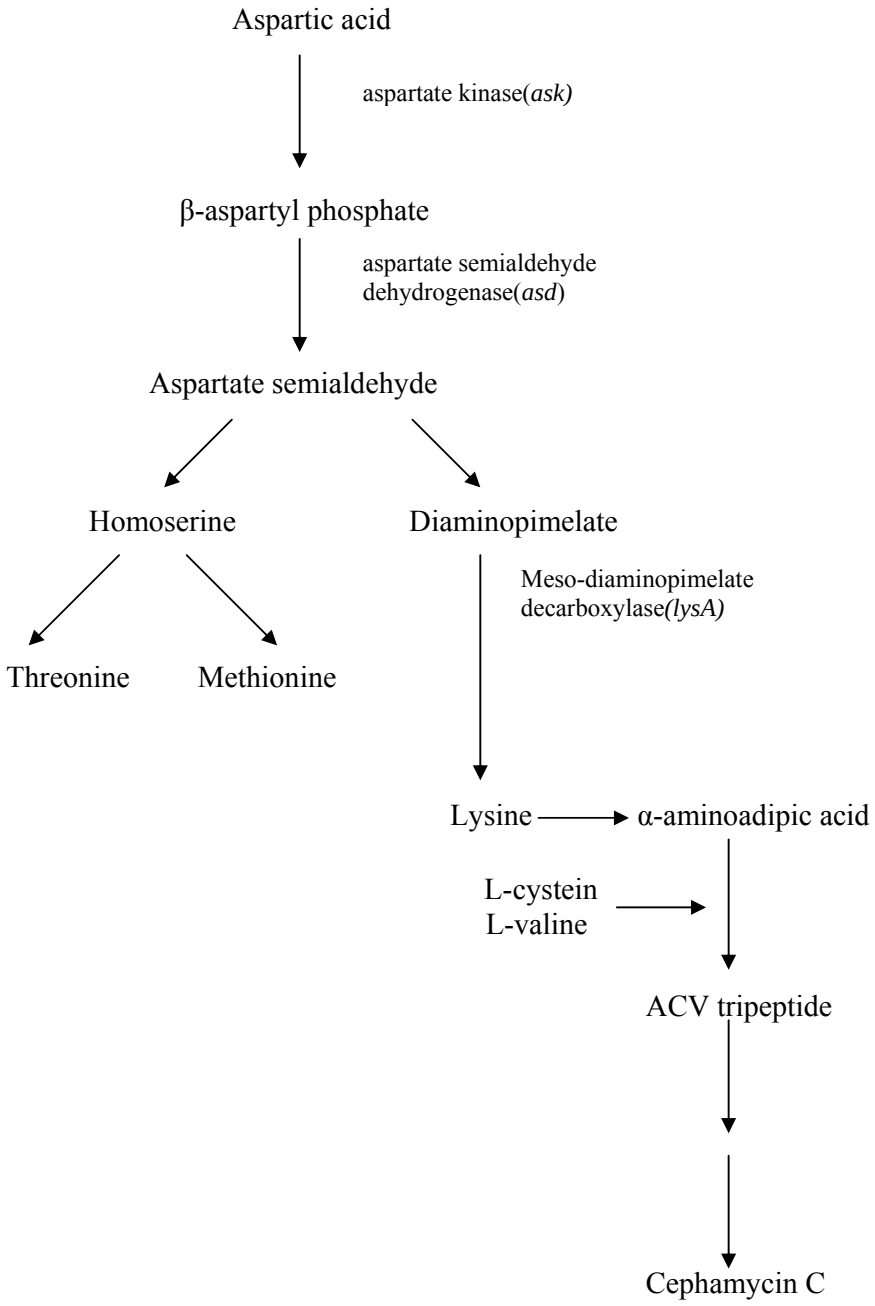


Figure 1.5. The aspartate pathway

The enzymes catalyzing the first two steps of this core pathway, aspartokinase (Ask, E.C.2.7.2.4) and aspartate semialdehyde dehydrogenase (Asd, E.C.1.2.1.11) are well conserved functionally. Different isoforms of Ask have been identified in various bacteria, but only one form of Asd has been found in all microorganisms examined so far. *S. clavuligerus ask* and *asd* genes have been cloned, characterized and heterologously expressed in our laboratory (Tunca *et al*., 2004).

1.6. *meso*-diaminopimelate decarboxylase (*lys*A) gene

The biosynthetic pathway from aspartate and regulatory mechanism of lysine production have been well studied in different bacteria. Among the genes of the aspartate pathway, *lys*A gene appears particularly interesting as it encodes for diaminopimelate (DAP) decarboxylase (E.C.4.1.1.20), the last enzyme of the pathway which catalyzes the decarboxylation of *meso*- DAP to lysine. DAP decarboxylase is a pyridoxal-5'-phosphate (PLP)-dependent enzyme. Like most enzyme-catalyzed decarboxylation reactions, the conversion of DAP to lysine is not reversible (Figure 1.6). The enzyme is of interest because of its importance in bacterial growth and survival as lysine is required in protein biosynthesis and essential for bacterial viability and development. As mentioned earlier, the lysine precursor DAP itself is used as structural cross-linking component of the peptidoglycan layer of gram-negative, gram-positive and mycobacterial cell walls (Gokulan *et al*., 2003).

*E. coli lys*A gene has been cloned and complete sequence of the gene and its regulatory region was determined (Straiger *et al*. 1983a). It has been determined that the *lys*A gene requires for its expression the presence of an activator protein which is the product of *lys*R gene. *Lys*R gene was found to be located immediately upstream from *lys*A gene (Straiger *et al*.,1983b). This regulation pattern of *E.coli lys*A gene was also demonstrated by *lys*A-*lac*Z fusions. The absolute requirement

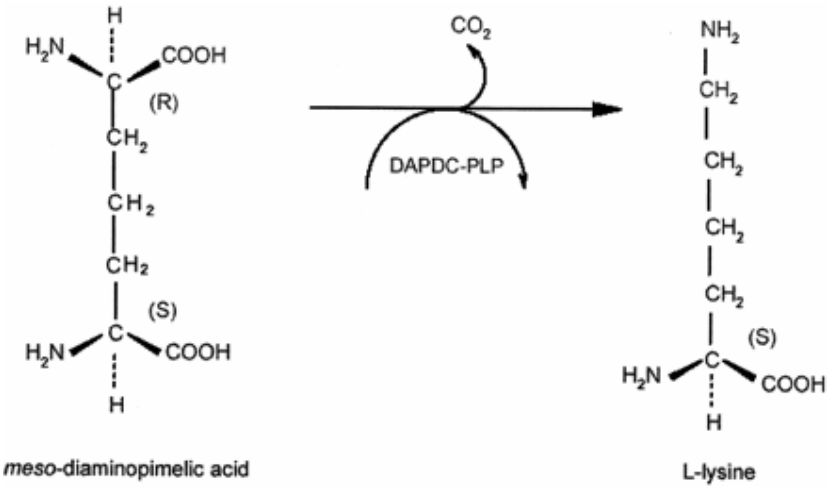


Figure 1.6. Decarboxylation of meso-DAP to lysine.

of an activation process for expression through *lys*A promoters was clearly shown (Straiger *et al*.,1983c). The *lys*R gene has also been determined by homology in *Streptomyces coelicolor* (U37580, Gen-Bank).

*lys*A gene from *Bacillus subtilis* was cloned and complementation was efficient in lysine-deficient *B. subtilis* host. The deduced amino acid sequence shared 29.7% identity with the sequence of DAP-decarboxylase from *E. coli* (Yamamoto *et al*., 1991).

*lys*A gene has been cloned also from *Corynebacterium glutamicum*. Sequence and enzymatic analyses in *C. glutamicum* indicated that the full expression of a cloned *lys*A gene apparently requires a large genomic fragment located immediately upstream from a functional promoter site (Yeh *et al*., 1988). For *C. glutamicum*, there is functional and molecular evidence that the *lys*A gene is expressed in polycistronic messenger with an upstream sequence, namely *orf*X gene (Marcel *et al*.,1990).

Cloning of *lys*A gene has also been reported from *Pseudomonas aeruginosa* (Martin *et al*., 1986). Comparison of the deduced amino acid sequence of the *lys*A gene product revealed extensive similarity with the sequences of the functionally equivalent enzymes from *E. coli* and *C. glutamicum* (Martin et al., 1988).

In *Brevibacterium lactofermentum*, *arg*S gene encoding an arginyl-tRNA synthetase was identified in the upstream region of the *lys*A gene. One single transcript of about 3,000 nucleotides corresponding to *arg*S-*lys*A operon was identified (Oguiza *et al*., 1993). An operon organization similar to that in Corynebacteria was described.

The nucleotide sequence of *lys*A gene from *Mycobacterium tuberculosis* was reported by Andersen and Hensen (1993). The deduced amino acid sequence was found to exhibit significant homology to DAP decarboxylase sequences from other bacterial species (Andersen and Hansen, 1993). It is interesting that the organization for *arg*S-*lys*A in *Brevibacterium* was the same in *M. tuberculosis* (Kröger *et al*., 1992). These similarities suggests a close phylogenetic relationship among the genera *Corynebacterium*, *Brevibacterium* and *Mycobacterium*, which all belong to high G+C subdivision of gram-positive bacteria (Oguiza *et al*., 1993).

*lys*R gene has been cloned and characterized as in an extremely thermophilic eubacterium, *Thermus thermophilus*. This gene was suggested to encode a regulatory protein of the *lys*A gene in this organism (Kosuge and Hoshino, 1997).

1.7.The present study

To date, no *lys*A genes have been cloned from the members of the genus *Streptomyces*. *lys*A gene sequences of *S. coelicolor* and *S. avermitilis* were identified through the genome projects of these species. The present study constitutes the first report on cloning in *E.coli* of the *lys*A gene from *S. clavuligerus*. The gene was characterized by documenting its complete nucleotide sequence. The *lysA* clone constructed in this work forms a basis for further studies in our laboratory which will involve its homologous expression in *S. clavuligerus* on a multicopy plasmid and determination of the effects of its overexpression on cephamycin C biosynthesis, regarding the significance of its role in supplying substrates to the biosynthetic enzymes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains and plasmid vectors

The characteristics and sources of bacterial strains and plasmid vectors used in this study are listed in Table 2.1. *S. clavuligerus* spore suspension was prepared by using the protocol described by Hopwood *et al*.(1985) and stored in 20% glycerol at – 80 ˚ C. *E .coli* DH5α strain was streaked onto LB agar (Appendix A), grown at 37 ˚ C, stored at + 4 ˚ C and subcultured monthly. Stock solutions of this strain was also prepared in LB containing 15% glycerol and stored at – 80 ˚ C. The plasmid vectors used was Promega pGEM-T and Stratagene pBluescript II KS (+) (Figure 2.1). '*lys*A' complementation was assessed on M9 minimal medium containing 100 µg/ mL ampicillin in the absence of lysine.

2.2. Culture media

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

2.3. Buffers and solutions

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

2.4. Chemicals and enzymes

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

Table 2.1. Bacterial strains and plasmid vectors used in the study

	Genotype/Phenotype	Source
Strains		
S. clavuligerus NRRL 3585	wildtype	Prof. J. Piret, Northeastern Univ., USA
E. coli DH5α	F'ϕd*lacZ*Δ(*lacZ*YA-argF), U169, *sup*E44λ$^-$, *thi*-1, *gyr*A, *rec*A1, *rel*A1 *end*A1, *hsd*R17	*E. coli* Genetic Stock Center
E. coli CGSC 4345	*Lac*Z118(Oc), *lac*I22, *lys*A23	*E. coli* Genetic Stock Center
Plasmids		
pBluescript II KS (+)	Amp^r, *lacZ*'	Stratagene
PGEM-T	Amp^r, *lacZ*'	Promega

2.5. Isolation of total chromosomal DNA from *S. clavuligerus*

Total chromosomal DNA was isolated as described by Hintermann (1981). One square cm of cell mass from *S. clavuligerus* culture on R2YE agar plate was transferred into a 250 mL baffled flask containing 50 mL Trypticase Soy Broth (Appendix A) supplemented with 0.5 % maltose (autoclaved seperately). The culture was grown at 28 ˚ C for 48 hours with constant agitation (250 rpm) in an orbital shaker. Mycelium was harvested by centrifugation at 10 000 rpm for 10 minutes at 4 ˚ C. Pellet was resuspended in 5 mL TSE buffer (Appendix B) containing 2 mg/mL lysozyme and incubated at 37 ˚ C for 1 hour by mixing

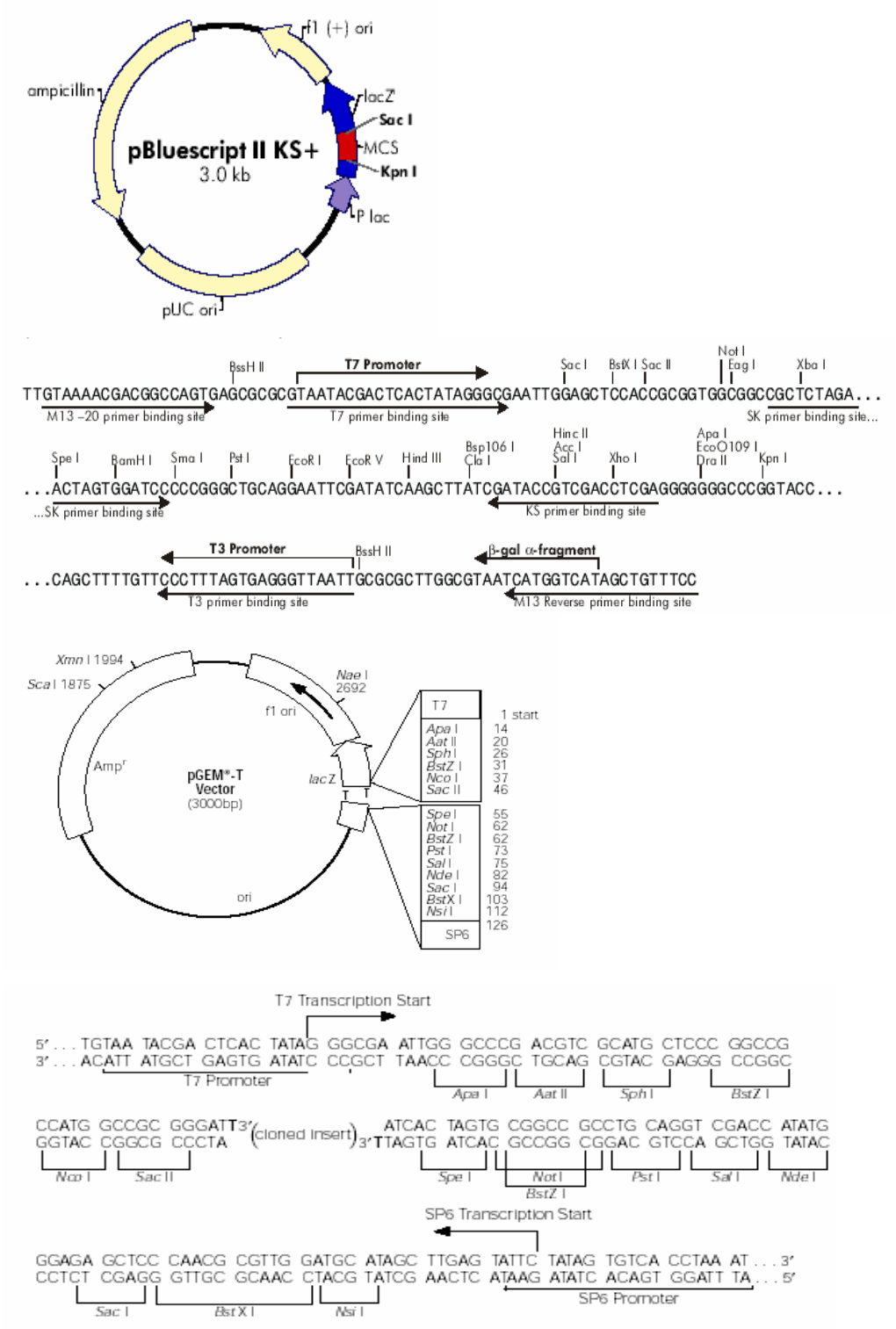


Figure 2.1. Maps and MCS sequences of pBlusecript II KS (+) and pGEM-T vectors.

occasionally by hand until granular mycelial pellets dissappear. 100 µL of 5 mg/mL solution of proteinase K (Appendix B) and 0.5 mL 10% SDS solution were added and the tube contents were mixed immediately by vortexing. The mixture was then incubated at 65 ˚ C for 25 minutes and cooled to room temperature. After cooling, 5 ml of phenol-chloroform solution saturated with TNE (Appendix B) was added and the mixture was shaked by hand for 5 minutes. Phases were seperated by spinning at 10 000 rpm for 10 minutes. The organic phase was removed and the aqueous phase was extracted once more with 5 mL phenol-chloroform solution, centrifuged and extracted once with 5 mL chloroform. After phases are separated, the aqueous phase was spun for 10 minutes at 10 000 rpm to pellet the remaining interphase. The supernatant was transferred into a new tube, 2 mg/mL RNase solution (Appendix B) was added to a final concentration of 20 µg/mL and incubated at 37 ˚ C for 1 hour. Next, the final concentrations of 1 M NaCl and 10% PEG 6200-8000 were added, the tube was mixed well by inversion and incubated on ice for 30 minutes. The tube was spun 5 minutes at 3000 rpm, the supernatant was decanted and the pellet was dissolved in 5 mL TE buffer (Appendix B). Then, 3 volumes of cold ethanol and 1/10 volumes of 3M unbuffered sodium acetate (pH: 4.8) were added and the mixture was kept at – 20 ˚ C overnight to precipitate DNA. The mixture was centrifuged at 10,000 rpm for 10 minutes and pellet was dissolved in 2 mL of TE buffer.

2.6. Preparation of *E.coli* competent cells

E.coli competent cells were prepared according to the protocol described by Sambrook *et al.* (1989) with slight modifications. 5- 50 mL of LB broth (Appendix A) was inoculated with *E. coli* from a fresh LB agar plate in a 250 ml flask and incubated overnight with shaking at 37 ˚ C to obtain a stationary phase culture. 300 µL from this seed culture was inoculated into a fresh flask containing 50 mL LB broth. The culture was incubated for 2- 2.5 hours at 37 ˚ C with vigorous shaking around 300 cycles/minute in an orbital shaker to obtain an exponentially-growing culture. Then the culture was asepticaly split into two sterile pre-chilled 40 ml screwcap centrifuge tubes and stored on ice for 10 minutes. Next, cells were

spun at 4000 rpm for 10 minutes at 4 ˚C in a Sorvall SS-34 rotor. After centrifugation, supernatants were decanted and each pellet was resuspended in 5 mL ice-cold 10 mM $CaCl_2$ by vortexing. The cells were spun down at 3000 rpm for 10 minutes at 4 ˚C.Lastly, fluid from cell pellets was decanted and each pellet was resuspended very gently in 1 mL ice-cold 75 mM $CaCl_2$. The competent cells were stored at – 80 ˚C.

2.7. Transformation of *E.coli* competent cells

Competent *E.coli* cells were transformed as described by Sambrook *et al*. (1989) with slight modifications. 100 µL of *E. coli* competent cells were transferred to a prechilled Eppendorf tube. Appropriate amount of DNA (1- 50 ng) in a maximum volume of 10 µL was added and mixed gently by pipetting. The tubes were left on ice for 30 minutes. Then, the tubes were placed in a 42 ˚ C water bath and heat-pulsed for 90 seconds. Next, the tubes were immediately put on ice ofr 2 minutes. 0.9 mL of LB broth was added to each tube and the cultures were incubated for 1.5 hours at 37 ˚ C with gentle shaking (180-200 rpm). Finally, 100 µL of transformed competent cells were spread onto selective LB agar plate and incubated overnight at 37 ˚ C.

2.8. Plasmid isolation from *E. coli*

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

E.coli plasmid DNA was also prepared by using the plasmid minipreps method described by Hopwood *et al*. (1985). Each strain was grown as patches on selective medium, LB agar containing 100 µg/mL ampicillin. About 1 square cm of cell mass was scraped with a sterile toothpick and put into Eppendorf tube containing 100 µL cold STE solution containing 2 mg/mL lysozyme (Appendix B). Each tube was mixed by vortexing to disperse the cells and the toothpick was

discarded. Then, the tubes were incubated on ice for 20 minutes. 3/5 volume of lysis solution (Appendix B) was added to each tube and vortexed immediately. The mixture was incubated at room temperature for 10 minutes to lyse the cells and then at 70 ˚ C for 10 minutes to denature DNA. Then, tubes were cooled rapidly in cold water. An equal amount of phenol-chloroform solution was added (water-saturated, Appendix B), vortexed hard until homogeneous and milky white mixture was obtained. Finally, the samples were spun for 5 minutes at 13 000 rpm to separate phases. 10 µL of supernatant was loaded directly on an agarose gel for electrophoresis.

2.9. Manipulation of DNA

2.9.1. Restriction endonuclease digestions

Restriction enzyme digestions were performed under the conditions specified by the manufacturers. For complete digestion, plasmid DNA was incubated for 3 hours and chromosomal DNA was incubated overnight at 37 ˚C. In general, 1 Unit enzyme was used for 1 µg DNA. For partial digestion of S. clavuligerus chromosomal DNA, the procedure of Sambrook *et al*. (1989) was used with small modifications: 594 µL of chromosomal DNA (261 µg) and 66 µL of appropriate enzyme buffer were mixed well by inverting the tube. The mixture was dispensed to introduce 120 µL into an Eppendorf tube (tube 1) and 60 µL into tubes 2 to 8 (tubes chilled on ice). 105.6 U of restriction enzyme (*Sau*3A) were added to tube 1 and mixed. The concentration of enzyme was 2 Units per µg DNA. 15 µL of the reaction mixture was transferred to tube 2 so that enzyme concentration became 1 U/µg DNA. After mixing in every step, the two fold serial dilution continued through to tube 8. Tubes 1-8 were incubated at 37 ˚C for 1 hour. The reaction was stopped by incubating the tubes at 70 ˚C for 10 minutes.

2.9.2. Agarose gel electrophoresis

For DNA analysis, agarose gels were prepared at concentrations of 0.8-1 %. Ethidium bromide at a final concentration of 0.5 μg/mL was added to the gels. Gels were run from 2 hours (at 6-9 Volts/cm) to overnight (1 Volt/cm). The electrophoresis buffer used was 1xTAE. The DNA bands were visualized on a UV transilluminator (UVP) and photographed by using Vilber Lourmat Gel Imaging System. Pst I digested λ DNA marker (AppendixA) or 100 bp DNA ladder (AppendixA) was used to determine the molecular weights of DNA bands.

2.9.3. Isolation of DNA fragments from gels

After separation of DNA fragments usually in a 0.9 % agarose gel prepared in TAE buffer, the desired fragments were extracted from the gel by using a Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA). The gel slice containing the DNA band was excised from the gel and weighed. DNA in such gels was recovered according to the Qiagen's instructions. After recovery, an aliquot was run on agarose gel to assess the yield.

2.10. Primer design

The primers were designed in this study according to the conserved nucleotide sequences of *lys*A genes from two different actinomycetes, *S. coelicolor and Corynebacterium efficiens* (Figure 2.2). The conserved regions are boxed and indicated with the arrows. Primers indicated by the arrows were designed according to *S. coelicolor lys*A gene sequence. The nucleotide sequences of the primers and expected product sizes are given in Table 2.2. The designed primers were synthesized by the Iontek Company (İstanbul, Turkey).

Table 2.2. Primers used for the amplification of *lys*A gene fragments

Name of the primer	Sequence of the primer	Expected product size (bp)
1 (L)	5'TCCACGGCAACAACAAGTCG3'	983
2 (R)	5' TCACCCGACGTCGAGACG 3'	983
3 (L)	5' ATGAGCCGTTCCGCACAG 3'	620
4 (R)	5' AACTTCTGGTCCTCGTGGG 3'	620

2.11. Polymerase chain reaction (PCR)

PCR mixtures contained 12 µL of dH_2O, 2.5 µL of 10 x PCR Mg^{+2} buffer, 50 pmols of each primer, 2.5 µL of 2 mM dNTP mix, 0.5 µg of genomic DNA, 2.5 µL of DMSO and 3 Units Taq Polymerase. The PCR cycles and conditions were as follows: PCR for the amplification of 983 bp *lys*A gene fragment was carried out at 35 cycles. First 5 cycles were carried out as 1 min at 94 ˚C, 1 min at 63 ˚C and 1 min at 72 ˚C for denaturation, annealing and extension, respectively. Remaining 30 cycles were carried out as 1 min at 94 ˚C, 1 min at 60 ˚C and 1 min at 72 ˚C. Initial denaturation was carried out at 94 ˚C for 10 minutes and final extension step was carried out at 72 ˚C for 10 minutes. PCR for the amplification of 620 bp *lys*A gene fragment was carried out at 35 cycles. All cycles were carried out as 1 min at 94 ˚C, 1 min at 63 ˚C and 1 min at 72 ˚C. Initial denaturation was carried out at 94 ˚ C for 10 minutes and final extension step was carried out at 72 ˚ C for 10 minutes.

Reaction mixtures were run in a 0.9 % agarose gel. PCR products were extracted from the gel as mentioned in Section 2.9.3. Sequence of the fragments were obtained as mentioned in Section 2.16.

2.12. Cloning of PCR products

Ligation of PCR products with PGEM-T vector was performed as follows: 5 µL 2X ligase buffer, 1 µL (50 ng/ µL) PGEM-T vector, 400 ng insert DNA, 1 µL T4 DNA ligase (3 unit/ µL) was mixed in a total volume of 10 µL. Ligation was carried out for 16 hours at 16 ˚ C.

2.13. Southern blot hybridization

2.13.1. Southern blotting

Southern blotting was performed as described by Sambrook *et al*. (1989). Following electrophoresis, the gel was first soaked in gel denaturation solution (Appendix B) with gentle agitation for 45 minutes and then neutralized in gel neutralization solution (Appendix B) with gentle agitation for 45 minutes. Then the gel was blotted for 20 hours in 20 x SSC (Appendix B) using a nylon membrane (Amersham) and air-dried for 1 hour. For DNA crosslinking, the membrane was exposed to UV light (300 nm) for 8 minutes.

2.13.2. Labelling of probe DNA with ECL random prime labelling and detection system

The probe DNA was labelled flourescently by using ECL Random Prime Labelling and Detection System (Amersham). The DNA to be labelled was denatured by heating for 5 minutes in a boiling water bath, then was chilled on ice. 10 µL of nucleotide mix, 5 µL of primer solution, 200 ng denatured DNA, 1 µL of enzyme solution (Klenow, 5 units/µL) and water to a final volume of 50 µL were added in order into a 1.5 mL microfuge tube. Reaction mixture was incubated at 37 ˚C overnight.

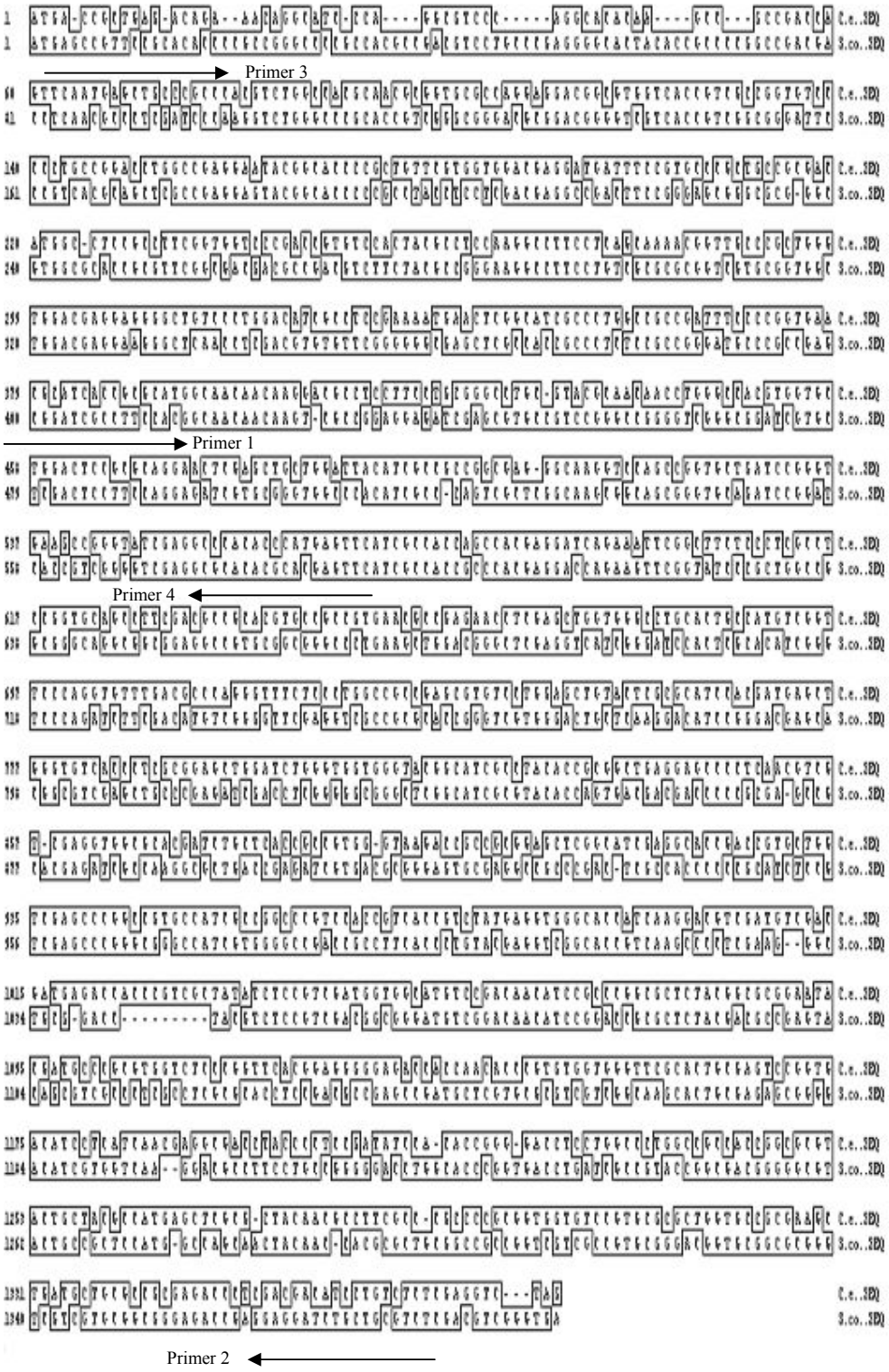


Figure 2.2. Alignment of *lys*A gene sequences of *Streptomyces coelicolor* (AL939123) and *Corynebacterium efficiens* (AB083132). Primers designed are indicated by arrows.

2.13.3. Prehybridization and hybridization

Blots for hybridization with the labelled probe were put in hybridization tubes. The membranes were prehybridized with prehybridization buffer (Appendix B) for 2 hours at 60 ˚C with constant shaking. After 2 hours, denatured labelled probe was added to prehybridization buffer and the membrane was hybridized overnight at 60 ˚C with constant shaking. The membrane was washed twice with wash solution 1(Appendix B) for 15 minutes at 60 ˚C and once with wash solution 2 (Appendix B) for 15 minutes at 60 ˚C.

2.13.4. Signal generation and detection

After stringency washes, the blot was placed in a clean container and rinsed with Buffer A (Appendix B) for 1 minute at room temperature. Buffer A was replaced with 20-fold dilution of Liquid Block in Buffer A and the blots were incubated in this solution for 30 minutes. The antiflourescein-HRP(horse radish peroxidase) conjugate was diluted 100-fold in a freshly prepared 0.5% (w/v) bovine serum albumin fraction V in Buffer A. Blots were incubated in this solution for another 30 minutes. The conjugate was drained off and unbound conjugate was removed by washing the blots for 3x10 minutes in excess 0.1% (v/v) Tween-20 in Buffer A. All the steps required gentle agitation at room temperature. Excess reagent from the blots was drained and the blots were placed in a clean container. Equal volumes of Detection Reagents 1 and 2 were mixed. This mixture was added directly on blots on the side carrying DNA. After 1 minute incubation at room temperature, excess Detection Reagents were drained and the blot was wrapped in a Saran wrap. Air pockets were removed gently. 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 2-3 hours and developed.

2.14. Construction of mini genomic library of *s. clavuligerus* in a plasmid vector

S. clavuligerus chromosomal DNA was completely digested with *Pst*I, *Eco*RI, *Eco*RV and *Hind*III, respectively. The digests were separated on 0.9 % agarose gel. After the transfer of DNA onto a nylon membrane and hybridization with the labelled *lys*A probe, the regions which gave positive signal were detected. The subpopulation of DNA fragments (in the molecular weigth range of 2.2-3.0 kb) which gave a positive signal were extracted form the gel and purified. pBluescript KS vector was digested with the same enzyme and treated with Calf Intestinal Alkaline Phosphatase at 37 ˚ C for 30 minutes. The enzyme was then inactivated at 65 ˚C for 45 minutes. After phenol-chloroform extraction, the purified digest was ligated to the vector to keep up the insert to vector molar ratios of 2:1 to 5:1, respectively. Ligation reactions contained 1 µL (10 Units) of T4 DNA ligase (MBI Fermentas), 1xligation buffer and approximately 25 ng/µL DNA. Reaction mixtures were incubated at 16 ˚ C for 16 hours.

E. coli DH5α competent cells were transformed with the ligation mixture and the recombinant colonies (white ones) were detected on LB agar containing ampicillin (100 µg/mL), X-Gal (40 µg/mL) and IPTG (40 µg/mL). To determine the efficiencies of libraries, plasmid isolation from 80 randomly chosen white colonies were performed.

2.15. Colony blot hybridization

Colony blot hybridization was performed according to the standard protocols (Sambrook *et a*l., 1989).

2.15.1. Transferring colonies onto nylon membranes

The putative recombinant colonies were streaked onto a master as well as a replica plate containing the selective antibiotic. 2-3 mm streaks were made in a grid

pattern, each colony being streaked in an identical position on both plates. The plates were inverted and incubated at 37 ˚C overnight. The master plate was sealed and stored at 4 ˚C until the results of the hybridization reaction were available. A piece of nylon membrane was placed on the replica for 10 minutes.

2.15.1. Lysis of colonies and binding of DNA onto nylon membranes

Nylon membrane as well as master and replica agar plate were marked to confer the positions of colonies to the same locations.The procedure described by Sambrook *et al*. (1989) was used to attach DNA content of bacterial colonies onto the nylon membrane, as described in Section 2.15.1: Four pieces of Whatmann 3 MM paper were cut in appropriate size. Each piece of paper was saturated with one of the following solutions: 10% SDS, denaturation solution (0.5 N NaOH, 1.5 M NaCl), neutralizing solution (1.5 M NaCl, 0.5 M Tris.Cl (pH 7.4)) and 2 xSSC. The nylon membranes were peeled off from the plates and were placed colony side up on the first 3MM paper saturated with 10% SDS, exposed for 3 minutes and transferred to the second paper saturated with denaturation solution and exposed for 5 minutes. After denaturation, membranes were placed on 3 MM paper saturated with neutralization solution and exposed for 5 minutes. Finally, they were placed on the paper saturated with 2 x SSC and exposed for 5 minutes. After membranes were air dried for 30 minutes at room temperature, DNA was fixed onto the membranes by UV exposure for 8 minutes.

2.15.2. Colony hybridization

Membranes were floated on the surface of a tray containing 2 x SSC and then submerged into this solution for 5 minutes. They were then transferred to hybridization tubes containing prewashing solution (Appendix B) and incubated there at 50 ˚C for 30 minutes with constant rotation. The bacterial debris was scrabed from the surfaces of membranes with tissue papers soaked in prewashing solution. Prehybridization, hybridization, stringency washing and detection steps were carried out as described before (Sections 2.12.2 and 2.12.3).

2.16. DNA Sequencing and Sequence Analysis

Sequencing of 983 bp and 620 bp fragments of *lys*A gene cloned into pGEM-T vector was made by İontek Company (İstanbul, Turkey). The sequence data were analysed with DNA Star programme and homologies and identities to previously characterized *lys*A genes were determined with a Blast search (www.ncbi.nlm.nih.gov/blast).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning of *lysA* gene from *S. clavuligerus* by constructing libraries

3.1.1. Preparation of a probe specific for *lysA* gene

A portion of the *S. clavuligerus lys*A gene was amplified by PCR to be used as a probe for screening the genomic library of the same organism. For this purpose, *lys*A gene sequences of two different *actinomycetes, namely S. coelicolor* (AL939123) *and C. efficiens* (AB083132) in the GeneBank database were aligned with each other and conserved regions of the gene were determined (Figure 2.2). After the alignment, instead of preparing degenerate primers, the *lys*A gene sequence of *S. coelicolor* was preferred to prepare primers 1 and 2 (Table 2.2) according to the conserved regions.

PCR reaction with primer 1 and 2 resulted in a 983 bp product using *S. clavuligerus* chromosomal DNA as a template (Figure 3.1). This fragment was purified from the gel and its nucleotide sequence was determined (Figure 3.2). Blast searches of the GeneBank database indicated that the sequence of 983 bp PCR product had 82 % identity to *S. coelicolor* and 80 % identity to *S. avermitilis lysA* genes.

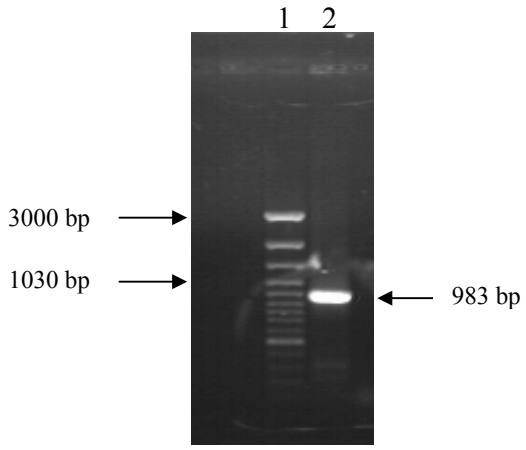


Figure 3.1. PCR product obtained with primers 1 and 2 designed for *lys*A gene. Lane 1: Marker (100 bp DNA ladder (Appendix C). Lane 2: 983 bp fragment

409ttcacggcaacaacaagtcccttgaggagatcgagcgcgccgtccgggccggggtcggacggatcgtcct
cgactcctcccaggagatcgtccgggtcgcccacgtcgcccaggccctcggggtgcgccagcgcggcctgatc
cggatcaccgtcggcgtcgaggcgcacacccacgagttcatcgccaccgcgcacgaggaccagaagttcggcc
tgccgctcgccggcggacaggcggcggagggcgtgcgccgcgcactcggcctcgacgggctggagctgatcgg
cctccactcccacatcggctcccagatcttcgacacctccggcttcgaggtcgccgcccaccgcgtcgtcggg
ctgctccagcaggtccgcgacgagcacggcgtcgaactccccgagatcgacctcggcggcggcctcggtatcg
cgtacacccccgacgacgacccgcgtgaaccgcgtcacatcgccaaggcgctccacgagatcgtcacccgtga
gtgcgaggacgccaagctgcgccctccgcggatgtccgtcgagccgggccgcgcgatcgtcggccccaccgcg
ttcaccctgtacgaggtcggcaccatcaagccgctgccgggactgcgcacctacgtcagcgtcgacgggggga
tgtccgacaacatccgcaccgctctgtacgacgccgagtacagcgtcgccctcgtctcccgggcatccgacgc
ggcagccatgctctcccgtgtcgtcggcaagcactgcgaaagtggtgacatcgtggtgaaggacgcgttcctt
ccccgcgacctcgcccccggcgatctgctcgccgtccccgcgaccggtgcgtactgccgctcgatggcccgca
actacaaccacgcgctgcgcccgcccgtggtggcggtgcgggacggtgcggcacgggtgatcgtccgccggga
gacggaggaggaaatcctgcgtctcgacgtcgggtga**1392**

Figure 3.2. Nucleotide sequence of 983 bp fragment of *lysA* gene

3.1.2. Construction of genomic libraries of *S. clavuligerus* and screening

For construction of the mini genomic libraries, *S. clavuligerus* DNA fragments were obtained by complete digestion with *Pst*I, *Eco*RI, *Eco*RV and *Hind*III (Figure 3.3). Southern blot analysis was carried out by using flourescently labelled 983 bp *lysA* gene fragment as the probe to detect the subpopulation of DNA fragments which might contain the whole *lysA* gene. Positive signals were obtained with *Pst*I and *Eco*RI digests (Figure 3.4).

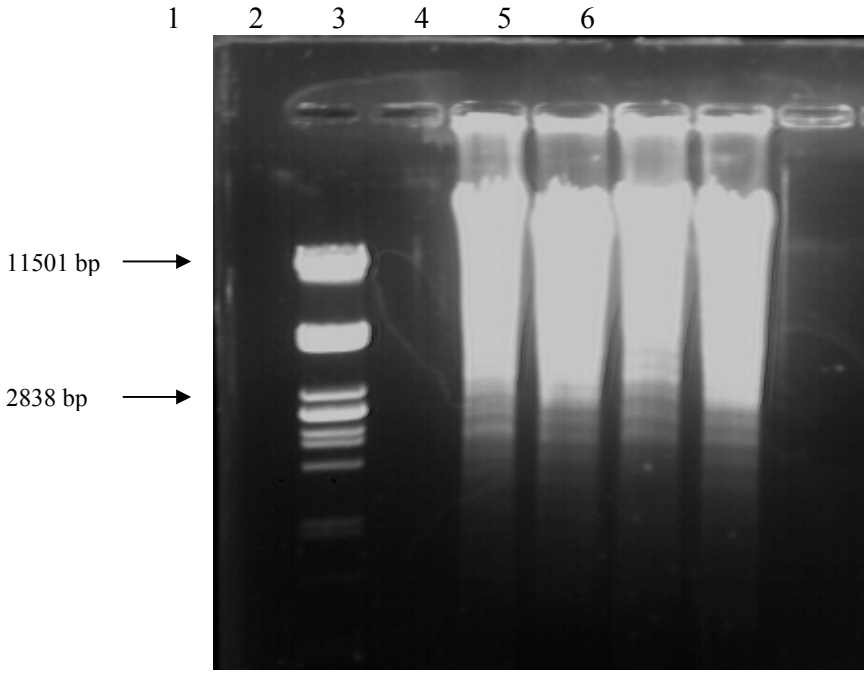


Figure 3.3. Complete digestion of *S. clavuligerus* chromosomal DNA. Lane 1: Lambda *Pst*I digest, Lane 2. empty, Lanes 3-6: Complete digestion of *S. clavuligerus* chromosomal DNA with restriction enzymes. Lane 3: *Pst*I digest, Lane 4: *Eco*RI digest, Lane 5: *Eco*RV digest, Lane 6: *Hind*III digest.

These regions of the gels were excised, DNA fragments were extracted, purified and ligated with pBluescript II KS linearized at its *Pst*I or *Eco*RI sites. The mini libraries were amplified by transforming *E. coli* DH5α with the ligation mixtures. Plasmid isolation from randomly chosen white colonies from *Pst*I and *Eco*RI mini libraries showed an insert size range of 2.7-3.2 kb and 2.1-2.7 kb, respectively, as expected (Figure 3.5). The mini libraries were screened under different stringency conditions by colony hybridization technique, however no positive clones were obtained.

Both *Pst*I and *Eco*RI mini libraries in pBluescript KS were inefficient since recombination frequency was around 15% in them. This value was rather low to find out the correct clone.

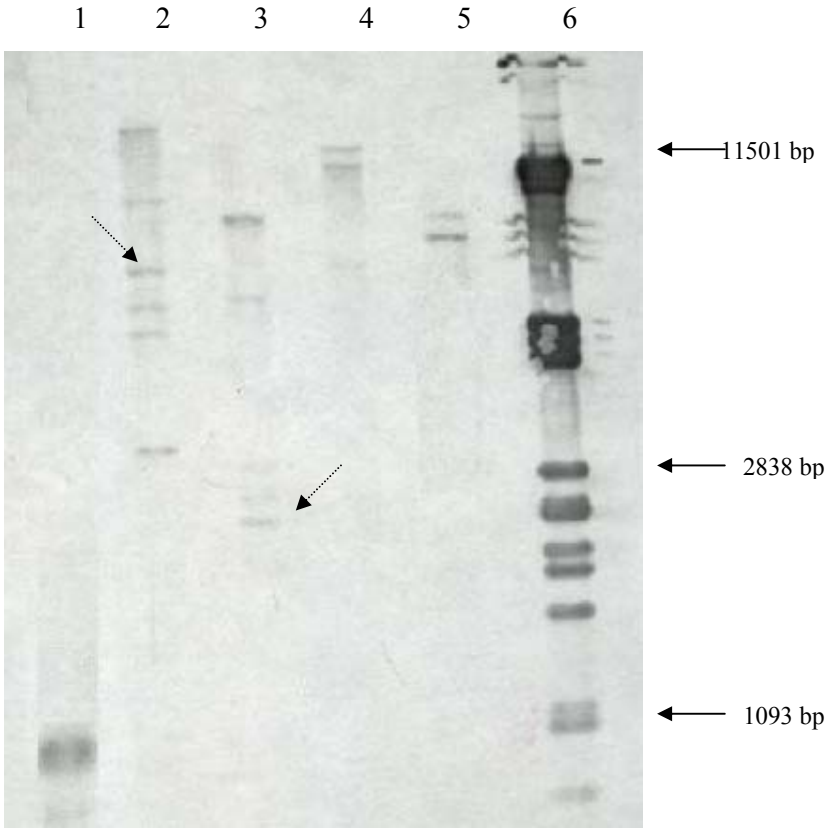


Figure 3.4. Southern blot hybridization of completely digested *S. clavuligerus* chromosomal DNA.

Lane 1: Probe DNA (983 bp PCR product), Lane 2: *Pst*I digest, Lane 3: *Eco*RI digest, Lane 4: *Eco*RV digest, Lane 5: *Hind*III digest, Lane 6: Lambda *Pst*I digest. The dashed arrows indicate regions of *Pst*I and *Eco*RI digests that gave positive signal with the probe.

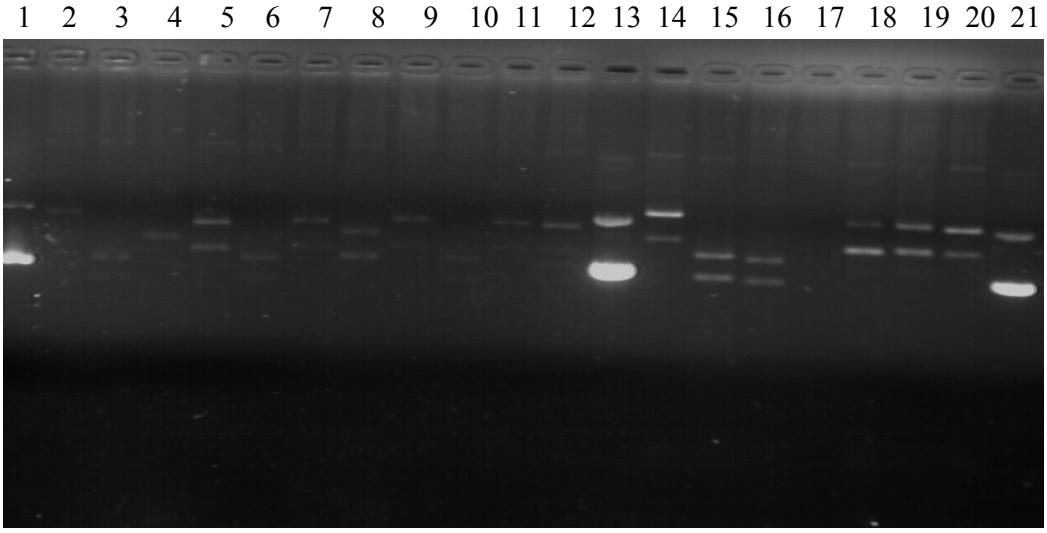


Figure 3.5. Recombinant plasmids carrying different DNA fragments.
Lanes 1, 13 and 21: Native pBluescript KS vector, Lanes 2-12 and 14-20: Putative recombinant plasmids.

It was therefore decided to construct another genomic library of *S. clavuligerus*. This time, *S. clavuligerus* chromosomal DNA was partially digested with *Sau*3A (Figure 3.6). The digests which contained more DNA fragments in the size range of 1.5-4.0 kb (obtained with $62{,}5x10^{-3}$, $31{,}25x10^{-3}$, $15{,}6x10^{-3}$ and $7{,}8x10^{-3}$ U *Sau*3A/ μg DNA) were ligated with pBluescript KS which was digested with *Bam*HI.

For screening, complemetation approach was used instead of homologous probing. The library was amplified by transforming a well-characterized *E.coli* lysine auxotroph, *E.coli* CGSC 4345 (*Lac*Z118(Oc), *lac*I22, *lys*A23) with the ligation mixture. A desired clone in which the recombinant lysA gene phenotypically complements the mutation could not be obtained from this screen.

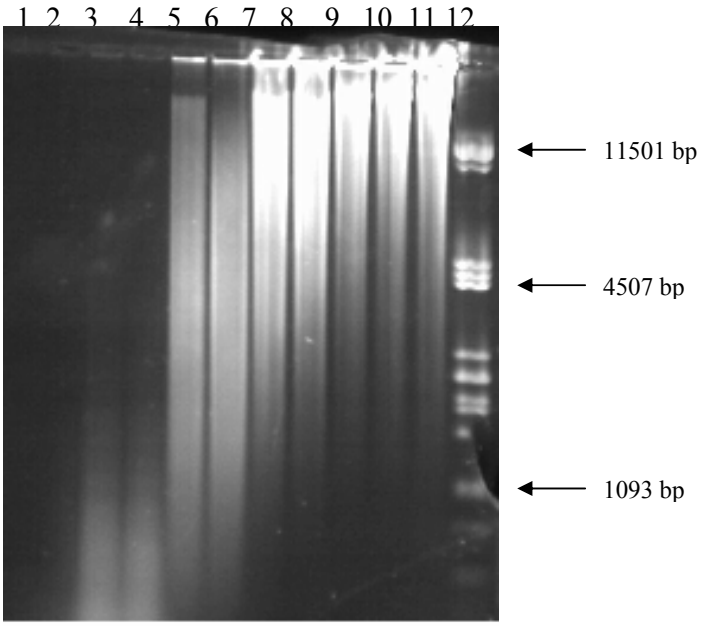


Figure 3.6. Partial digestion of *S. clavuligerus* chromosomal DNA with *Sau*3A.

Lanes 1-2: empty; 3: 0.25 U *Sau*3A/µg DNA, 4: 0.125 U *Sau*3A/µg DNA, 5: $62{,}5x10^{-3}$ U *Sau*3A/µg DNA, 6: $31{,}25x10^{-3}$ U *Sau*3A/µg DNA, 7: $15{,}6x10^{-3}$ U *Sau*3A/µg DNA, 8: $7{,}8x10^{-3}$ U *Sau*3A/µg DNA, 9: $3{,}9x10^{-3}$ U *Sau*3A/µg DNA, 10: $1{,}95x10^{-3}$ U *Sau*3A/µg DNA, 11: $9{,}76x10^{-4}$ U *Sau*3A/µg DNA, 12: Lambda DNA *Pst*I digest.

3.2. Cloning of *lysA* gene by PCR-based approach

Our attempts to isolate a *lys*A gene clone either by screening of the genomic libraries of *S. clavuligerus* with a homolog probe or complemetation approach gave no positive results. The size of the probe which was amplified from the *S. clavuligerus* chromosomal DNA was 983 bp and had the sequences of the central and C-terminal regions of the *lys*A gene. We therefore decided to use PCR approach to amplify the N-terminal portion of the gene. For this purpose, another set of primers was designed according to the conserved regions of the alignments shown in (Figure 2.2). PCR with primers 3 and 4 (Table 2.2) yielded a 620 bp fragment by *using S. clavuligerus* chromosomal DNA as a template (Figure 3.7).

After purification from the gel, the fragment's nucleotide sequence was determined (Figure 3.8).

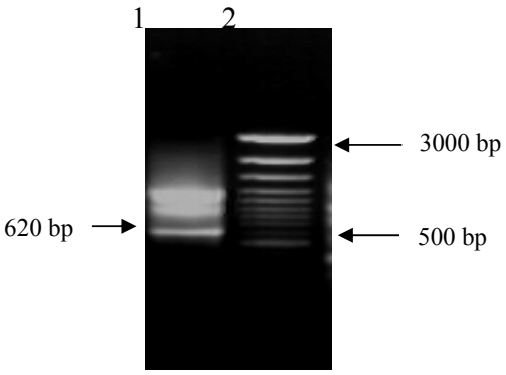


Figure 3.7. PCR product obtained with primers 3 and 4 designed for *lysA* gene. Lane 1: 620 bp fragment, Lane 2: Marker- Lambda *Pst*I digest (Appendix C)

Blast search of the sequence indicated that the 620 bp fragment corresponded to the N-terminal region of *lys*A gene. When 983 bp and 620 bp fragments' sequences were aligned, an overlapping region between the bases 391 and 620 were found (Figure 3.9). Thus complete nucleotide sequence of *S. clavuligerus lys*A gene was obtained by joining and aligning the nucleotide sequences of 983 bp and 620 bp PCR products.

1atgagccgttccgcacaccccgcagggcccccacacgccgacgtcctccccgagggccactacaccgccccg
cccgcagacctcaaccgcctcgacgccaagatctggtcccgtaccgtcgaccggaacgaggacggggttgcca
ccgttggcggcatcgatgtgacccggctcgccgaggagttcggcacccccggctacatcctggacgaggacga
cttccgcgaccgcgcccgcgcctgggccaccgccttgggccacgaggccgacgtcttctacgccgggaaggcg
ttcctcagccgcgccgtggtccgctggctgcacgaggaagggctcaatctcgatgtgtgctccggcggcgagc
tgctcaccgccctcgacgccgggatgcccgccgaccgcatcgcctttcacggcaacaacaagtcccttgagga
gatcgagcgcgccgtccgggccggggtcggacggatcgtcctcgactcctcccaggagatcgtccgggtcgcc
cacgtcgcccaggccctcggggtgcgccagcgcggcctgatccggatcaccgtcggcgtcgaggcgcacaccc
acgagttcatcgccaccgcgcacgaggaccagaagtt **620**

Figure 3.8. Nucleotide sequence of 620 bp *lysA* gene fragment

Since N-terminal portion of the gene was obtained with primers 3 and 4 and the C-terminal with primers 1 and 2, the whole gene was tried to be amplified with

primers 2 and 3. However, since T_m values for these two primer pairs were not compatible, our attempts to amplify the whole gene in a single PCR were not successful.

Figure 3.9. Alignment of the nucleotide sequences of 983 bp and 620 bp DNA fragments of *lysA* gene. Bold characters show the overlapping region of two fragments amplified.

983 bp and 620 bp PCR fragments (Figure 3.1 and 3.7) were seperately cloned into pGEM-T vector and amplified in *E.coli* DH5α cells. Figure 3.10 shows the recombinant plasmids obtained from the ligation of pGEM-T and 983 bp insert. One of the plasmids which was designated pGEM-T1 was digested with *Pst*I and *Sph*I to release the 983 bp insert DNA (Figure 3.11). Recombinant plasmids obtained from ligation of 620 bp fragment to pGEM-T are shown in Figure 3.12. One of them was digested with *Pst*I and *Sph*I to verify the presence of the 620 bp insert and it was designated pGEM-T2 (Figure 3.13).

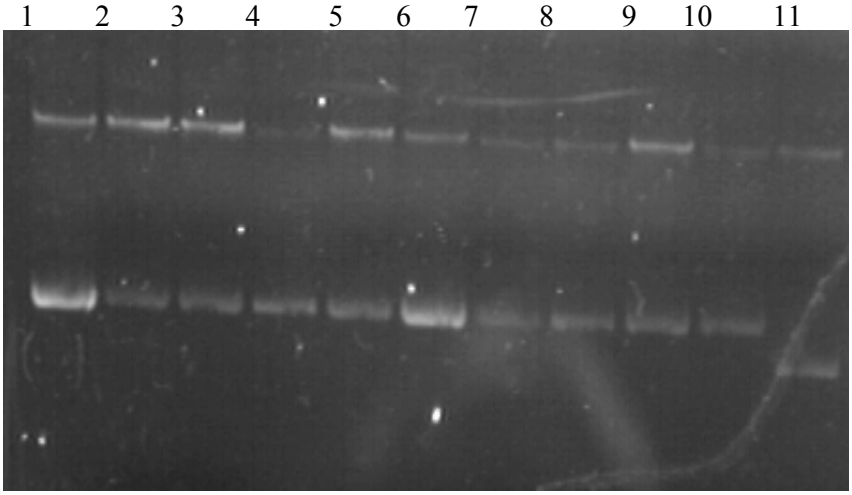


Figure 3.10. Analysis of the plasmids of the putative recombinants carrying 983 bp PCR product. Lanes 1-10: Recombinant pGEM-T vector carrying 983 bp PCR products, Lane 11: Native pGEM-T vector.

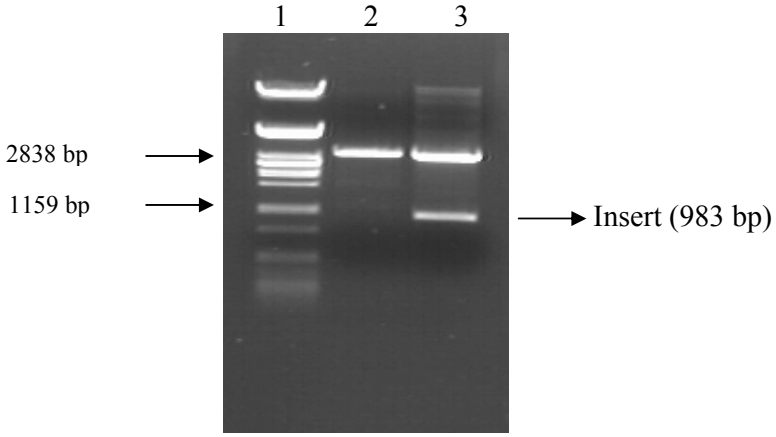


Figure 3.11. Verification of the cloning of the 983 bp PCR product. Lane 1: Lambda DNA *Pst*I digest, Lane 2: pGEM-T vector digested with *Pst*I and *Sph*I, Lane 3: Recombinant pGEM-T1 digested with *Pst*I and *Sph*I.

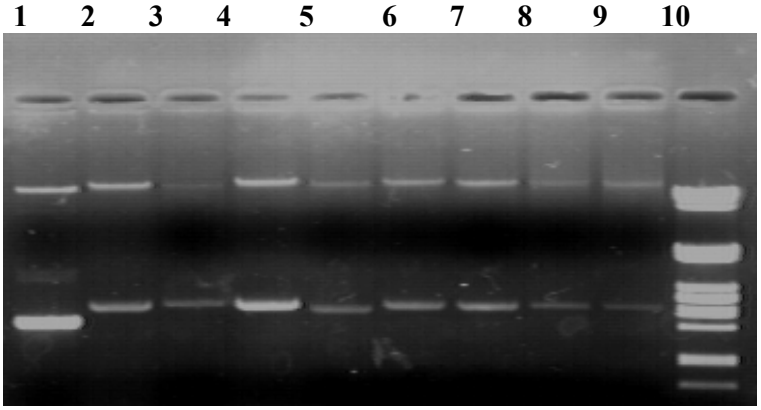


Figure 3.12. Analysis of the plasmids of the putative recombinants carrying 620 bp PCR product. Lane 1: Native pGEM-T vector, Lanes 2-9: Recombinant pGEM-T molecules carrying 620 bp PCR products, Lane 10: Lambda DNA *Pst*I digest.

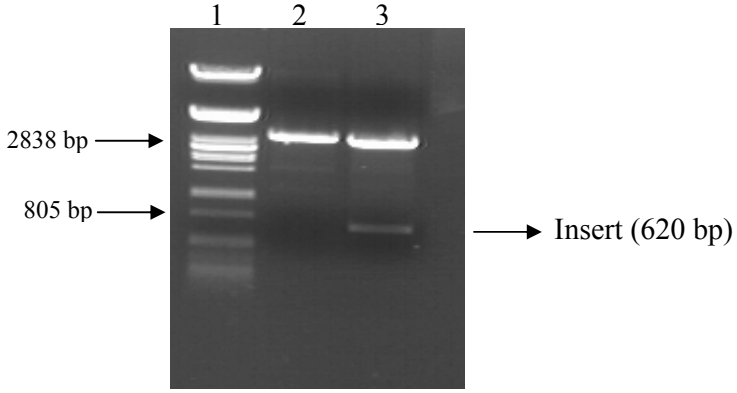


Figure 3.13. Verification of the cloning of the 620 bp PCR product.Lane 1: Lambda DNA *Pst*I digest, Lane 2: pGEM-T vector digested with *Pst*I and *Sph*I, Lane 3: Recombinant pGEM-T2 digested with *Pst*I and *Sph*I.

3.3. Organization and characteristics of *S. clavuligerus lys*A gene

The nucleotide sequences of the inserts in the pGEM-T1 and pGEM-T2 were combined to obtain the complete *S. clavuligerus lys*A gene sequence (Figure 3.14). The gene sequence showed 83 % identity to the sequence of *S. coelicolor lys*A gene and 81 % identity to *S. avermitilis lys*A gene. Also some short fragments of the sequence showed 89 % to 95 % identity with *lys*A gene sequences of other *Actinomycetes* family members; namely *M. tuberculosis*, *C. glutamicum* and *C. efficiens*.

The gene starts with an `atg` codon and terminates at position 1392 with a `tga` stop codon (Figure 3.14). Computer aided analysis indicated that *lys*A gene product is a 463 residue protein with a calculated molecular mass of 49.9 kDa. The mean G+C % content of the gene is 70.98 % (Figure 3.15). This value is quite typical for streptomyces ORFs and approaches the average of 72.12 % for ORFs recently reported for the *S. coelicolor* genome (Bentley *et al*., 2002). Since the GC content of *Streptomyces* species is quite high, G or C occurs in the third codon position about 90 % of the time. However, as already noted (Minton *et al*., 1984; Hadero and Crawford, 1986; Martin *et al*., 1988) there is a preference for C over G (the Arg, Thr, Ala, Gly, Arg and Val codons). Also, there is an excess of acidic residues over basic ones (68 and 50, respectively). Among the basic amino acids, arginine is overrepresented (mostly by CGC codon) relative to lysine, as compared with *E. coli* DAP-decarboxylase protein characterized by Straiger *et al*. (1983b).

ATGAGCCGTTCCGCACACCCCGCAGGGCCCCCACACGCCGACGTCCTCCCCGAGGGCCACTACACCGCCCCGCCCGCAGAC
M S R S A H P A G P P H A D V L P E G H Y T A P P A D

CTCAACCGCCTCGACGCCAAGATCTGGTCCCGTACCGTCGACCGGAACGAGGACGGGGTTGCCACCGTTGGCGGCATCGAT
L N R L D A K I W S R T V D R N E D G V A T V G G I D

GTGACCCGGCTCGCCGAGGAGTTCGGCACCCCCGGCTACATCCTGGACGAGGACGACTTCCGCGACCGCGCCCGCGCCTGG
V T R L A E E F G T P G Y I L D E D D F R D R A R A W

GCCACCGCCTTGGGCCACGAGGCCGACGTCTTCTACGCCGGGAAGGCGTTCCTCAGCCGCGCCGTGGTCCGCTGGCTGCAC
A T A L G H E A D V F Y A G K A F L S R A V V R W L H

GAGGAAGGGCTCAATCTCGATGTGTGCTCCGGCGGCGAGCTGCTCACCGCCCTCGACGCCGGGATGCCCGCCGACCGCATC
E E G L N L D V C S G G E L L T A L D A G M P A D R I

GCCTTTCACGGCAACAACAAGTCCCTTGAGGAGATCGAGCGCGCCGTCCGGGCCGGGGTCGGACGGATCGTCCTCGACTCC
A F H G N N K S L E E I E R A V R A G V G R I V L D S

TCCCAGGAGATCGTCCGGGTCGCCCACGTCGCCCAGGCCCTCGGGGTGCGCCAGCGCGGCCTGATCCGGATCACCGTCGGC
S Q E I V R V A H V A Q A L G V R Q R G L I R I T V G

GTCGAGGCGCACACCCACGAGTTCATCGCCACCGCGCACGAGGACCAGAAGTTCGGCCTGCCGCTCGCCGGCGGACAGGCG
V E A H T H E F I A T A H E D Q K F G L P L A G G Q A

GCGGAGGGCGTGCGCCGCGCACTCGGCCTCGACGGGCTGGAGCTGATCGGCCTCCACTCCCACATCGGCTCCCAGATCTTC
A E G V R R A L G L D G L E L I G L H S H I G S Q I F

GACACCTCCGGCTTCGAGGTCGCCGCCCACCGCGTCGTCGGGCTGCTCCAGCAGGTCCGCGACGAGCACGGCGTCGAACTC
D T S G F E V A A H R V V G L L Q Q V R D E H G V E L

CCCGAGATCGACCTCGGCGGCGGCCTCGGTATCGCGTACACCCCCGACGACGACCCGCGTGAACCGCGTCACATCGCCAAG
P E I D L G G G L G I A Y T P D D D P R E P R H I A K

GCGCTCCACGAGATCGTCACCCGTGAGTGCGAGGACGCCAAGCTGCGCCCTCCGCGGATGTCCGTCGAGCCGGGCCGCGCG
A L H E I V T R E C E D A K L R P P R M S V E P G R A

ATCGTCGGCCCCACCGCGTTCACCCTGTACGAGGTCGGCACCATCAAGCCGCTGCCGGGACTGCGCACCTACGTCAGCGTC
I V G P T A F T L Y E V G T I K P L P G L R T Y V S V

GACGGGGGGATGTCCGACAACATCCGCACCGCTCTGTACGACGCCGAGTACAGCGTCGCCCTCGTCTCCCGGGCATCCGAC
D G G M S D N I R T A L Y D A E Y S V A L V S R A S D

GCGGCAGCCATGCTCTCCCGTGTCGTCGGCAAGCACTGCGAAAGTGGTGACATCGTGGTGAAGGACGCGTTCCTTCCCCGC
A A A M L S R V V G K H C E S G D I V V K D A F L P R

GACCTCGCCCCCGGCGATCTGCTCGCCGTCCCCGCGACCGGTGCGTACTGCCGCTCGATGGCCCGCAACTACAACCACGCG
D L A P G D L L A V P A T G A Y C R S M A R N Y N H A

CTGCGCCCGCCCGTGGTGGCGGTGCGGGACGGTGCGGCACGGGTGATCGTCCGCCGGGAGACGGAGGAGGAAATCCTGCGT
L R P P V V A V R D G A A R V I V R R E T E E E I L R

CTCGACGTCGGG**TGA**
L D V G **.**

Figure 3.14. Nucleotide and deduced amino acid sequence of *S. clavuligerus lys*A gene. Start and stop codons are in bold face.

a) Codon usage

gca	Ala(A)	7	cag	Gln(Q	8	uug	Leu(L)	1	uaa	Ter(.)	0
gcc	Ala(A)	33	+++	Gln(Q)	8	+++	Leu(L)	44	uag	Ter(.)	0
gcg	Ala(A)	16	gaa	Glu(E)	5	aaa	Lys(K)	0	uga	Ter(.)	1
gcu	Ala(A)	1	gag	Glu(E)	29	aag	Lys(K)	9	+++	Ter(.)	1
+++	Ala(A)	57	+++	Glu(E)	34	+++	Lys(K)	9	aca	Thr(T)	0
aga	Arg(R)	0	gga	Gly(G)	3	aug	Met(M)	6	acc	Thr(T)	19
agg	Arg(R)	0	ggc	Gly(G)	27	+++	Met(M)	6	acg	Thr(T)	1
cga	Arg(R)	0	ggg	Gly(G)	12	uuc	Phe(F)	10	acu	Thr(T)	0
cgc	Arg(R)	23	ggu	Gly(G)	4	uuu	Phe(F)	1	+++	Thr(T)	20
cgg	Arg(R)	11	+++	Gly(G)	46	+++	Phe(F)	11	ugg	Trp(W)	3
cgu	Arg(R)	7	cac	His(H)	18	cca	Pro(P)	1	+++	Trp(W)	3
+++	Arg(R)	41	cau	His(H)	0	ccc	Pro(P)	13	uac	Tyr(Y)	10
aac	Asn(N)	7	+++	His(H)	18	ccg	Pro(P)	9	uau	Tyr(Y)	0
aau	Asn(N)	1	aua	Ile(I)	0	ccu	Pro(P)	1	+++	Tyr(Y)	10
+++	Asn(N)	8	auc	Ile(I)	23	+++	Pro(P)	24	gua	Val(V)	0
gac	Asp(D)	31	auu	Ile(I)	0	agc	Ser(S)	4	guc	Val(V)	30
gau	Asp(D)	3	+++	Ile(I)	23	agu	Ser(S)	1	gug	Val(V)	11
+++	Asp(D)	34	cua	Leu(L)	0	uca	Ser(S)	0	guu	Val(V)	2
ugc	Cys(C)	4	cuc	Leu(L)	25	ucc	Ser(S)	14	+++	Val(V)	43
ugu	Cys(C)	0	cug	Leu(L)	16	ucg	Ser(S)	1	nnn	???(X)	0
+++	Cys(C)	4	cuu	Leu(L)	2	ucu	Ser(S)	0	TOTAL		464
caa	Gln(Q)	0	uua	Leu(L)	0	+++	Ser(S)	20			

(b)Amino acid composition

Amino acids	Number	Mole Percentage
Strongly Basic (+) Amino Acids (K, R)	50	10.8
Strongly Acidic (-) Amino Acids (D, E)	68	14.7
Hydrophobic Amino Acids (A, I, L, F, W,V)	181	39
Polar Amino Acids (N, C, Q, S, T, Y)	70	15

(c) Base composition

Bases	Number	Mole Percentage
Adenosine	208	18.69
Guanidine	447	32.11
Thymidine	195	14.01
Cytosine	541	38.86
(A+T)	404	29.02
(G+C)	988	70.98

Figure 3.15. Characteristics of deduced nucleotide and amino acid sequence of *S. clavuligerus lys*A product. a) codon usage, b) amino acid composition, c) base composition.

The primary structure of *S. clavuligerus* DAP-decarboxylase enzyme was aligned with those from other bacteria (Figure 3.16). The deduced amino acid sequence of *S. clavuligerus* DAP-decarboxylase indicated that it has considerable homology to known DAP-decarboxylases of other microorganisms, especially those of actinomycetes, *S. coelicolor*, *C. efficiens* and *M. tuberculosis* (Figure 3.16; Table 3.1).

Several conserved regions were found among all bacteria examined (Figure 3.16). Region 1 was first identified by Poulin *et al*. (1992) with the lysine residue being common in all decarboxylases including bacteria, yeast, animals, plants and human. As shown in Figure 3.16, Lys (K) residue in region 1 is conserved in both gram-positive and -negative bacteria.

Table 3.1. Percent similarity and divergence of *S. clavuligerus* DAP decarboxylase enzyme to DAP decarboxylases from other organisms. S.c: *Streptomyces clavuligerus*, S.co: *Streptomyces coelicolor* (AL939123), P.a: *Pseudomonas aeruginosa* (AE004940), B.s: *Bacillus subtilis* (D90189), C.e: *Corynebacterium efficiens* (AB083132), M.t: *Mycobacterium tuberculosis* (Z73419), E.c: *Escherichia coli* (AE005512), S.t: *Salmonella typhimurium* (AE008838).

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8		
1		86.0	30.0	36.3	48.7	54.7	21.4	19.0	1	S.c..PRO
2	15.3		30.0	37.2	50.4	57.6	20.9	21.1	2	S.co..PRO
3	128.5	128.5		26.4	27.9	29.8	25.5	26.7	3	P.a..PRO
4	114.0	110.4	141.3		34.0	32.4	22.3	20.9	4	B.s..PRO
5	79.6	75.6	137.5	115.9		56.5	22.1	22.8	5	C.e.PRO
6	62.5	56.3	126.4	121.6	60.9		20.7	20.2	6	M.t..PRO
7	162.0	170.5	132.4	161.9	166.7	178.3		87.9	7	E.c..PRO
8	162.0	168.7	127.6	170.5	161.7	174.4	13.0		8	S.t..PRO

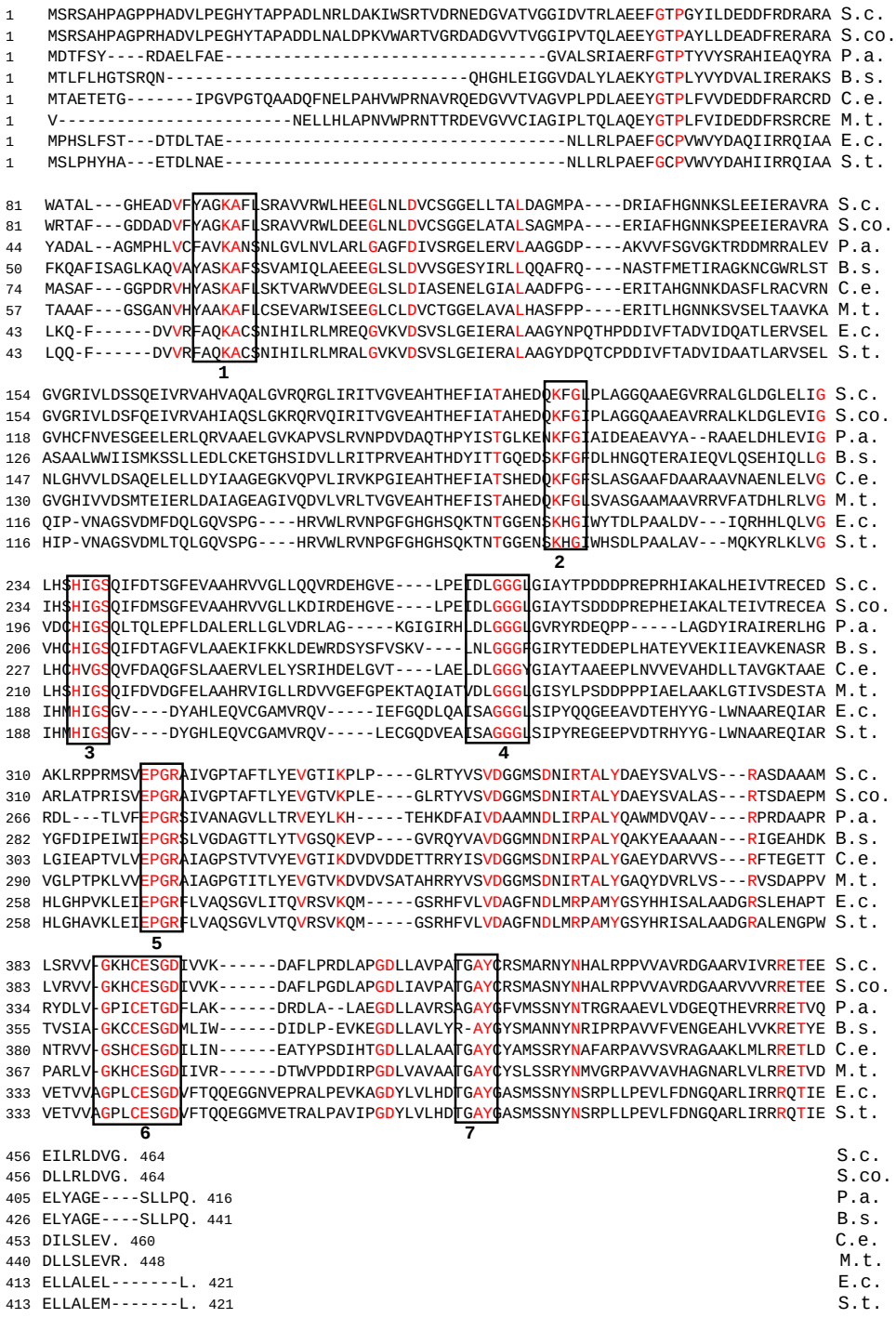


Figure 3.16. Alignment of *S. clavuligerus* DAP decarboxylase with those from other organisms. Amino acids identical in all organisms are indicated with red. Abbreviations for the microorganisms are the same to those used in Table 3.1.

DAP decarboxylase, like most of the amino acid decarboxylases, is a pyridoxal phosphate (PLP)-dependent enzyme (Martin *et al*., 1988). In all PLP-requiring enzymes, PLP is covalently bound to the ε-amino group of lysine residue. PLP-binding consensus sequences are S-X-H-K or simply H-K in many decarboxylases. But none of the DAP decarboxylases presented in Figure 3.15 appear to have these consensus sequences. A His-Lys dipeptide is found only in the sequence of *P. aeroginosa* (position 297) where it is located in a nonconserved region. In region 2, Lys residue is conserved in all decarboxylases (Figure 3.16). This residue is suggested to be responsible for Schiff's base formation with PLP (Poulin *et al*., 1992).

Straiger *et al*. (1983b) localized a PLP mutation in the *E. coli* DAP decarboxylase to a span of 107 amino acids from residues 214 to 321, including the conserved regions 4 and 5. Mutation in this area resulted in reduction in PLP binding, suggesting that they are involved in the PLP- and/or substrate-binding. In *S. clavuligerus*, this span corresponds to residues 265 to 370, which also includes the conserved regions 4 and 5 (Figure 3.16). The conserved motif in region 6 includes an identical cysteine residue in all the enzymes analyzed. This Cys residue may be involved in the catalytic site of all decarboxylases. In the work of Yamamoto *et al*. (1989), a *B. subtilis lys*A-*dna*N fusion able to complement a *B. subtilis* lysA mutant was subcloned. *B.subtilis lys*A-*dna*N fusion contained the N-terminal 314 amino acids of the B. subtilis DAP decarboxylase lacking two of the C-terminal conserved regions (region 6 and 7) (Figure 3.16). This truncated fusion made DAP decarboxylase activity 20-40 fold lower than that produced by wild-type chromosomal *lys*A gene. This reduction in the activity could be in part due to the deletion of the Cys residue at region 6.

After complete nucleotide sequence of the gene was identified, a restriction map was drawn by using the DNAStar programme. When the restriction map of *S. clavuligerus lys*A gene was examined, a unique restriction enzyme site (*Acc*III at position 551) in the overlapping region of 620 bp and 983 bp *lys*A gene fragments

was identified (Figure 3.15). This unique restriction site will be used to join two *lys*A gene fragments cloned in the pGEM-T1 and pGEM-T2 recombinant vectors. *Acc*III does not have a site in pGEM-T vector (Figure 2.1). *Pst*I and *Sph*I enzymes have unique sites in the multiple cloning site of pGEM-T vector and they do not cut *S. clavuligerus lys*A gene internally. Therefore, it will be possible to cut the pGEM- pGEM-T2 recombinant plasmid with *Acc*III and *Pst*I/*Sph*I to release insert from position 1 to 551. The pGEM-T1 recombinant plasmid will also be cut with the same enzymes to remove the insert from position 551 to 1392. The released insert (nucleotides1 to 551) from the recombinant pGEM-T2 will be ligated to recombinant pGEM-T1, having its insert representing the sequence between nucleotides 551 to 1392 to obtain the complete *lys*A gene. After joining of the two recombinant fragments, it will be possible to achieve expression of *S. clavuligerus lys*A gene in *E. coli*.

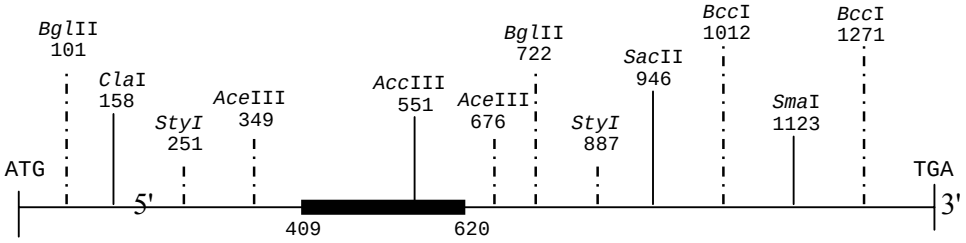


Figure 3. 17. Restriction map of *S. clavuligerus lys*A gene. The thick solid line shows the overlapping region between 620 bp and 983 bp fragments cloned. Solid lines indicate the restriction enzymes having unique site in the gene. Dashed lines indicate restriction enzymes having two sites in the gene.

The present research in our laboratory has focused on joining two *S. clavuligerus lys*A gene fragments contained in recombinant pGEM-T vectors. After heterologous expression of *S. clavuligerus lys*A gene in *E. coli*, integration of extra copies of *lys*A gene into chromosome of *S. clavuligerus* via homologous recombination remains to be undertaken to observe the effects of these manipulations on cephamycin C production. As the carbon flow through the

lysine-specific branch of aspartate pathway is rate-limiting in cephamycin C biosynthesis, the results of this research will be very helpful in a better understanding of the mechanism and an assesment of the contribution of carbon flux to the antibiotic production of this pathway in the producer organism *S. clavuligerus*. The characterization of this gene together with the two other key genes previously cloned and characterized in our laboratory (*ask* and *asd*, coding for two key enzymes aspartokinase and aspartate semialdehyde dehydrogenase of the aspartate pathway) will provide valuable information for both basic sciences and related biotechnological applications.

CHAPTER 4

CONCLUSION

* Two different fragments of *lys*A gene encoding for DAP decarboxylase enzyme were cloned from cephamycin C-producer *S. clavuligerus*. The recombinant vectors carrying the 983 bp and 620 bp gene fragments were designated as pGEM-T1 and pGEM-T2, respectively.

* 620 bp fragment started with the start codon 'atg' and 983 bp fragment ended with the stop codon 'tga'. As these two cloned fragments had an overlapping central region, complete nucleotide sequence of *S. clavuligerus lys*A gene could be determined. Nucleotide sequencing revealed a protein of 463 amino acids (M_r 49 907).

* *lys*A gene from *S. clavuligerus* has been sequenced and characterized for the first time.

* The GC content of the gene was determined as 70.98 %. The gene sequence showed 83 % identity to the sequence of *S. coelicolor lys*A gene and 81 % identity to the sequence of *S. avermitilis lys*A gene.

* Comparison of the deduced amino acid sequence of *S. clavuligerus* DAP decarboxylase with other DAP decarboxylase sequences available in database revealed the sites important in the catalytic activity of the enzyme.

* A few motifs possibly involved in catalytic activity of DAP decarboxylase were found around the amino terminus and middle of the protein. These protein regions seem to be conserved among gram-positive and –negative bacteria studied so far.

* Examination of the restriction map of the gene revealed a unique restriction enzyme site (*Acc*III) within the overlapping region of the two fragments cloned seperately in pGEM-T vectors. This site is now being used to join two fragments in pGEM-T1 and pGEM-T2 vectors in order to obtain the whole *lys*A gene as a single clone in *E. coli*.

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APPENDIX A

COMPOSITION AND PREPARATION OF CULTURE MEDIA

1. Media

1.1. Liquid Media

Luria Broth(LB)

	g/L
Luria Broth	15.5
NaCl	9.5

Sterilized at 121 ˚ C for 15 minutes.

Tryptone Soy Broth (TSB)	g/L
Tryptic Soy Broth(Difco)	30

Sterilized at 121 ˚ C for 15 minutes.

After autoclaving, add:

Maltose (40%) to a final concentration of 0.5%

1.2. Solid Media

LB Agar

	g/L
Luria Broth	15.5
NaCl	9.5
Agar	15

Sterilized at 121 ˚ C for 15 minutes.

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

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

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

Sterilized at 121 ˚ C for 15 minutes.

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

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

*Trace element solution	mg/L
$ZnCl_2$	40
$FeCl_2.6H_2O$	200
$CuCl_2.2H_2O$	10
$MnCl_2.4H_2O$	10
$Na_2B_4O_7.10H_2O$	10
$(NH_4)_6Mo_7O_{24}.4H_2O$	10

M9 Minimal Medium

Per Liter:

To 750 mL of sterile distilled water (cooled to 50 ˚ C), add:

5X M9 salts	200 mL
1 M $MgSO_4$	2 mL
20 % glucose	20 mL
1 M $CaCl_2$	0.1 mL

Sterile distilled water to 1 liter

5X M9 Salts

	g/L
$Na_2HPO_4.7H_2O$	64
KH_2PO_4	15
NaCl	2.5
NH_4Cl	5

Sterilized at 121 ˚ C for 15 minutes.

APPENDIX B

BUFFERS AND SOLUTIONS

Phenol-Chloroform Solution (Buffered, Hintermann, 1981)

Phenol	500 g
Chloroform	500 mL
TNE buffer	400 mL

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

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

Phenol	500 g
Chloroform	500 mL
Distilled water	400 mL

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

Rnase (Hintermann, 1981)

Tris-HCl (pH 7.5)	20mM
EDTA	2 mM
Rnase A	2 mg/mL

The solution was heated 10 minutes at 100 ˚ C and stored at –20 ˚ C.

Proteinase K (Hintermann, 1981)

Proteinase K	5 mg
TE buffer	1 mL

Incubated at room temperature for 15 minutes, stored in 1 mL aliquots at –20 ˚ C.

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	100 mL

Tris-EDTA Buffer (TE)

Tris-HCl (1 M, pH 8.0)	10 mL
EDTA (0.4 M, pH 8.0)	5 mL
Distilled water added to	1000 mL

TNE Buffer (Hintermann, 1981)

Tris-HCl (1 M, pH 8.0)	50 mM
EDTA	5 mM
NaCl	100 mM

Lysis Solution

0.3 M NaOH

2% SDS

TSE Buffer (Hintermann, 1981)

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

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

X-Gal	20 mg
Dimethylformamide	1 mL

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

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
Distilled water	1 mL

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

SSC (20 X)

NaCl	175.32
Sodium citrate dihydrate	88.23

Sterilized at 121 ˚C for 15 minutes.

Buffer A

100 mM Tris-HCl

600 mM NaCl

pH adjusted to 7.5

Prehybridization and hybridization buffer

5X SSC

0,1 % (w/v) SDS

5 % (w/v) dextran sulphate (Sigma D-6001)

20 fold dilution of liquid block (supplied by ECL kit)

All components are combined and made up to the required volume. Gentle heating and stirring is required to dissolve all components. Stored at –20 ˚C in 25 mL aliquots.

Wash solution 1

1x SSC

0,1 % (w/v) SDS

Wash solution 2

0.5 x SSC

0,1 % (w/v) SDS

Prewashing solution

5 x SSC

0.5 % (w/v) SDS

1 mM EDTA (pH 8.0)

APPENDIX C

CHEMICALS AND SUPPLIERS

Chemicals

Luria Broth	Sigma
Agar	Difco
Glucose	Sigma
Sucrose	Merck
Maltose	Merck
K_2SO_4	Merck
$MgCl_2.6H_2O$	Amnesco
Yeast Extract	Difco
Casaminoacids	Difco
KH_2PO_4	Merck
$CaCl_2.2H_2O$	Sigma
$ZnCl_2$	Sigma
$FeCl_2.6H_2O$	Sigma
$CuCl_2.2H_2O$	Sigma
$MnCl_2.4H_2O$	Sigma

$Na_2B_4O_7.10H_2O$	Sigma
$(NH_4)_6.Mo_7O_{24}.4H_2O$	Sigma
NH_4Cl	Sigma
$Na_2HPO_4.7H_2O$	Sigma
$MgSO_4$	Sigma
L-Proline	Sigma
TES	Sigma
Tryptic Soy Broth	Difco
Agarose	Sigma
Ethidium Bromide	Sigma
EDTA	Sigma
NaOH	Merck
Glycerol	Merck
Ethanol	TEKEL
Isopropanol	Merck
Isoamyl alcohol	Merck
SDS	Merck
Polyethylene glycol 6000-8000	Sigma
Phenol	Merck
Chloroform	Merck
Dextran sulphate	Sigma
Sodium Acetate	Merck
Glacial Acetic Acid	Merck
$CaCl_2$	Sigma

Sodium Citrate dihydrate	Merck
Tris-HCl	Merck
NaCl	Merck
Tween-20	Merck
Tris Base	Sigma
X-Gal	MBI Fermentas
IPTG	MBI Fermentas
Bovine Serum Albumin	Sigma
Dimethylformamide	Merck

Enzymes

RNase A	Sigma
Lysozyme	Sigma
T4 DNA Ligase	Promega
T4 DNA Ligase	MBI Fermentas
Proteinase K	Sigma
Calf-intestinal alkaline phosphatase	Promega
*Bam*HI	MBI Fermentas
*Sau*3A	Sigma
*Eco*RI	MBI Fermentas
*Eco*RV	MBI Fermentas
*Hind*III	Sigma
*Pst*I	MBI Fermentas
*Sph*I	MBI Fermentas
Taq DNA Polymerase	MBI Fermentas

DNA size markers

GeneRuler 100 bp DNA Ladder Plus MBI Fermentas

The DNA Ladder yields the following 14 discrete fragments (in base pairs):

3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100.

*Pst*I digested Lambda DNA MBI Fermentas

The DNA Marker yields the following 29 discrete fragments (in base pairs):

11501, 5077, 4749, 4507, 2838, 2556, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 200, 164, 150, 94, 87, 82, 15.

Kits

ECL Random Prime Labelling And Detection System	Amersham
DNA Extraction Kit	Qiagen
Plasmid isolation Kit	Qiagen
p-GEM T Vector	Promega
Nylon Membranes	Amersham