

GENETIC ENGINEERING OF GLYCOLYTIC PATHWAY BY DISRUPTING
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE GENE IN AN
INDUSTRIAL STRAIN OF *STREPTOMYCES CLAVULIGERUS*

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INDUSTRIAL STRAIN OF *STREPTOMYCES CLAVULIGERUS***

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ABSTRACT

GENETIC ENGINEERING OF GLYCOLYTIC PATHWAY BY DISRUPTING GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE GENE IN AN INDUSTRIAL STRAIN OF *STREPTOMYCES CLAVULIGERUS*

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Streptomyces clavuligerus is a gram-positive, filamentous bacterium which produces several important secondary metabolites, including isopenicillin N, cephamycin C and the β -lactamase inhibitor, clavulanic acid (CA). CA is being used in combination with commonly used β -lactam antibiotics in order to fight against bacterial infections that are resistant to such antibiotics. Glyceraldehyde-3-phosphate (GAP) which is an intermediate product of glycolytic pathway is also used in CA biosynthesis as a crucial substrate. In this study, *S. clavuligerus gap1* encoding GAP dehydrogenase enzyme was targeted to canalize all produced GAP to CA biosynthetic pathway. In this way, a significant improvement of CA yield of an industrial strain of *S. clavuligerus* was aimed at. In the present study, *gap1* amplified from *S. clavuligerus* genome was cloned into *E. coli*, and then disrupted by kanamycin resistance gene (*aphII*) cassette. The construct was cloned into the industrial strain; however, any *gap1*-disrupted mutant resulted from homologous recombination couldn't be obtained following plasmid curing/selection process. However, for such metabolic engineering studies, a faster and more efficient plasmid curing technique was developed for *S. clavuligerus* which can be used for selection of disrupted mutants. For further study, after *gap1::aphII* mutant of *S. clavuligerus* is obtained, its CA yield will be compared with that in the parental strain through fermentation experiments.

Keywords: *Streptomyces clavuligerus*, clavulanic acid, glyceraldehyde-3-phosphate dehydrogenase, gene disruption, homologous recombination

ÖZ

GLİKOLİTİK YOLAKTAKİ GLİSERALDEHİD 3-FOSFAT DEHİDROGENAZ GENİNİN BLOKASYONU YOLUYLA ENDÜSTRİYEL BİR *STREPTOMYCES* *CLAVULİGERUS* SUŞUNUN GENETİK MANİPÜLASYONU

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Gram-pozitif, filamentöz bir bakteri olan *Streptomyces clavuligerus*, biyoteknolojik açıdan önemli izopenisilin N, sefamisin C ve β -laktamaz inhibitörü olan klavulanik asit (KA) gibi birçok sekonder metaboliti sentezleme yeteneğine sahiptir. KA, yaygın olarak kullanılan β -laktam antibiyotiklerine dirençli bakteriyel enfeksiyonların tedavisinde bu antibiyotikler ile bir arada kullanılmaktadır. Glikolitik yolda ara ürün olarak ortaya çıkan gliseraldehid 3-fosfat (GAP) klavulanik asit biyosentezinde önemli bir substrat olarak kullanılmaktadır. Bu çalışmada, hücrede üretilen GAP moleküllerinin KA biyosentetik yolağına yönlendirilmesi için GAP dehidrogenaz enzimini kodlayan *gap1* geni hedef alınmıştır. Bu yolla, endüstriyel bir *S. clavuligerus* suşunun KA veriminin önemli ölçüde artırılması amaçlanmıştır. *S. clavuligerus* genomundan çoğaltılan *gap1* geni *E. coli*'ye klonlandıktan sonra kanamisin dirençliliği sağlayan *aphII* gen kaseti ile bloklanmıştır. Bu yapı endüstriyel suşa klonlanmış, ancak homolog rekombinasyon sonucu *gap1*-bloklı mutantları plasmid giderme/seçilim işlemi ile elde edilememiştir. Ancak, bu tip *S. clavuligerus* metabolik mühendisliği çalışmalarında kullanılmak üzere, mutant seçiminde kullanılacak hızlı ve etkili bir plasmid giderme tekniği geliştirilmiştir. Çalışmanın devamında, *gap1*-bloklı mutant elde edildikten sonra fermentasyon deneyleri ile endüstriyel suşa kıyasla KA verimliliği karşılaştırılacaktır.

Anahtar Kelimeler: *Streptomyces clavuligerus*, klavulanik asit, gliseraldehid 3-fosfat dehidrogenaz, gen blokasyonu, homolog rekombinasyon

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
NRRL	Agricultural Research Service Collection
bp	Base pair
dH ₂ O	Distilled water
dNTP	Deoxynucleotide Triphosphate
ORF	Open Reading Frame
OD	Optical Density
rpm	Revolution per minute
kb	Kilo base
RT	Room temperature
spp.	Species
CA	Clavulanic acid
GAP	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
WHO	World Health Organization
LB	Luria Broth
LA	LB Agar
TSB	Tryptic Soy Broth
TSA	TSB Agar
EtBr	Ethidium Bromide

CHAPTER 1

INTRODUCTION

1.1. Antibiotics

Antibiotics (from the Ancient Greek: anti means “against”, bios means “life”) are substances or compounds that kill bacteria or inhibit their growth. They are secondary metabolites mostly synthesized by bacteria, filamentous fungi and plants (Fabbretti *et al.*, 2011). Antibiotics increase survivability of the producer against microorganisms in the same ecological niche by eliminating them from competition for food and space (Vining, 1990; Fisher *et al.*, 2005).

The practical importance of antibiotics is tremendous. They are widely used in human therapy, veterinary, agriculture, scientific research and several other areas (Berdy, 2005). Antibiotics are used to prevent infections after surgery or at open wounded areas (Cimochowski *et al.*, 2001) or they are used for elimination of pathogens and other harmful microorganisms. At veterinary medicine, animals are protected by antibiotics from diseases (Gustafson and Bowen, 1997). Antibiotic usage increases yield in agriculture by preventing harmful microorganisms from damaging plants (McManus *et al.*, 2002).

1.2. Classification of antibiotics and action mechanisms

Antibiotics are categorized based on their mechanism of action, chemical structure or spectrum of activity. Bacterial functions or growth processes are main targets of antibiotics (Calderón *et al.*, 2007). Antibiotics that target bacterial cell wall (penicillins, cephalosporins), or cell membrane (polymixins) causes bacterial cells to burst due to osmotic pressure or to leak out cell contents (bacteriolytic); thereby killing them. Antibiotics that interfere with essential bacterial enzymes (quinolones, sulfonamides) are usually bactericidal. Rifampin inhibits nucleic acid biosynthesis. Aminoglycosides, macrolides, erythromycin and tetracyclines target protein synthesis, and are usually bacteriostatic (Finberg *et al.*, 2004). Another type of classification is based on their target specificity: Narrow-spectrum antibiotics target particular types of bacteria, such

as gram-negative or gram-positive bacteria, whereas broad-spectrum antibiotics affect a wide range of bacteria (Walsh, 2003). Figure 1.1 shows targets of antibiotics on a bacterial cell with specific examples.

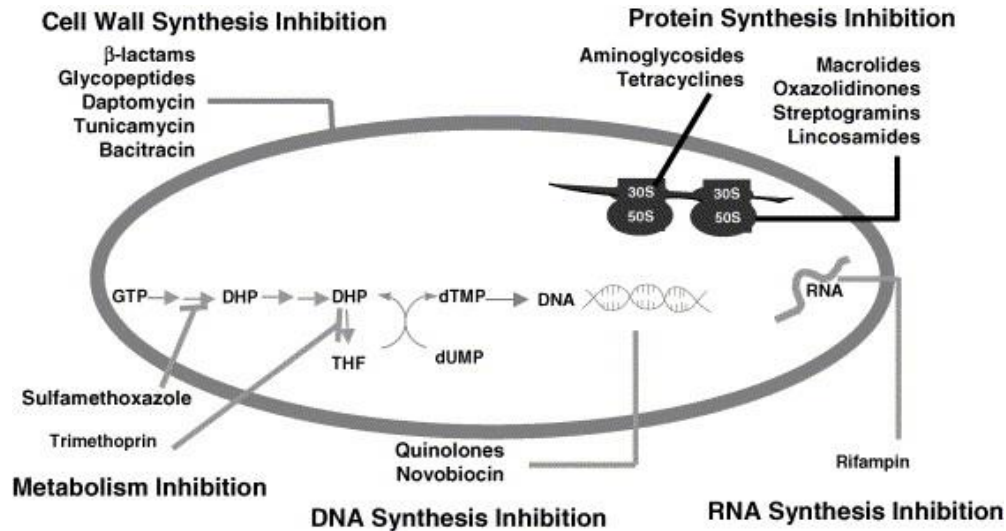


Figure 1.1. Targets of antibiotics on a bacterial cell (Singh and Barrett, 2006).

1.3. Antibiotics market and current situation

In October 2010, a comprehensive global report on antibiotics market “Antibiotics - A Global Strategic Business Report” was announced by Global Industry Analysts Incorporation. The global antibiotics market is forecast to reach \$40.3 billion by the year 2015. According to another report published online by Bccresearch; Antibiotic Resistance and Antibiotic Technologies: Global Markets, the market size is going to increase with 9.6% 5-year compound annual growth rate. This increase trend is driven by intensive research in new areas of treatment, favorable regulatory environment and emergence of new drug classes. Although the projections are optimistic, there is a potential threat which may affect the sector negatively. The emergence of antibiotic

resistance is becoming a more serious problem in that it decreases the effectiveness of antibiotics.

1.4. Antibiotic resistance

World Health Organization (WHO) defines antimicrobial resistance as resistance of a microorganism to an antimicrobial medicine to which it was previously sensitive (<http://www.who.int/mediacentre/factsheets/fs194/en>). Microorganisms can gain resistance to a stress condition by carrying a spontaneous or induced genetic mutation. In addition, genes providing this resistance feature can be transferred between microorganisms by conjugation, transduction or transformation (Usha *et al.*, 2010). Furthermore, the microorganism having the resistance gene increases in number by natural selection under specific stress conditions, such as antibiotic usage (Levy, 1994). Increased number of resistant microorganisms for certain antibiotics in a population causes those antibiotics become less effective in treatment of infections (Cowen, 2008).

There are four main mechanisms providing antibiotic resistance; (i) inactivation or modification of antibiotic molecules, (ii) altered antibiotic binding site of target, (iii) the use of alternative pathways rather than target pathway of antibiotic, and (iv) reduced accumulation of antibiotic inside the cell by decreasing influx and increasing efflux of the antibiotic (Fisher *et al.*, 2005; Tenover and Zhang, 2006; Zhang, 2008). Some bacteria have gained penicillin resistance by expressing β -lactamases that enzymatically deactivates the antibiotic. Another group of bacteria, such as methicillin-resistant *Staphylococcus aureus*, have gained resistance to the same antibiotic by the altered penicillin binding site (Lowy, 2003). On the other hand, some sulfonamide-resistant bacteria use folate uptake system as mammalian cells, so sulfonamides which target endogenous synthesis of folic acid fail to stop DNA synthesis (Sköld, 2000; Mayers, 2009). Furthermore, fluoroquinolone resistance is provided by a kind of transmembrane efflux protein which decreases intracellular quinolone concentration (Morita *et al.*, 1998).

Any antibiotic resistance might lead a biological cost to bacterial cells. In the absence of antibiotic, the resistant bacteria have reduced fitness when compared to nonresistant ones. The reduction in fitness can be expressed as reduced growth, virulence or transmission (Andersson, 2006).

1.5. β -lactam antibiotics and their importance

β -lactam antibiotics include a wide range of antibiotics all of which contains a β -lactam ring in their molecular structure (Figure 1.2). The first discovered antibiotic, penicillin, and its derivatives are all β -lactam antibiotics which is the most widely used group of

antibiotics. In 2003, it was reported that about 65% of the commercially available antibiotics in use were β -lactam compounds (Elander, 2003). In 2013, the sales of β -lactam antibiotics including penicillins, cephalosporins, cefoxitin, monobactams, clavulanic acid and carbapenems is already over \$20 billion per year (Ozcengiz and Demain, 2013).

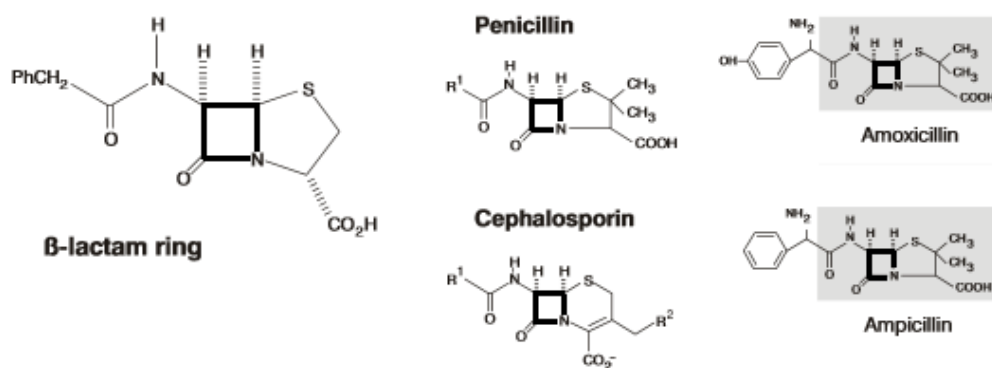


Figure 1.2. Core structure of β -lactam antibiotics (Bold region indicates β -lactam ring; Rosário and Grumach, 2006).

β -lactam antibiotics are highly specific for prokaryotes and have almost no effect on mammalian cells (Brakhage, 1998). Their target is cell wall synthesis in bacteria (Glazer and Nikaido, 1998). Peptidoglycans, main cell wall compounds, are synthesized from their monomers by transglycosylases as glycan strands outside of cell membrane. These glycan chains are attached to each other by crosslinks created by transpeptidases (also known as penicillin-binding proteins or PBPs). Therefore, the crosslinks creates a strong network to protect bacterial cells. The active sites of these transglycosylases and transpeptidases are the binding sites for β -lactam ring structure. This binding results in acylation of enzyme active sites which interferes crosslinking reaction. As a result of the absence of crosslinks and high internal osmotic pressure, the cell wall breaks apart (Walsh, 2000).

β -lactam antibiotics have been used since the first half of 20th century. Widespread use of these compounds for long time period has caused selective pressure in favor of β -lactam resistant bacteria (Blazquez *et al.*, 2002, Fisher *et al.*, 2005). As mentioned earlier, the β -lactam resistance mechanism has been developed in three ways; the decreased access to the bacterial cell, β -lactamase production to destroy antibiotic molecules, or altered penicillin-binding proteins (PBPs) such as low-affinity PBPs which fail to bind to antibiotic molecules (Williams, 1999). Low affinity PBPs are modified enzymes involved in cell wall synthesis (Paradkar *et al.*, 1996). Resistance due to β -lactamase production is more common than others (Fisher *et al.*, 2005). β -lactamase enzyme opens β -lactam ring thereby deactivating antibacterial properties of the target antibiotic (Figure 1.3).

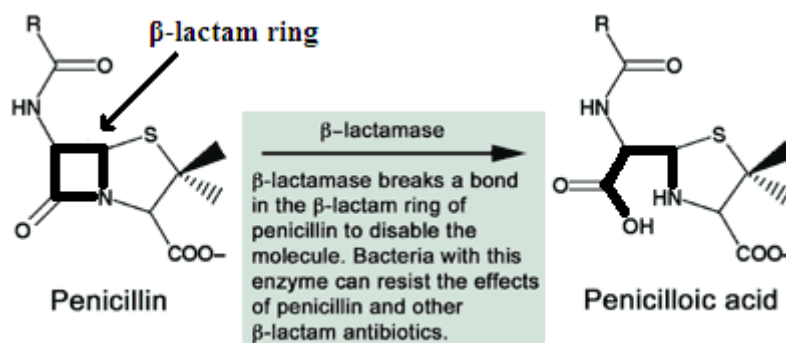


Figure 1.3. Opening of β -lactam ring of penicillin by β -lactamase (modified from http://www.wiley.com/college/pratt/0471393878/student/activities/bacterial_drug_resistance/).

β -lactamases are classified in four classes based on their mechanisms of action; class A (penicillinases), class B (metallo-enzymes), class C (cephalosporinases) and class D (oxacillinases) β -lactamases. A, C and D classes show a transient serine acylation/deacylation strategy and share a similar folding whereas β -lactamases of class B need zinc for their action (Ambler, 1980; Fisher *et al.*, 2005).

β -lactamase enzymes are either inhibited or destroyed by β -lactamase inhibitors. Although these inhibitors have insignificant degree of antimicrobial activity, they are potent inhibitors of β -lactamases. Due to their low antibiotic activity, they are combined with a β -lactam antibiotic to increase its effectiveness. These β -lactamase inhibitors, such as clavulanic acid, sulbactam, tazobactam bind to the β -lactamase enzymes more efficiently than the actual β -lactam antibiotic, so while the inhibitor keeps β -lactamases busy, the antibiotic can function properly without any degradation threat. Several antibiotic/beta-lactamase inhibitor combinations exist on the market; among them, sulbactam is usually combined with ampicillin (Unasyn), Tazobactam is usually combined with piperacillin (Zosyn), and clavulanic acid or clavulanate are usually combined with amoxicillin (Augmentin™) or ticarcillin (Timentin™).

1.6. Clavulanic acid

Clavulanic acid (CA) is a very strong β -lactamase inhibitor produced by *S. clavuligerus*. Although it has an intrinsic antimicrobial activity against a wide spectrum of bacteria due to the presence of a β -lactam ring, the strength of activity is not enough for effective use as an antibiotic (Brown, 1986; Lee *et al.*, 2002). Yet, there is no record of bacteria that produce only CA in the literature. Therefore, the production of this β -lactamase inhibitor along with other β -lactam antibiotics by *Streptomyces clavuligerus* (*S. clavuligerus*) proves its use as a β -lactamase inhibitor to compete with β -lactam resistant bacteria sharing the same environment (Challis and Hopwood, 2003).

CA is a member of serine type (or classes A, C, and D) β -lactamase. It is used as β -lactamase inhibitor against class A and D type β -lactamases, and it is ineffective against class B β -lactamases (Walsh, 2003). The potassium clavulanate form of CA is used in the compositions of Augmentin™ and Timentin™ antibiotic drugs currently in the market (Watve *et al.*, 2000; Santos *et al.*, 2009).

Clavulanic acid market exceed over \$1 billion, and Augmentin™ increased the market size above \$2 billion in 2003 (Ozcengiz and Demain, 2013).

1.7. β -lactamase inhibition mechanism by CA

CA has a bicyclic β -lactam ring with 3R, 5R stereochemistry according to X-ray studies which is a shared feature with penicillins (Howarth *et al.*, 1976; Brown *et al.*, 1984). This feature allows CA to be used as a substrate by β -lactamases (Liras and Rodríguez-García, 2000; Fisher *et al.*, 2005).

CA is a suicide inhibitor which means it binds to β -lactamases irreversibly by covalent bond. Serine hydroxyl group (Ser70) in the catalytic site of β -lactamases is the target of

CA. Hydrogen bonding between C7 of CA and Ser70 of β -lactamase is the initial step of a cascade of reactions (Figure 1.4). CA- β -lactamase binding occurs via acylation which opens β -lactam ring of CA. With the opening of the five-membered oxazolidinone ring, the inhibitor is linearized, and after isomerization reactions, a stable molecule is formed which is the irreversibly inhibited β -lactamase enzyme. Since CA occupies the active site of β -lactamase enzyme, the antibiotic can freely act on its targets without any threat of degradation by β -lactamases (Chen and Herzberg, 1992; Padayatti *et al.*, 2005).

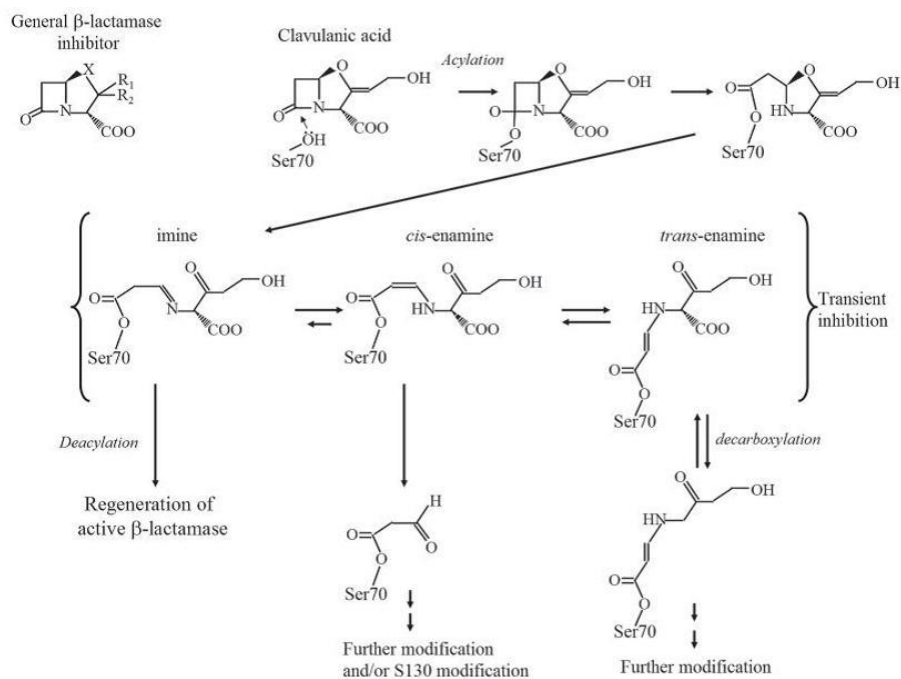


Figure 1.4. Inhibition mechanism of β -lactamases by CA (Padayatti *et al.*, 2005).

1.8. The genus *Streptomyces*

Members of *actinomycete* genera are gram positive bacteria with high GC content in their DNA (Kieser *et al.*, 2000). They are well known for production of a wide range of secondary metabolites like antibiotics, antitumor compounds, immunosuppressants, herbicides, antiviral and antiparasitic agents. There are 23,000 biologically active secondary metabolites produced by microorganisms has been identified up to now, and 10,000 of them are produced by the order of *Actinomycetes*. *Streptomyces* spp. produce 7,600 of these 10,000 secondary metabolites (Sacramento *et al.*, 2004; Olano *et al.*, 2008). Streptomycin, chloramphenicol, aminoglycoside, macrolides, anthracyclines, tetracyclines and β -lactam antibiotics are examples of these compounds mostly produced by *Streptomyces* spp. (Goodfellow *et al.*, 1988).

Streptomyces spp. are filamentous, spore forming and strictly aerobic bacteria which belong to *Actinomycetes* order (Paradkar *et al.*, 2003). High GC content genome (more than 70 mole %) and large linear plasmids (10-600 kb) are distinctive features of *Streptomyces* species (Kieser *et al.*, 2000, Paradkar *et al.*, 2003). The classification of *Streptomyces* according to Bergey's Manual of Systematic Biology, 2004 is shown below (Table 1.1).

Table 1.1. The classification of *Streptomyces* (Bergey's Manual of Systematic Biology, 2004)

Classification

Domain <i>Bacteria</i>	OrderI <i>Actinomycetales</i>
Phylum BXIV <i>Actinobacteria</i>	Suborder XIV <i>Streptomycineae</i>
Class I <i>Actinobacteria</i>	Family I <i>Streptomycetaceae</i>
Subclass V <i>Actinobacteridae</i>	Genus I <i>Streptomyces</i>

Streptomyces life cycle involves many steps of morphological changes (Figure 1.5). *Streptomyces* forms mycelium of branched hyphae by tip extension (Flärdh, 2003). Due to this structure of growth, they look like filamentous fungi morphologically rather than bacteria. Productivity in large scale fermentations depends on the characteristic mycelial growth by improving their industrial value as secondary metabolite producers (Flärdh, 2003).

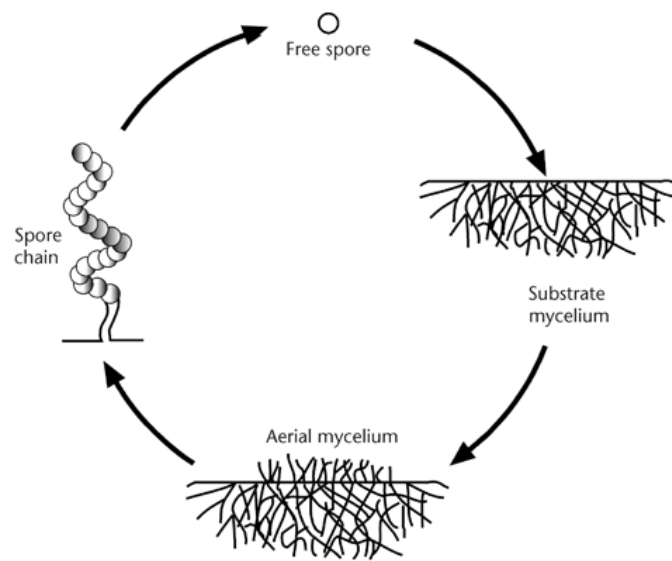


Figure 1.5. The lifecycle of *Streptomyces* (modified from Trujillo, 2008).

The life cycle starts with germination of spore. In vegetative growth phase, a complex network of multinucleoid filaments creates the hyphae or substrate mycelium (Manteca *et al.*, 2008). The next phase is the formation of aerial hyphae by breaking the surface barrier (Goriely and Tabor, 2003). In this phase, secondary metabolites are synthesized by cultures grown in/on solid media (Chater, 1989). Extensive cellular divisions in the aerial phase result in formation of uni-nucleoidal compartments from which *Streptomyces* spores are produced (Goriely and Tabor, 2003).

Secondary metabolite production of *Streptomyces* is strictly related and regulated with morphological changes (Paradkar *et al.*, 2003). Low specific growth rate causes signals which activate a cascade of regulatory events resulting in chemical and morphological differentiation (Jonsbu *et al.*, 2002). Antibiotic production starts with stationary phase in liquid culture and aerial mycelium formation on solid media (Rodríguez *et al.*, 2008; Bibb, 2005).

Restriction modification system of *Streptomyces* spp. generates a strong restriction barrier which makes these cells hardly transformable. This feature prevents DNA from non-*Streptomyces* sources to be taken into *Streptomyces* cells (Matsushima *et al.*, 1987).

1.9. *S. clavuligerus*: a biologically important bacterium

S. clavuligerus was first isolated from South American soil samples and described as a cephalosporin producer. The word “clavuligerus” means “bearing little clubs” which comes from club-like side chains of the bacteria. It was recorded to Agricultural Research Service Collection and American Type Culture Collection as NRRL 3585 and ATCC 27064 respectively (Higgins and Kastner, 1971).

S. clavuligerus is known to produce 21 secondary metabolites (Ortiz *et al.*, 2007) including holomycin; a member of the pyrrothine class antibiotics, an antibiotic related to tunicamycin; a glucosamine-containing antibiotic (Kenig and Reading, 1979), and β -lactam metabolites with antibiotic, antifungal and β -lactamase-inhibitory activities, which is why it has been studied over three decades (Thai *et al.*, 2001). Two major groups of β -lactam products are sulfur containing and oxygen containing compounds. Isopenicillin N, desacetoxycephalosporin C and cephamycin C are examples of sulfur containing compounds which have antibiotic activity, and five clavam compounds including alanylclavam (antibacterial and antifungal activities) and CA are oxygen containing compounds (Paradkar and Jensen, 1995). Besides, BLIP (β -lactamase inhibitory protein), BLP (BLIP-homologous protein), a CA sensitive β -lactamase, and non- β -lactam antibiotics such as N-propionylholothin, tunicamycin and holomycin (antitumor activity) are also produced by *S. clavuligerus* (Okamura *et al.*, 1977; Kenig and Reading, 1979; Santamarta *et al.*, 2002).

Genome sequence of *S. clavuligerus* NRRL3585 has been published recently (Song *et al.*, 2010). On the other hand, *S. clavuligerus* ATCC 27064 genome has been published by another group (Medema *et al.*, 2010). Identification of numerous cryptic secondary metabolite gene clusters with unknown functions by sequencing of *Actinomycetes* genomes could allow production of new antibiotics to be used clinically (Zerikly and Challis, 2009).

1.10. CA gene cluster in *S. clavuligerus*

CA gene cluster of *S. clavuligerus* is found adjacent to cephamycin C gene cluster in its genome, and both gene clusters are arranged as a 60 kb supercluster (Ward and Hodgson, 1993; Kurt *et al.*, 2013; Ozcengiz and Demain, 2013) (Figure 1.6).

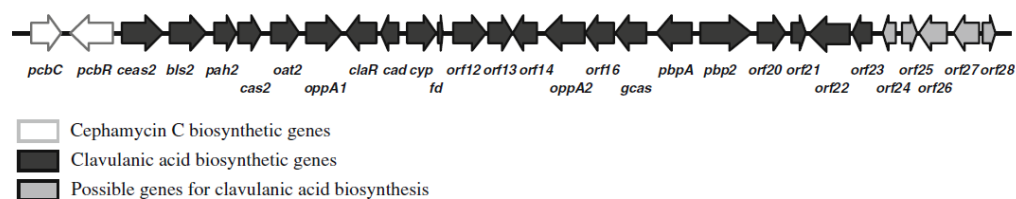


Figure 1.6. CA biosynthetic gene cluster of *S. clavuligerus* (Song *et al.*, 2010).

According to several studies, more than 22 genes are responsible for the biosynthesis, transport, and regulation of CA (Hodgson *et al.*, 1995; Jensen *et al.*, 2000; Li *et al.*, 2000; Mellado *et al.*, 2002; Jensen *et al.*, 2004; Song *et al.*, 2010). Open reading frames of CA gene cluster and their functions are listed in Table 1.2. The roles of some of these genes have not been identified yet. Several genes involved in the biosynthesis of CA are duplicated in *S. clavuligerus* genome. The paralog gene cluster is on a large plasmid pSCL4 (Song *et al.*, 2010; Medema *et al.*, 2010; Kurt *et al.*, 2013).

Table 1.2. Open reading frames of CA gene cluster and their functions

Gene	Gene Product- Putative Function	Reference
<i>ceaS</i>	N2-(2-carboxyethyl) arginine synthase	(Khaleeli <i>et al.</i> , 1999)
<i>bls2</i>	β -lactam synthase	(Bachman <i>et al.</i> , 1998; McNaughton <i>et al.</i> , 1998)
<i>pah2</i>	proclavamate amidinohydrolase	(Wu <i>et al.</i> , 1995)
<i>cas2</i>	clavamate synthase	(Elson <i>et al.</i> , 1987; Baldwin <i>et al.</i> , 1990; Salowe <i>et al.</i> , 1990; Marsh <i>et al.</i> , 1992)
<i>oat2</i>	ornithine acetyltransferase	(Kershaw <i>et al.</i> , 2002)
<i>oppA1</i>	oligopeptide binding protein	(Hodgson <i>et al.</i> , 1995; Jensen <i>et al.</i> , 2000; Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004; Lorenzana <i>et al.</i> , 2004)
<i>clar</i>	ClaR regulatory protein (DNA binding protein)	(Paradkar <i>et al.</i> , 1998; Pérez-Redondo <i>et al.</i> , 1998)
<i>cad</i>	clavulanic acid dehydrogenase	(Nicholson <i>et al.</i> , 1994)
<i>cyp</i>	P450 mono-oxygenase	(Jensen <i>et al.</i> , 2000; Li <i>et al.</i> , 2000; Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004)
<i>fd</i>	ferredoxin	(Li <i>et al.</i> , 2000; Jensen <i>et al.</i> , 2004)
<i>orf12</i>	acetyl transferase	(Li <i>et al.</i> , 2000; Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004)
<i>orf13</i>	efflux pump	(Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004)
<i>orf14</i>	acetyltransferase	(Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004)
<i>oppA2</i>	oligopeptide binding protein	(Hodgson <i>et al.</i> , 1995; Jensen <i>et al.</i> , 2000; Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004; Lorenzana <i>et al.</i> , 2004)
<i>orf16</i>	hypothetical protein	(Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004)
<i>gcas</i>	N-glycyl-clavaminic acid synthetase	(Arulanantham <i>et al.</i> , 2006)

Table 1.2 (continued)

<i>pbpA</i>	PBP	(Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004)
<i>pbp2</i>	PBP	(Mellado <i>et al.</i> , 2002; Jensen <i>et al.</i> , 2004)
<i>orf21</i>	Sigma factor	(Song <i>et al.</i> , 2009; Jnawali <i>et al.</i> , 2011)
<i>orf22/23</i>	Two component CA positive regulator	(Jnawali <i>et al.</i> , 2008; Song <i>et al.</i> , 2009)

1.11. CA biosynthesis in *S. clavuligerus*

All steps of CA biosynthesis with enzyme, gene, substrate and product names are represented in Figure 1.7. The first step of CA biosynthesis is condensation of L-arginine and glyceraldehyde-3-phosphate (GAP, a 3-carbon intermediate of glycolysis) by carboxyethylarginine synthase (CEAS) (Khaleeli *et al.*, 1999). The product of this reaction (N2-(2-carboxyethyl) arginine) is used as substrate by β -lactam synthase (BLS) to produce deoxyguanidinoproclavaminic acid which is a monocyclic intermediate (Bachman *et al.*, 1998, McNaughton *et al.*, 1998). Clavamate synthase (CAS) catalyzes a hydroxylation reaction to form guanidinoproclavaminic acid (Baldwin *et al.*, 1993; Paradkar *et al.*, 1998). Proclavaminic acid is obtained by removal of guanidine group from guanidinoproclavaminic acid with catalysis of proclavamate amidinohydrolase (PAH) (Wu *et al.*, 1995). Clavaminic acid formation occurs as a two-step reaction catalyzed by clavamate synthase (CAS) to form oxazolidine rings (Elson *et al.*, 1987; Salowe *et al.*, 1990; Baldwin *et al.*, 1990). Clavaminic acid is the common intermediate for both 5S clavams and CA. N-glycyl-clavaminic acid synthetase (GCAS) converts clavaminic acid into N-glycyl-clavaminic acid via an ATP dependent reaction. Although the reactions between N-glycyl-clavaminic acid to clavulanate-9-aldehyde are still unknown, a double epimerization and an oxidative deamination reaction are predicted for this conversion. Clavulanate-9-aldehyde is a highly unstable intermediate, and CA dehydrogenase (CAD) reduces this molecule to stable form (CA) by a NADPH dependent reaction (Song *et al.*, 2010). The path from clavaminic acid to CA includes modification of the side-chain and inversion of ring stereochemistry from to 3S, 5S to 3R, 5R. For β -lactamase inhibitor activity, the inversion is crucial. Other *S. clavuligerus* clavams lacks this activity due to their 5S configuration (Ozcengiz and Demain, 2013).

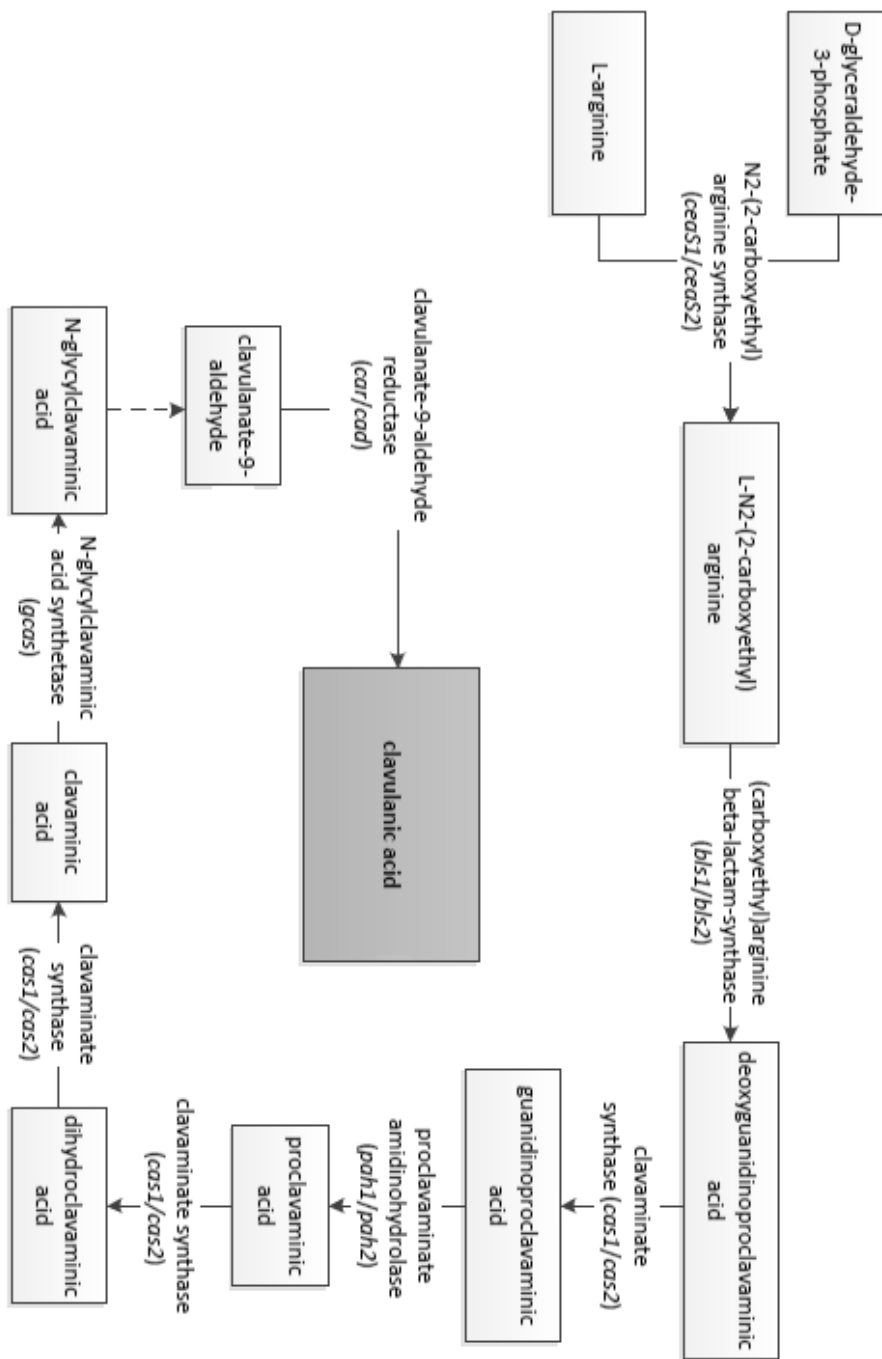


Figure 1.7. CA biosynthesis pathway (Adapted from; Mellado *et al.*, 2002; Tahlan *et al.*, 2007; Liras *et al.*, 2008, Zelyas *et al.*, 2008; Jnawali *et al.*, 2011; <http://www.genome.jp/kegg/pathway/map/map00331.html>).

1.12. Regulation of CA production

Several factors affect the production yield of CA. First of all, mechanisms initiating and regulating secondary metabolite production have the highest impact for CA production. Enzyme activities of metabolic and structural pathways which provide intermediates or a suitable environment for CA production are also crucial for high CA production yields. Another key element in the regulation of CA biosynthesis is nutritional conditions in the environment. As the last regulatory point, the efficiency of transport of all elements required for CA production inside cell should also be mentioned (Khetan *et al.*, 1999).

CcaR and ClaR are SARP (Streptomyces antibiotic regulatory protein) type positive regulators of CA biosynthesis. CcaR controls antibiotic biosynthesis in *S. clavuligerus* by regulating the transcription of biosynthetic genes (Kurt *et al.*, 2013). The *ccaR*-disrupted *S. clavuligerus* mutants are unable to produce cephamycin C and clavulanic acid, and the functionality is restored upon trans-complementation. Carboxyethyl-arginine-synthase (*ceaS2*) expression, which produces the precursor for CA biosynthesis, almost stops at *ccaR*-disrupted mutants. CcaR acts on promoter of *ceaS2*, and its coding enzyme is lost at *ccaR* mutants (Tahlan *et al.*, 2004). CcaR regulates early genes of CA biosynthesis through this promoter due to polycistronic expression. Deletion of 688 bp of intergenic *cmcH-ccaR* region showed that CA production decreased 30-40%, which means this region is not essential for *ccaR* expression, but enhances the expression (Kurt *et al.* 2013). CcaR is an autoregulatory protein which binds to its own promoter as well as acts on *claR* expression, so indirectly regulates CA production (Santamarta *et al.*, 2011; Kurt *et al.*, 2013). ClaR is a transcriptional activator of LysR family, and strictly required for CA production. It acts on expression of late genes of CA biosynthesis because clavaminic acid, the last intermediate in the pathway, is accumulated in *claR*-null mutants. Oligopeptide permease (*oppA1*), clavulanic-9-aldehyde reductase (*car*) and a cytochrome P450 (*cyp*) are other genes regulated by ClaR (Santamarta *et al.*, 2002; Liras *et al.*, 2008; Santamarta *et al.*, 2011).

Expression of *ccaR* is *bldG*-dependent, so *bldG* has higher place above *ccaR* and *claR* in the antibiotic regulatory cascade in *S. clavuligerus*. This gene encodes an anti-anti-sigma factor which regulates both morphological differentiation and secondary metabolite production in *S. clavuligerus*. Anti-anti-sigma factors works with an anti-sigma factor regulating the activity of the target sigma factor by a series of phosphorylation/dephosphorylation reactions on conserved serine residues (Liras *et al.*, 2008; Ozcengiz and Demain, 2013).

Some small diffusible signal molecules like γ -butyrolactone A-factor are produced in accordance with certain physiological or environmental signals. These factors are part of quorum sensing mechanism. These signaling molecules are autoregulators and important in initiation of antibiotic production in *Streptomyces* (Horinouchi and Beppu, 1994). Butyrolactone receptor protein (encoded by *brp* gene) has a repressor role on

CA and cephamycin production since *brp* null mutants of *S. clavuligerus* are also overproducers of these products (Liras *et al.*, 2008). Binding of these molecules to their cytoplasmic receptors activates a cascade to inhibit specific DNA targets, which encode SARP proteins. Due to repressor function of these proteins, γ -butyrolactone removes repression and allows expression of target genes (Ozcengiz and Demain, 2013).

Nutritional regulation mechanisms of CA biosynthesis are nitrogen metabolite regulation, carbon catabolite repression, phosphate regulation, and induction. Antibiotic production decreases with the decay of antibiotic synthetases, feedback inhibition, and repression of these enzymes (Saudagar *et al.*, 2008)

It was reported that increase in total nitrogen concentration results in higher production of CA (Butterworth, 1984; Gouveia *et al.*, 1999).

Streptomyces clavuligerus cannot use glucose as a carbon source because it lacks a glucose transport system on cell membrane. It was observed that glucose utilizing mutants can be obtained by adding genes related to glucose uptake system (Aharonowitz and Demain, 1978; Garcia-Dominguez *et al.*, 1989). Due to this inability of utilizing glucose, previous reports showing increased secondary metabolite production in other organisms with excess glucose usage is not applicable for *S. clavuligerus* (Saudagar *et al.*, 2008). It can use lipids as carbon source for production of CA (Butterworth, 1984). According to a study for the utilization of vegetable oil in CA production, the medium with soybean oil and starch gave the best CA yield (Maranesi *et al.*, 2005).

Glycerol addition to medium increases both CA yield and production time interval in batch culture (Chen *et al.*, 2002), but high concentrations of glycerol, glutamic acid, ammonium and phosphate in the medium inhibit biosynthesis of CA (Romero *et al.*, 1984). On the other hand, the absence of glycerol prevents CA production which shows that glycerol is essential for CA biosynthesis (Saudagar *et al.*, 2008).

Phosphate is a growth-limiting nutrient and affects antibiotic synthesis. Growth-limiting concentrations of inorganic phosphate are used for industrial antibiotic production. Non-growing, antibiotic producing cells can be converted back to growing, non-producing state by addition of phosphate to the medium (Liu *et al.*, 1975). The inhibitory effect of phosphate on CA production was previously reported (Aharonowitz and Demain, 1978; Romero *et al.*, 1984; Lebrihi *et al.*, 1987).

Another factor which determines CA yield is the availability of precursor molecules, such as arginine, ornithine and lysine (Saudagar *et al.*, 2008). CA production increases with increased arginine concentration in the medium, but at a certain point, excess arginine becomes ineffective due to the limited availability of 3-carbon precursor which makes it a rate limiting molecule (Ives and Bushell, 1997). Ornithine can be

converted to arginine which can be used at CA biosynthesis (Kershaw *et al.*, 2002). Although ornithine is not an actual precursor of CA, it is more effective than arginine on stimulation of CA biosynthesis pathway (Chen *et al.*, 2003).

1.13. Strain improvement for CA production

Random mutagenesis and selection techniques have been traditionally used to improve CA yields in industry. With increasing knowledge about the biosynthesis and regulation of CA production, target specific genetic engineering methods have been used more commonly instead of random mutagenesis (Paradkar *et al.*, 2001). Addition of extra copies or increasing expression of CA biosynthetic genes can increase CA yields in a more rational way. The mutants of *S. clavuligerus* with amplified *ceaS2* gene doubled the amount of CA production (Pérez-Redondo *et al.* 1999). In addition, introduction of extra copies of *ccaR* positive transcriptional regulator gene into *S. clavuligerus* resulted in about 3 fold increase in CA yield (Pérez-Llarena *et al.*, 1997; Kurt *et al.*, 2013). *S. clavuligerus* strain carrying *claR* gene on multicopy plasmid produced CA at a rate double the amount of wild type (Pérez-Redondo *et al.* 1998). Overexpression of both *ccaR* and a rate limiting enzyme CAS2 in *S. clavuligerus* increased CA yield 23.8 fold which is the highest improvement (Hung *et al.*, 2007, Song *et al.*, 2010). Mellado *et al.* (2002) reported that introduction of extra copies of CA biosynthetic genes; *cyp*, *fd*, *orf12*, and *orf14* affected CA yield positively in *S. clavuligerus*. Overproduction of AdpA, a pleiotropic regulator which is responsible for morphological differentiation and antibiotic production enhances CA production of *S. clavuligerus* almost 2 fold (López-García *et al.* 2010). Deletion of the *lat* gene involved in cephamycin C biosynthesis causes carbon flux into CA biosynthesis. This led to cessation of cephamycin C production while increasing CA yield 2- to 2.5-fold. Elimination of *cvm1* in these mutants also increased CA yield 10% (Paradkar *et al.*, 2001). In a similar way, rate limiting CA precursor glyceraldehyde-3-phosphate (GAP) which is 3-carbon intermediate of glycolytic pathway was totally canalized into CA biosynthesis by disruption of *gap1* encoding GAP dehydrogenase and by producing pyruvate from glycerol from an alternative pathway to bypass glycolysis (Figure 1.8). This metabolic engineering approach increased CA yield 2 fold. Furthermore, these mutants grown on arginine rich medium showed increased CA production a further threefold of the wild type strain (Li and Townsend, 2006). Moreover, overexpression of *ccaR* and *claR* regulators in this mutant further increased CA yield about 2.5 and 6 fold, respectively (Jnawali *et al.*, 2010). Additional copies of glycerol utilization gene cluster (*gylR-glpF1K1D1*) increased the yield of glycerol utilization in *S. clavuligerus* mutants which led to increase in CA yield 7.5 fold (Baños *et al.*, 2009). *orf21*, putative sigma factor overexpression in *S. clavuligerus* NRRL 3585 showed 1.4-fold increase whereas integration of *ceaS2*, *bls2*, *cas2* and *pah2* into same strain resulted in 8.7 fold improvement (Jnawali *et al.*, 2011; Ozcengiz and Demain, 2013).

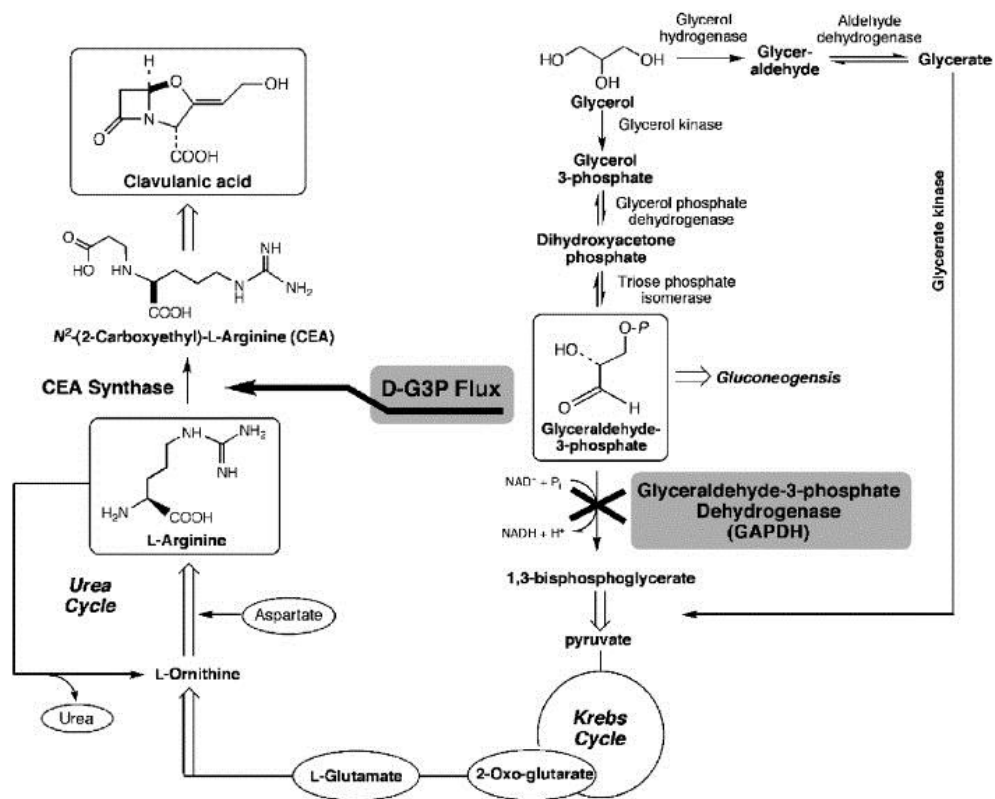


Figure 1.8. Flow of all GAP into CA biosynthesis upon disruption of GAPDH gene (Li and Townsend, 2006).

1.14. Present Study

S. clavuligerus is a well-known CA producer used in industry. With an inspiring knowledge about the stimulatory effect of increased flow of glyceraldehyde-3-phosphate to clavulanic acid biosynthesis pathway from glycolytic pathway of *S. clavuligerus* by the disruption of *gap1* (Li and Townsend, 2006) (Figure 1.8), this study aims to replace disrupted *gap1* with the original copy in the genome of an industrial CA overproducer strain of *S. clavuligerus* by means of homologous recombination in order to further increase its clavulanic acid yield. This would be a more direct and rational approach of strain improvement when compared to random mutagenesis, commonly used in industry.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains, culture conditions and plasmids

Bacterial strains and plasmids used in this study are shown in Table 2.1. *Escherichia coli* (*E. coli*) cultures were grown in either Luria Broth liquid medium (LB, Appendix A) on a rotary shaker (200 rpm) or on agar plates (LA, Appendix A) at 37 °C. *E. coli* was maintained on agar plates at 4 °C for up to 2 weeks or stored in -80 °C for a year after 30 sec centrifugation at 13,200 rpm and dissolved in 50% glycerol. *S. clavuligerus* cultures were grown in either Tryptone Soy Broth (TSB, Appendix A) in baffled flasks on a rotary shaker (220 rpm) or on agar medium (TSA, Appendix A) at 28 °C. *S. clavuligerus* mycelium was mixed with 50% glycerol with 1:1 ratio and stored at -80 °C for long term maintenance. *S. clavuligerus* spores were produced from vegetative cells after incubation on sporulation medium (Appendix A) at 28 °C for 4-6 days.

Table 2.1. Bacteria and plasmids used in this study

Strains and Plasmids	Description	Source and Reference
Strains		
<hr/>		
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF) U169 supE44 λ thi-1 gyrA recA1 relA1 endA1 hsdR17	<i>E. coli</i> Genetic Store Center

Table 2.1. (continued)

ET12567	F <i>dam</i> ⁻ 13::Tn9 <i>dcm</i> -6 <i>hsdM</i> <i>hsdR</i> <i>lac</i> YI	Prof. Keith Chater, John Innes Centre, UK
IJgneo	DH5α containing <i>gap1::aphII</i> in pIJ773	This study
L21	DH5α containing <i>gap1::aphII</i> in aac(3)IVKS(+)	This study
L21minus	ET12567 containing <i>gap1::aphII</i> in aac(3)IVKS(+)	This study
<i>S. clavuligerus</i>		
NRRL 3585	Wild-type, cephamycin C and clavulanic acid producer	Prof. J. Piret, Northeastern University, USA
DEPA	Clavulanic acid overproducer	DEPA Pharmaceuticals, İzmit, Turkey
ID+	pL21	This study
<i>S. lividans</i>	Wild type	Prof. J. Piret, Northeastern University, USA
<i>Klebsiella pneumoniae</i>		
ATCC 29665	Penicillin sensitive indicator organism	Prof. P. Liras, INBIOTEC, Leon, Spain
Plasmids		
pGEM-T Easy	Amp ^R <i>lacZ'</i>	Promega
pTC192-Km	1.3 kb <i>Hind</i> III-blunt ended fragment with the <i>aphII</i> gene in pTC192	Prof. P. Liras, INBIOTEC, Leon, Spain
aac(3)IVKS(+)	<i>apr-oriT</i> cassette from pIJ773 in <i>Xba</i> I site of pBluescriptII KS(+)	Previously created in our laboratory
pIJ486	Thio ^R , <i>Streptomyces</i> plasmid, pIJ101 replicon	Prof. Keith Chater, John Innes Centre, UK
pGgap	<i>gap1</i> in pGEM-T	Previously created in our laboratory

Table 2.1. (continued)

pIJgap	<i>gap1</i> in pIJ773	Previously created in our laboratory
pIJgneo	<i>gap1::aphII</i> in pIJ773	This study
pL21	<i>gap1::aphII</i> in aac(3)IVKS(+)	This study

2.2. Culture media, buffers, solutions, chemicals and enzymes

Ingredients and preparation of culture media are found in Appendix A. Buffers and solutions with their compositions and preparations are listed in Appendix B. Chemicals and enzymes used in this study are listed in Appendix C with their suppliers.

2.3. Genomic DNA isolation from *S. clavuligerus* by salting out procedure

Salting out method (Pospiech and Neumann, 1995) was used for *S. clavuligerus* genomic DNA isolation. Overnight grown cells in 30 ml TSB (Appendix A) were centrifuged at 3500 rpm for 2 min. The pellet was resuspended in 5 ml SET buffer (Appendix B) containing 100 μ l lysozyme (50 mg/ml) (Appendix C) and incubated at 37 °C for 1 h. After addition of 140 μ l proteinase K (20 mg/ml) (Appendix C) and 10% SDS (Appendix C), the mixture was incubated at 55 °C for 2 h while mixing by inversion time to time. Then, 2 ml NaCl (5 M) (Appendix C) was added to the solution and mixed by inversion. It was allowed to be cooled down to 37 °C at RT. Chloroform (5 ml) (Appendix C) was added, and mixed by inversion. After incubation at 20 °C for 30 min, the mixture was centrifuged at 4500 rpm for 15 min. The supernatant was mixed with 0.6 volume isopropanol (Appendix C) by inversion. A single DNA mass emerged in liquid isopropanol. This DNA was transferred to a new tube and washed once with 5 ml ethanol (Appendix C). After removal of ethanol by air drying, remaining DNA was dissolved in 1 ml TE buffer (Appendix B) at 55 °C and stored at +4 °C.

2.4. Plasmid isolation from *E. coli*, *S. clavuligerus* and *S. lividans*

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

Manual plasmid isolation from *E. coli* cells were performed according to plasmid miniprep method described by Hopwood *et al.* (1986).

Qiagen Plasmid Purification Midi Kit (Qiagen Inc., Valencia, CA) was used for plasmid isolation from *S. lividans* and *S. clavuligerus* with some modifications at initial steps of manufacturer's instructions.

2.4.1. Manual plasmid isolation from *E. coli*

E. coli cells were scratched from 0.5 cm² area LA medium surface (Appendix A) and put into an eppendorf tube containing 100 µl cold STE solution (Appendix B) and then vortexed. The tube was incubated on ice for 20 min. Then, 60 µl lysis solution (Appendix B) was added to the mixture and vortexed. With 10 min incubation at RT, the lysis of cells was completed. Incubation at 70 °C for 10 min followed by immediate cooling under tap water provided DNA denaturation in the mixture. After addition of 160 µl phenol-chloroform solution (Appendix B) and vortexing, homogeneous and cloudy mixture was obtained. Centrifugation at 13,200 rpm for 5 min created three separate phases. The upper transparent phase contains plasmids, so it is gently transferred into another tube without disturbing other phases. After running 10 µl of this liquid in 1 % agarose gel, the isolated plasmids were visualized.

2.4.2. Plasmid isolation from *Streptomyces* spp.

Streptomyces culture in 50 ml TSB with 0.5% (w/v) maltose (Appendix A) was incubated at 28 °C, 220 rpm for 2.5 days. Cells were harvested from 15 ml of the culture by centrifugation at 6000 rpm for 20 min. After washing with 10.3% sucrose (Appendix A), the pellet was stored at -20 °C o/n. Addition of 1.5 ml P1 buffer (Qiagen Plasmid Purification Midi Kit) with 1 mg/ml lysozyme (Appendix C) was followed by vortexing until no visible clumps left. Following 30 min of incubation at 37 °C, 1.5 ml P2 (Qiagen Plasmid Purification Midi Kit) buffer was added. Qiagen Plasmid Purification Midi Kit (Qiagen Inc., Valencia, CA) procedure was applied for the rest of the process.

2.5. Competent *E. coli* preparation

E. coli competent cells were prepared by using RbCl₂ method (Hanahan *et al.*, 1982). Four milliliters of 16 h-grown *E. coli* culture was inoculated into 50 ml LB medium with selective agents if necessary, and left to incubation at 37 °C, 220 rpm. When its absorbance at 600 nm was between 0.4 and 0.7 the cells were transferred to centrifuge tube and incubated in ice for 15 min. Supernatant was discarded after the cells were centrifuged at 3500 rpm for 5 min. The pellet was resuspended in 5 ml Competence Buffer 1 (Appendix B). The supernatant was discarded after centrifugation, and the pellet was dissolved in 2 ml Competence Buffer 2 (Appendix B). The suspension was transferred to eppendorf tubes as 100 µl aliquotes. After incubation on ice for 30 min, they were immediately frozen in liquid nitrogen and stored at -80 °C.

2.6. Heat-shock transformation of *E. coli* competent cells

Competent *E. coli* cells were transformed by heat-shock method (Sambrook *et al.*, 1989) with slight modifications. Ligation product (10-20 µl) or plasmid DNA (up to 50 ng) was added into 100 µl competent *E. coli* cells, and incubated in ice for 30 min. As the heat shock step, the cells were incubated at 42 °C for 60-90 sec and immediately returned back to ice for 5 min further incubation. Addition of 900 µl LB and 1 h incubation at 37 °C allowed competent cells to regenerate. The cells were harvested by centrifuging at 3200 rpm for 10 min, and the pellet was dissolved in 100 µl LB (Appendix A). The cell suspension was spread on LA (Appendix A) plate with selective agent(s) and incubated at 37 °C for 16 h.

2.7. Delivery of DNA into *S. clavuligerus*

2.7.1. Intergeneric conjugation between *E. coli* and *S. clavuligerus*

A modified conjugation procedure based on methods described by Mazodier *et al.* (1989) and Flett *et al.* (1997) was used. *E. coli* ET12567 culture containing the desired plasmid which will be transferred to *S. clavuligerus* cells was grown in 10 ml LB (Appendix A) with selective agents and 25 µg/ml chloramphenicol (Appendix C) at 37 °C for 16 h. The culture (1.5 ml) was added into 50 ml LB (Appendix A) in 250 ml flask, and incubated in a rotary shaker at 37 °C, 200 rpm until OD₆₀₀ reached to 0.4-0.6. The cells were washed twice with equal volume of LB (Appendix A) at 3500 rpm for 5-10 min. This step would remove antibiotics. The final pellet was resuspended in 0.1 volume of LB (Appendix A).

S. clavuligerus spores were harvested from sporulation medium (Appendix A) by using 3-5 ml TTX solution (Appendix B) and incubated at 50 °C for 10 min to apply heat-shock to spores or *S. clavuligerus* mycelia were harvested from TSA (Appendix A) by using 3-5 ml 20% sterile glycerol. *S. clavuligerus* mycelia mixed homogenously in glycerol as much as possible by pipetting.

E. coli suspension (500 µl) was mixed with equal volume of *S. clavuligerus* spores/mycelia by pipetting. The cell mixture was spread on Mannitol-Soybean flour (MS) agar (Appendix A) and incubated at 30 °C for 16-20 h. Sterile dH₂O containing 0.5 mg/ml nalidixic acid and 1 mg/ml selective agent was spread on plates as 10 ml aliquotes and incubation continued for further 2-4 days. Grown colonies were spread on TSA (Appendix A) with the selective agents.

2.7.2. Electroporation

Electroporation which creates gaps on cell surface, and keeps cells in stress by application of electric current requires preparation of electrocompetent cells. For this purpose, 24 h-grown culture was harvested by centrifugation at 10000 rpm. First pellet was resuspended in 100 ml ice-cold sucrose (10%), then in 50 ml ice-cold glycerol (15%), and finally in 10 ml 15% glycerol mixed with lysozyme (100 µg/ml) following every centrifugation. After 30 min incubation at 30 °C, following two centrifugations, the pellet was resuspended in 10 ml ice-cold 15% glycerol twice. With final centrifugation, pellet was dissolved in 1-5 ml solution composed of 30% PEG1000, 10% glycerol and 6.5% sucrose. The cells were rapidly frozen in liquid nitrogen as 50 µl aliquots and stored at -80 °C.

Introduction of DNA into cells started with mixing 1-3 µl DNA and electrocompetent cells in ice-cold cuvette. After application of *S. aureus* program, 750 µl ice-cold CRM medium was added immediately and incubated at 30 °C for 3 h in an orbital shaker in order to allow cells to normalize. Cells were collected by 5 min centrifugation at 10,000 rpm, the pellet was dissolved in 200 µl CRM medium and spread on TSA plates with selective agents. Following 30 °C incubation for 3-4 days, colonies would appear.

2.7.3. Protoplast transformation

First phase is preparation of protoplasts. Seed culture was grown in 50 ml TSB medium containing 0.5% maltose at 28 °C, 220 rpm for 48-60h until its OD₆₀₀ was 0.5-0.7 with 10X dilution. The seed culture (5 ml) was inoculated into 50 ml of 2:3 ratio TSB:YEME medium and left to incubation at 28 °C, 220 rpm for 24h. This second culture (25 ml) was centrifuged at 3000xg for 15 min and washed twice 10.3% sucrose. Pellet was resuspended in 2 ml PE buffer containing 1 mg/ml lysozyme and incubated

at 30 °C until protoplast formation was verified under light microscope. Protoplasts were filtered through non-absorbent sterile cotton plugs, and then washed 3 times with PE buffer at 2000xg for 10 min. After resuspension of pellet in 1 ml PE buffer, it was divided as 50 µl aliquots, and stored at -80 °C.

Second phase is the PEG-mediated heat shock transformation. Thawed protoplasts in ice were incubated at 42 °C for 10 min. DNA (2-10 µl, 0.05-1.5 µg) was added to 50 µl heat shocked-protoplasts, and then mixed with 500 µl 50% PEG1000 (in PE buffer) immediately. After no more than 3 min incubation at RT, it was diluted with 1.5 ml PE buffer, centrifuged at 2000xg for 10 min. Pellet was resuspended in small amount of PE buffer and spread on R2YE regeneration medium. After 48 h incubation at 26 °C, 2.5 ml soft nutrient agar (0.5% agar) mixed with 8 µg/ml thiostrepton and 50 µg/ml of kanamycin was laid on plate. Following 2-4 days, colonies would appear.

2.8. DNA manipulations

2.8.1. Digestions with restriction endonucleases

Restriction endonucleases (Roche, Fermentas and New England Biolabs) were used for cloning purposes and verification of recombinants. Restriction enzyme digestions were performed under the conditions specified by the manufacturers.

2.8.2. End filling by Klenow Fragment and T4 DNA Polymerase

Fermentas Klenow Fragment and T4 DNA polymerase enzymes were used to fill the sticky ends of digested DNA fragments to form blunt ends for creating proper ligation ends. The reactions were carried out according to the instructions provided by the manufacturer.

2.8.3. Alkaline phosphatase treatment

To prevent self-ligation of linearized vector DNA, alkaline phosphatase treatment was performed to remove phosphate groups from 3' ends of the DNA, so that ligase cannot produce phosphate backbone at that point. rAPid Alkaline Phosphatase (Roche) (Appendix C) was used for this purpose in accordance with the procedure provided by the manufacturer.

2.8.4. DNA ligations

Ligation reaction was performed as follows: 0.5 µl of T4 DNA ligase (Promega or Fermentas), 5 µl of 2X ligation buffer (supplied by the manufacturer), and vector and insert with 1:1 to 1:7 vector:insert molar ratio were mixed and the volume was completed to 10 µl with dH₂O. The volume increased to 20 µl by doubling the amounts of ingredients in cases of using insert DNA with low concentrations. For blunt end ligations, T4 DNA ligase (Fermentas) was used with 5 % (w/v) PEG 4000 supplied by the manufacturer. The ligation reaction was performed with 16 h incubation at +4 °C or 1 h incubation at 22 °C.

2.9. Agarose gel electrophoresis

Plasmid DNAs were run in 1% agarose gels by applying 90-100V for 40-60 min. Genomic DNAs were run in either 1% or 0.8% agarose gel. TAE buffer (1X) (Appendix B) was used as running buffer and also in gel composition with agarose. The gels were stained by incubation in ethidium bromide (EtBr) solution (EtBr dissolved in 1X TAE buffer (Appendix A) as 0.4 ng/ml final concentration) for 5-10 min at RT. Visualization of DNA bands on the gel were carried out by a UV transilluminator (UVP) and photographed by using Vilber Lourmat Gel Imaging System. Lambda (λ) DNA/*Pst*I DNA ladder (Fermentas) and O'GeneRuler™ 100 bp Plus DNA Ladder (Fermentas) were used as the size markers (Appendix C).

2.10. Extraction of DNA fragments from agarose gels

Desired DNA bands were excised from the gel and weighed in Eppendorf tubes. Purification of DNA slices were performed by using Qiagen Gel Extraction kit or Genemark Gel Extraction kit (<http://www.genemark.com.tr>) in accordance with the supplied procedure by the manufacturers. The purified DNA concentrations were measured by using NanoDrop® ND-2000 (ThermoScientific).

2.11 Polymerase chain reaction (PCR)

PCR reaction mixture was prepared as either 25 or 50 µl volumes. PCR reactions were performed by using Taq or Pfu DNA polymerases. The composition of PCR reaction mixture and reaction conditions are given in Table 2.2 and Table 2.3 respectively. PCR products were run in 1% agarose gel to visualize amplification product.

Table 2.2. PCR reaction components with their concentrations and volumes

Reaction Components	Final Concentration	Volume (μl)
Taq Buffer (10X) Or Pfu Buffer (10X)	1X	2.5
dNTP Mixture (10 mM)	200 nM	1
Forward Primer (10 μM)	0.2 μM	0.5
Reverse Primer (10 μM)	0.2 μM	0.5
Template DNA	50 ng	1.5
MgCl ₂ (25 mM)	3 mM	3
DMSO (100 %)	4 %	1
Taq DNA Polymerase or Pfu DNA Polymerase (5 u/μl)	2.5 u/μl	0.5
dH ₂ O	-	14.5
<i>Total</i>		25

Table 2.3. PCR reaction conditions for amplification with *gap1* and *aphII* primers

PCR Cycle	Temp (°C)	Time	Temp (°C)	Time
Primer pairs	<i>gap1</i>		<i>aphII</i>	
Initial Denaturation	94	5'	95	10'
Denaturation	95	45"	95	1'

Table 2.3. (continued)

Annealing	55 45"	56 1'
Extension	72 2.5'	72 2'
Final Extension	72 10'	72 10'
Cycle Number	30	30

2.11.1 The sequence of *gap1* and *aphII* genes and the PCR Primers

The nucleotide sequence of *gap1* (DQ178995 accession number at GenBank database) and *aphII* gene (AAA73390.1 protein id) and their primers sequences with melting temperatures used in this study are shown in Figure 2.1, 2.2 and Table 2.4, respectively.

Table 2.4. Primers used in this study

Primers	Sequence (5'-3')	T_m (°C)
gap1F	AACGAGGAGATCGGTTTCGTGA	61.0
gap1R	TCAGAGCCGTGTCCCTCAGA	63.0
aphIIF	CTGCTAAAGGAAGCGGAACA	57.0
aphIIR	AGCCAACGCTATGTCCTGAT	59.0

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1 atctccgtcg cgtatgtcac cgactcggtc acgggcggcc atggcgtctt cctcgacgac
61 acccggctgg tgactcgggg cggcaccgcc gccgcccagg ggttcgagac ctcgctcggc
121 gcgtgggtcgg cgacggagtc gccggccggc agccccgctc cgcaggggtg gtggacgcgc
181 agccaggagc tgttcccgac cgctggagcg gttactacgc tggacactgt cctgttgggc
241 ttcggtctcg aacacctgac cgatgagggg gctcgcgcaa gggtgctcgg acgggcgctg
301 gggggcgtca aacgctgact ccgagcccct cgaaacccgt catccgaagg ggccccagcc
361 caggtgatcg gcggtccttg ccctctgtcg agggtaggg gccgccatcg ccacgtacgg
421 gccctccgat gtcacttcgg gcccttcagg gaggtagggt cgtaacgggt cggggacatc
481 gcaatacaac tcgccgggag gttttgatca cccggcgtae caacgaggag atcggttcgg
541 gacgatccgc gtaggcatca acggctttgg ccgcatcggg cgtaactact tccgcccggc
601 gctggagcag ggtgcagaca tcgagatcgt ggctgtcaac gacctgggtg acaccgcgac
661 cacggctcac ctgctgaagt acgacacccat tctgggacgc ctcaaggccg aggtcaccca
721 caccgcccgc accatcaccc tcgacggcca cacgatcaag gtgctctccg agcgcaccc
781 cgcgacatc ccgtggggcg agctggggcg cgacatcgtg atcgagtoga cgggcatctt
841 caccaagaag gccgacgccg agaagcacct cgcggcgccg gcccaagaagg tcctgatctc
901 ggctcccggc aaggacgagg acatcacccct ggtgatgggt gtcaaccagg acacctacga
961 cccggcgtag caccacgtca tctccaacgc ctctgcacc accaactgtg tgggcccgat
1021 ggcgaagggt ctcgacgaga acttcggcat cgtccgcggt ctgatgacga cggtcacgc
1081 ctacaccaac gaccagcgca tectggactt cccgcactcg gacctgcgcc gcgcccgcgc
1141 cgcgcccggg aacatcatcc cgaccaccac gggtgcccgc aaggccaccg cgctggctct
1201 cccgcagctc aagggaagc tggacggcat cgcgatgcgc gtcccgggcc ccaccggctc
1261 cgcgaccgac ctggctcgtc agctgagccg cgaggtcacc aaggacgagg tcaacgcgcg
1321 gttcaagaag gccgcccagg gcgagctcca gggcatcctg agctacaccg aggaccgat
1381 cgtctcctcg gacatcgtcg gcgacccggc ctctgcacc ttogactcct ccctgacccat
1441 ggtccaggag ggcaactcgg tgaagatcct cggctgggtac gacaacgagt ggggctactc
1501 caaccgcctc gtcgacctca cggctctcgt cggcgagcag ctctgagggg cacggctctg
1561 accagcacct atcaacacag tacggttctg atcagctcag tgaagggcgn aaanngggg
1621 ccccg

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Figure 2.1. *gap1* sequence (darkened) of *S. clavuligerus* ATCC 27064 with PCR primer binding sites.

2.11.2. Colony PCR

For verification of recombinant colonies, a small amount of colonies grown on solid media was used as template DNA in PCR reaction. Drawn cells from solid media was dissolved in 50 µl dH₂O and incubated at 94 °C for 15 min. This suspension (1.5-2 µl) was used instead of template DNA in a standard PCR reaction.

2.12. DNA sequencing reactions

RefGen Biotechnology Inc. (Ankara, Turkey) provided this service by using the chain termination method with BigDye Cycle Sequencing Kit V3.1 (Applied Biosystems) of ABI 3130xl Genetic Analyzer (Applied Biosystems).

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1  AATTC AAGCTT CACGCT GCCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAA 60
1  aphIII ||||| CTGCTAAAGGAAGCGGAA 18
61  CACGTAGAAAGCCAGTCCGCAGAAAACGGTGTCTGACCCCGGATGAATGTCAGCTACTGGGC 120
19  ||
   CA →
121  TATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTAC 180
181  ATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCAGGAAATGCCAGCTGG 240
241  GCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCC 300
301  AAGGATCTGATGGCGCAGGGGATCAAGATCCCCGGGGATCTGATCAAGAGACAGGATGAG 360
361  GATCGTTTCGGATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGG 420
421  AGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGT 480
481  TCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGGCC 540
541  TGAATGAAGTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGCGTTCCTI 600
601  GCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGACTGGCTGCTATTGGGCGAAG 660
661  TGCCGGGGCAGGATCTCCTGTCACTCACCTTGCTCCTGCCGAGAAAAGTATCCATCATGG 720
721  CTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCAATCGACCACCAAG 780
781  CGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTGATCAGGATG 840
841  ATCTGGACGAAGAGCAICAGGGGCTCGCGCCAGCCGAACTGTTGCCAGGCTCAAGGCGC 900
901  GCATGCCCGACGGCGAGGATCTCGTGTGACCCATGGCGATGCCTGCTTGCCGAATATCA 960
961  TGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACC 1020
1021 GCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGG 1080
2  |||||
   ---TCAGGACATAGCGTTGGCT aphIIR
1081 ← CTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGAATTCGACGCGCAICGCCTTCT 1140
1141 ATCGCCTTCTTGACGAGTTCTTCTGATCGGGACTCTGGGGTTCGAAATGACCGACCAAGC 1200
1201 GACGCCAACCTGCCATCACGAGATTTCGAATCCACCGCCGCTTCTATGAAAGGTTGGG 1260
1261 CTTCCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCT 1320
1321 GGAGTTCCTGCCCCACCCCGGGCTCGATCCCTCGTGCAGTCTAGAGGATCCCCGGGTAC 1380
1381 CGAGCTCG 1388

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Figure 2.2. *aphII* sequence (boxed) with PCR primer binding sites on *EcoRI* cut small fragment of pTC192-Km vector.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Disruption of *gap1* with *aphII*

The *gap1* was previously PCR amplified from the genome of *S. clavuligerus* DEPA strain and cloned into pGEM-T cloning vector, named as pGgap, and then into pIJ773 vector, named as pIJgap, in our laboratory (unpublished). The *gap1* primers were designed according to the *gap1* sequence registered under DQ178995 accession number at GenBank database.

pIJgap was digested with *BlpI* restriction enzyme in order to divide *gap1* into two parts (Figure 3.1; Appendix D). This digestion opened a position to insert disruption fragment into *gap1* thereby creating a nonfunctional gene. Sticky ends were filled by Klenow fragment to create proper ligation sites.

Kanamycin resistance gene (*aphII* or *neo*) which is the disruption fragment was extracted from pTC192-Km vector by *EcoRI* digestion. 5' sticky ends were filled by Klenow Fragment. Then, *aphII* fragment was ligated to *BlpI* digested pIJgap, and transferred to DH5 α competent cells by transformation. Colonies were selected by their resistance to both kanamycin (50 μ g/ml) and ampicillin (100 μ g/ml). Plasmids were isolated from 20 colonies by manual plasmid isolation method and run alongside with pIJgap on agarose gel to find the plasmid with increased size due to disruption cassette insertion. Because of two antibiotics used in selection of recombinants, all plasmids were seen higher in size than pIJgap on agarose gel (data not shown). Thus, one of the putative recombinant plasmids was isolated by using Fermentas GENEJET Plasmid Miniprep Kit and run with pGgap and pIJgap on agarose gel (Figure 3.2).

The isolated plasmid was linearized by *EcoRI* digestion to determine its size correctly on agarose gel (Figure 3.3). The recombinant plasmid and strain were labelled as pIJgneo and *E. coli* IJgneo, respectively. PCR amplification with *gap1* primers for genomic DNA of *S. clavuligerus* DEPA and recombinant plasmid pIJgneo also showed increased size in amplified product (Figure 3.4). As the last step of confirmation, pIJgneo was sequenced by using *gap1* primers. It was seen that *gap1* and *aphII* were ligated in the same direction. The selected colony was stored at -80 °C.

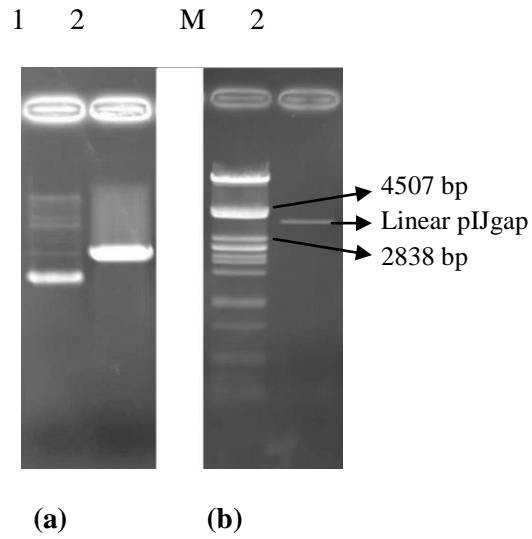


Figure 3.1. *BlpI* digestion of pIJgap; (a); 1: intact pIJgap, 2: *BlpI* digested linear pIJgap, (b); M: λ *PstI* DNA ladder, 2: *BlpI* digested linear pIJgap plasmid after gel purification.

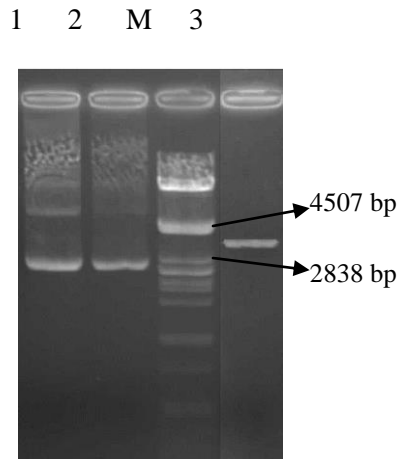


Figure 3.2. Size determination of pIJgneo after isolation by Mini Kit; M: λ *PstI* DNA ladder, 1: pGgap (4055 bp), 2: pIJgap (3992 bp), 3: Putative recombinant plasmid (pIJgneo) isolated from the selected colony (expected size of the band is 5387 bp).

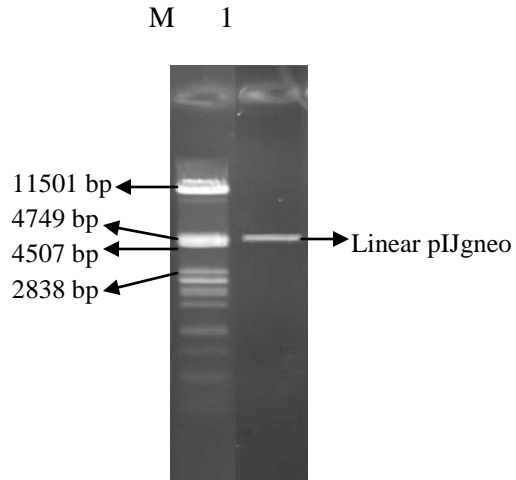


Figure 3.3. *EcoRI* digestion of pIJgneo for verification of recombination; M: λ /*PstI* DNA ladder, 1: linearized pIJgneo (expected size is 5387 bp).

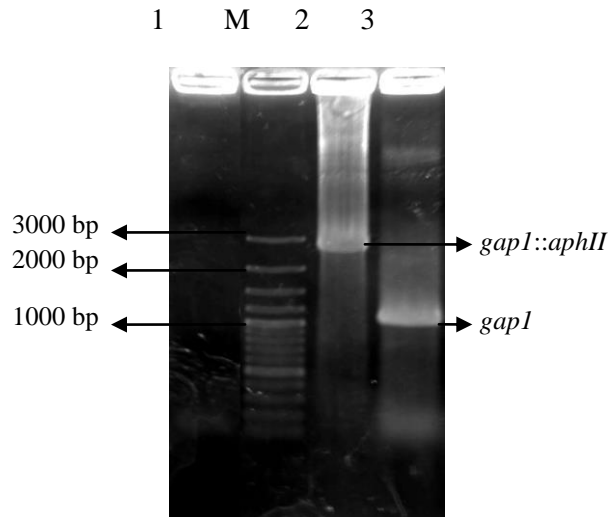


Figure 3.4. PCR amplification with *gapI* primers for genomic DNA of *S. clavuligerus* DEPA and recombinant plasmid pIJgneo; M: O'GeneRuler™ 100 bp Plus DNA Ladder, 1: PCR mixture with no template DNA, 2: pIJgneo as template DNA, 3: pIJgap as template DNA.

3.2. Cloning of *gap1::aphII* into destination vector [aac(3)IVKS(+)]

A suicidal vector was designed for transfer of *gap1::aphII* into *S. clavuligerus* DEPA from *E. coli* ET12567 through conjugation. [aac(3)IVKS(+)] was chosen as base vector to construct the suicidal vector. This vector carries *ori-T* cassette which is required for mobilization of the plasmid during conjugation. Also, apramycin resistance gene on this vector would facilitate the selection of exconjugants and *gap1*-disrupted mutant of *S. clavuligerus* DEPA. For construction of suicidal plasmid, *gap1::aphII* cassette was extracted from pIJneo by *ApaI-SpeI* digestion. [aac(3)IVKS(+)] was linearized through same digestion enzymes to create compatible ends for ligation (Figure 3.5). These two compatible ends were ligated, and transferred into *E. coli* DH5 α competent cells by transformation. LA medium containing apramycin (50 μ g/ml) and kanamycin (50 μ g/ml) was used as selection medium following transformation. Plasmids were isolated from putative recombinant colonies by manual plasmid isolation, and the suicidal plasmid size (expected: 6672 bp) was compared on agarose gel with a control plasmid 6.6 kb in size (Figure 3.6). Plasmids isolated from the selected colonies 1,2 and 12 were digested with *ApaI-SpeI* enzymes to verify insertion of *gap1::aphII* disruption cassette into the vector (Figure 3.7). Finally, the recombinant plasmids were sequenced with *gap1* primers which confirmed the success of suicidal plasmid construction, and the colony 1 was selected for use in the following experiments. The recombinant plasmid and strain were named as pL21 and L21, respectively.

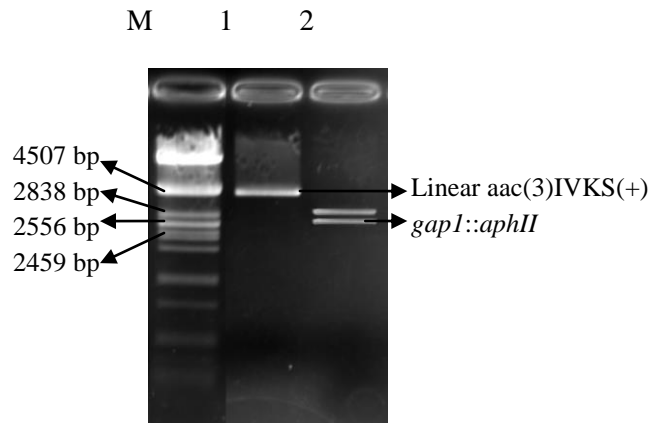


Figure 3.5. *ApaI-SpeI* digestion of aac(3)IVKS(+) and pIJneo; M: λ /*PstI* DNA ladder, 1: linearized aac(3)IVKS(+), 2: *gap1::aphII* and the remaining part of pIJneo

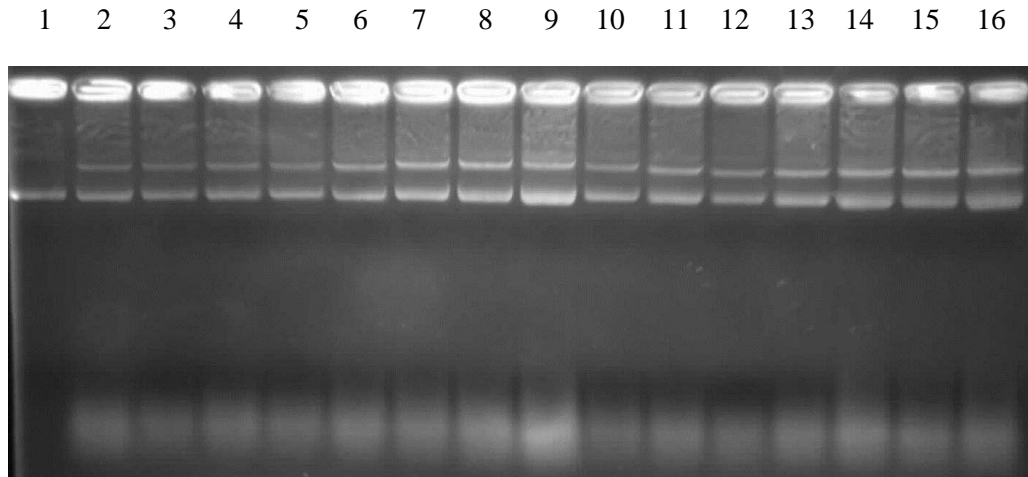


Figure 3.6. Size comparison of putative recombinant suicidal plasmids; 1: a control plasmid (6.6 kb in size), 2-16: putative recombinant suicidal plasmids.

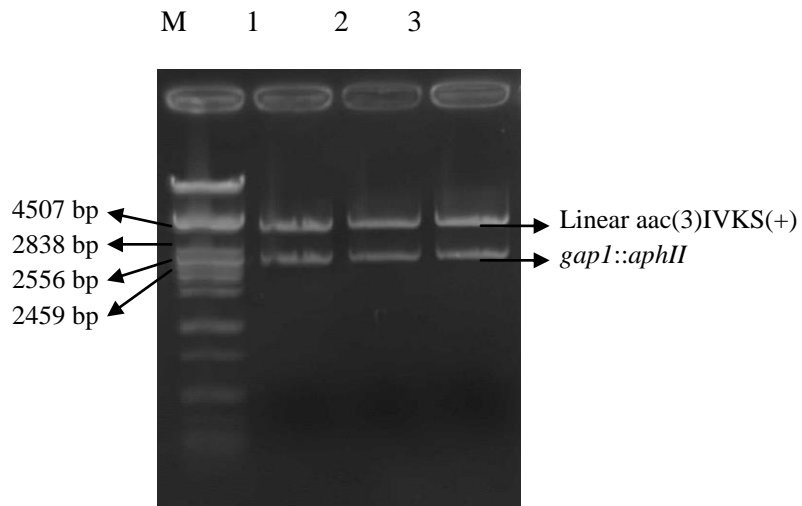


Figure 3.7. *ApaI-SpeI* digestion of putative recombinant suicidal plasmids; M: λ /*PstI* DNA ladder, 1,2,3: Linearized *aac(3)IVKS(+)* vector and the released *gapI::aphII* from putative recombinant suicidal plasmid 1,2 and 12, respectively.

3.3. Delivery of *gap1::aphII* into *S. clavuligerus* DEPA strain

S. clavuligerus has a strong DNA modification system, and the most significant feature of this system is that it detects and destroys methylated foreign DNA. Therefore, any DNA which will be transferred into *S. clavuligerus* should be demethylated. *E. coli* ET12567 cells are methylation deficient (*dam*⁻), so any plasmid construct amplified in its system would not be methylated. This strain also contains pUZ8002 plasmid which carries *tra* genes required for conjugation. Any plasmid containing *oriT* region will be mobilized in these cells. For this purpose, pL21 was isolated from *E. coli* L21, and it was delivered into *E. coli* ET12567 competent cells by transformation. LA medium with apramycin (50 µg/ml), kanamycin (50 µg/ml) and chloramphenicol (25 µg/ml) was used for selection of transformants. After transformation, one of the resulting transformant colonies was selected, and the plasmid isolated from this selected colony was compared to pL21 in terms of its size and *ApaI-SpeI* digestion profile (Figure 3.8). This verified transformant was named as L21minus. L21minus cells would be expected to interact with *S. clavuligerus* DEPA cells by conjugation, and transfer demethylated pL21 plasmid.

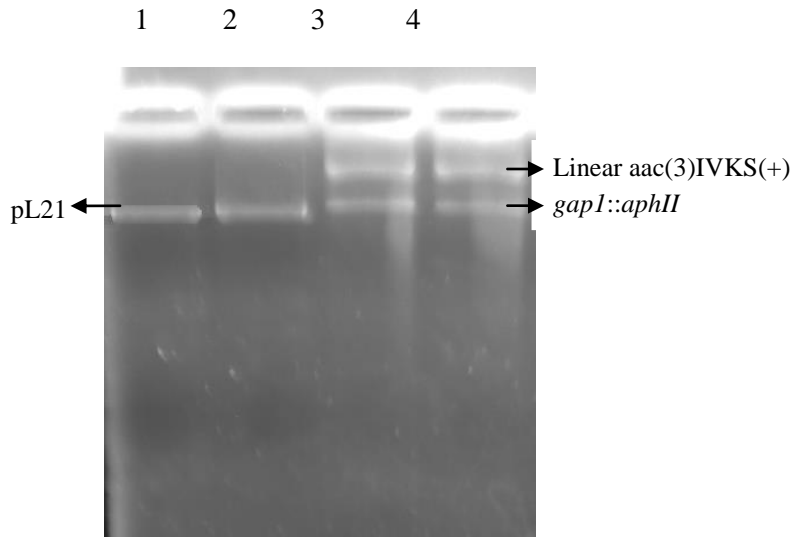


Figure 3.8. Comparison of the plasmids isolated from *E. coli* L21 and L21minus based on their size and *ApaI-SpeI* digestion profile; 1: demethylated pL21, 2: methylated pL21, 3: *ApaI-SpeI* digested demethylated pL21, 4: *ApaI-SpeI* digested methylated pL21.

It was a difficult task to get a pL21 containing strain of *S. clavuligerus* DEPA strain due to quite unsteady nature of *Streptomyces* spp. In addition to strong DNA modification system, genetic instability is a common feature in *Streptomyces* spp. resulting in alterations of DNA frequently and causing loss of certain functions (Dharmalingam and Cullum, 1996; Kieser *et. al.*, 2000). DEPA strain which was subjected to excessive random mutagenesis was particularly expected to be highly unstable. Different methods were applied to introduce pL21 into *S. clavuligerus* DEPA cells, including: (i) conjugation, (ii) electroporation and (iii) protoplast transformation.

3.3.1. The use of conjugation for *gapI::aphII* delivery

Conjugation procedure which has been the widely used method in our laboratory is the easiest one among the previously-mentioned methods to deliver DNA into *Streptomyces*. However, several attempts of intergeneric conjugation between *E. coli* L21minus and *S. clavuligerus* DEPA cells have resulted in either no viable *Streptomyces* colonies or false positive colonies.

After conjugation, kanamycin (1 mg/ml) and nalidixic acid (0.5 mg/ml) were used in the selective solution laid on MS agar. The problem could be that *S. clavuligerus* DEPA cells were naturally resistant to kanamycin, or sensitive to nalidixic acid or MS medium could not provide a suitable growth environment for this extensively mutagenized strain. Tests on MS agar showed that DEPA cells could grow on MS agar without antibiotic and survive up to 1 mg/ml nalidixic acid in the selective solution. On the other hand, it could not survive even the half concentration of kanamycin present in the selective solution. Also, DEPA strain showed no growth on TSA plates with different concentrations of kanamycin (15, 25 and 50 µg/ml), and apramycin (25 and 50 µg/ml). Thus, it was deduced that antibiotic resistance was not the cause of the false positives.

It was considered that the expression level of kanamycin resistance could be lower in *S. clavuligerus* DEPA cells than in *E. coli* since both bacteria have distinct metabolisms. The low resistance to these antibiotics would lead death of successful exconjugants. Thus, lower concentrations of kanamycin down to 0.2 mg/ml were used in the selective solution, but none of the colonies obtained could be grown in liquid TSB medium.

Conjugation procedure was repeated with vegetative *S. clavuligerus* cells although the original protocol recommends the use of spores. This was resulted in no viable colonies after conjugation.

It is known that bacteria cannot hold two plasmids belonging to the same incompatibility group. There is no available background information on genetic make up of *S. clavuligerus* DEPA strain, thus it was impossible to discard the possibility of

existence of any plasmid of the same incompatibility group in this organism. Qiagen Midi Plasmid Purification Kit was used to see plasmid profile, if any, of *S. clavuligerus* DEPA cells (Figure 3.9). There appeared to be no plasmids other than a very big one.

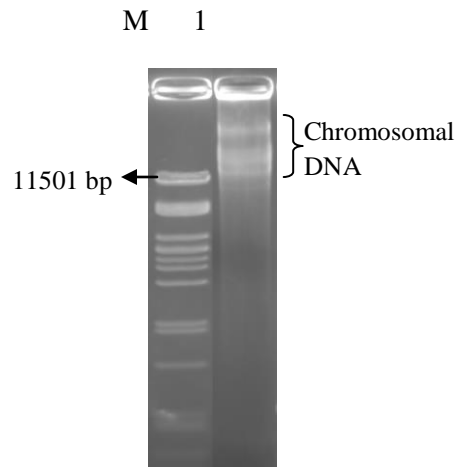


Figure 3.9. Plasmid profile of *S. clavuligerus* DEPA strain; M: λ /*Pst*I DNA ladder, 1: Elute obtained from *S. clavuligerus* DEPA cells by Qiagen Plasmid Purification Midi Kit.

The problem was thought to be related to unique genetic features of DEPA strain, so the conjugation process was also applied for *S. clavuligerus* wild type (NRRL 3585) strain. No significant difference was obtained.

3.3.2. The use of electroporation for *gap1::aphII* delivery

Electroporation is another method to deliver DNA into bacterial cells. The plasmids were isolated from *E. coli* ET12567 cells and used for electroporation with different amounts up to 1 μ g per cuvette. No viable colonies of *S. clavuligerus* DEPA were

obtained after a few attempts. It was seen that the procedure requires high labor work, needing several optimizations for DNA concentration, pulse amplitude, frequency, etc.

3.3.3. The use of protoplast transformation for *gap1::aphII* delivery

Protoplast transformation is an effective method to directly deliver DNA into *Streptomyces* cells although it is more labor intensive during protoplast preparation (Kieser *et.al.*, 2000). This method is similar to *E. coli* heat-shock transformation, and pIJ486 has been generally used as suicidal vector due to its unstable nature in the absence of selective antibiotic(s) (Paradkar *et.al.*, 2001). For this purpose, demethylated plasmids were isolated from *E. coli* L21minus cells. The *gap1::aphII* fragment was extracted from the demethylated pL21 by *ApaI-SpeI* digestion, and demethylated pIJ486 isolated from *S. lividans* was digested with *HindIII* (Figure 3.10).

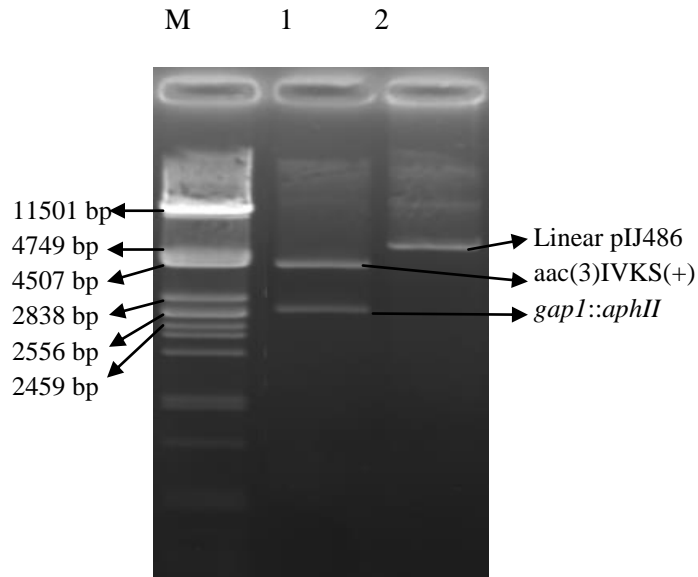


Figure 3.10. *ApaI-SpeI* digestion of pL21 and *HindIII* digestion of pIJ486: M: λ /*PstI* DNA ladder, 1: Linearized *aac(3)IVKS(+)* and the released *gap1::aphII*, 2: *HindIII* digested linear pIJ486.

To create compatible ends for ligation, sticky ends were converted to blunt ends by Klenow fragment and T4 DNA polymerase for *gap1::aphII* fragment and linearized pIJ486, respectively. Blunt end ligations are resulted in either direct or reverse attachment of insert and vector, but in this case, it is not important since the fragment is required for exchange in genome via homologous recombination, and *aphII* was inserted inside this fragment with its own promoter. The ligation product was then introduced into *S. clavuligerus* protoplasts by transformation. Afterwards, the cells were plated on R2YE regeneration medium, and following 48h incubation at 26 °C, soft agar with 8 µg/ml thiostrepton, and 50 µg/ml kanamycin was laid on plates. Colonies on R2YE medium were transferred to TSA plate with 50 µg/ml kanamycin and thiostrepton, but no colony grew. The transformation step was repeated with only kanamycin selectivity, but even with lower concentrations of kanamycin (25 and 15 µg/ml), the result did not change.

As a backup strategy, the ligation product was used for protoplast transformation of *S. lividans*. After isolation from *S. lividans* system, it could be used for transformation of *S. clavuligerus* DEPA strain. Thereby, the plasmid could be compatible for *Streptomyces* system, and it would be easier to transform *S. clavuligerus* DEPA protoplasts with intact plasmid at high concentrations. Nevertheless, no viable *S. lividans* colonies could be obtained through this transformation. In addition, pL21 intact plasmid was also used for protoplast transformation of both *S. lividans* and *S. clavuligerus* DEPA strains, but no positive result was obtained.

The procedure used was adopted from the procedure of Kieser *et al.* (2000) in an earlier MSc thesis in our laboratory (Caydasi, 2006). The application of the original procedure (Kieser *et al.*, 2000) with 16-20 h incubation prior to addition of selective soft agar and use of a slightly different buffer produced no positive result.

Considering that protoplast transformation might require adjustments in standard protoplast transformation procedure to be used for *S. clavuligerus* DEPA strain, several conditions were changed. In an article reported by Garcia-Dominguez *et al.* (1987), different parameters of protoplast transformation for *S. clavuligerus* were modified. According to these findings, several parameters were also changed in this study; different PEG1000 concentrations (40-60%), time of exposure to plasmids at RT (1-3 min), heat shock exposure time (1-10 min), and application of lower centrifugation force (1000xg down from 2000xg) to increase viability of protoplasts since they are very fragile to physical forces. Unfortunately, no colonies were obtained after repeating the procedure with these adjustments. In addition to these, protoplasts were washed with the buffer prior to transformation. This would remove restriction enzymes in the medium released from protoplasts, thus introduced DNA would be unharmed. Also, as suggested by Kieser *et al.* (2000), the use of neither denatured DNA nor the *gap1::aphII* linear fragment prior to introduction into protoplasts to facilitate homologous recombination rate did not provide any help.

In a previous study (Caydasi, 2006), several parameters for delivery of DNA into wild type *S. clavuligerus* NRRL 3585 were modified, still only a single colony could be obtained. It might be expected that for DEPA strain with unidentified mutations in its genome, genetic modifications would be more time-consuming and labor-intensive so that new optimizations and adjustments of protocols will be needed.

3.3.4. Verification of *gap1::aphII* delivery into *S. clavuligerus* DEPA via conjugation

The conjugation procedure was repeated several times as it is the fastest and easiest method to perform as compared to the other two. Thus, after several trials of conjugation with vegetative *S. clavuligerus* DEPA cells, only one colony was obtained on TSA plate in the presence of kanamycin (50 µg/ml) and apramycin (50 µg/ml). This colony was named as *S. clavuligerus* ID+. Colony PCR with *gap1* and *aphII* primers showed amplification of *gap1*, *gap1::aphII*, and *aphII* (Figure 3.11 and 3.12). Also, a third band (II) was seen in between *gap1::aphII*-sized band (I) and *gap1*-sized band (III) on the gel. To characterize the bands I and II, they were extracted from the gel separately, and subjected to PCR amplification with *aphII* primers. It was shown that both bands include kanamycin resistance gene (Figure 3.13). Also, these two bands were sequenced with *gap1* primers, and both ends of these amplified PCR products belonged to *gap1* of *S. clavuligerus*. It could be deduced that there is a deletion either at upstream of *aphIIF* primer or downstream of *aphIIR* primer binding sites. This deletion could be related to host defense system or genetic instability of *Streptomyces*.

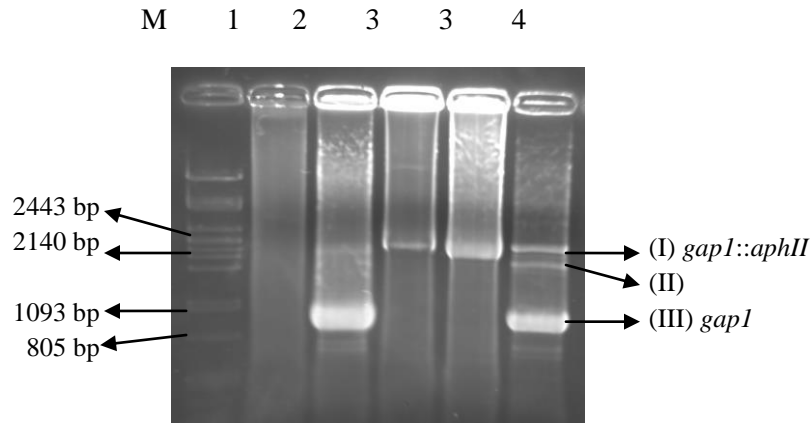


Figure 3.11: Colony PCR with *gap1* primers to confirm presence of *gap1::aphII*; M: λ /*PstI* DNA ladder, 1: Negative control with no template DNA, 2: *gap1* PCR amplicon obtained by using *S. clavuligerus* DEPA genomic DNA as template, 3: *gap1::aphII* PCR amplicon obtained by using pL21 as template, 4: PCR amplicons obtained by using *S. clavuligerus* ID+ DNA as template.

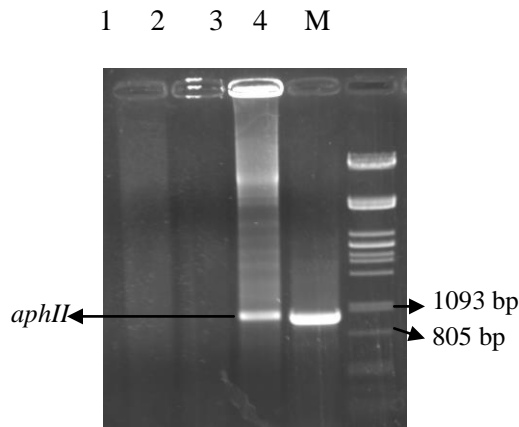


Figure 3.12. Colony PCR with *aphII* primers to confirm presence of *aphII*; M: λ /*PstI* DNA ladder, 1: Negative control with no template, 2: No PCR amplicon obtained by using *S. clavuligerus* DEPA genomic DNA as template, 3: *aphII* PCR amplicon obtained by using pL21 as template, 4: *aphII* PCR amplicon obtained by using *S. clavuligerus* ID+ DNA as template.

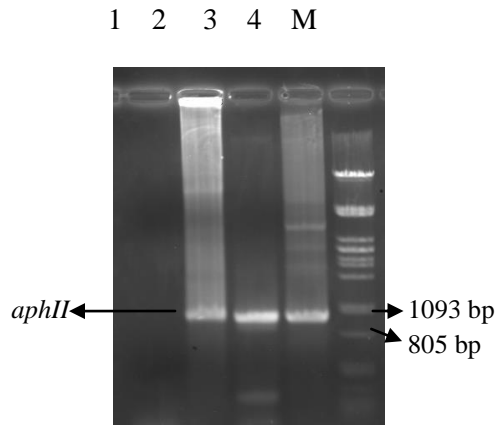


Figure 3.13. Nested PCR of band I and band II with *aphII* primers: M: λ PstI DNA ladder, 1: Negative control with no template DNA, 2: Positive control of PCR (*aphII* PCR amplicon obtained by using demethylated pL21 as template DNA), 3: *aphII* PCR amplicon obtained by using second longest band (II) in ID+ lane of Figure 3.11 as template DNA, 4: *aphII* PCR amplicon obtained by using the longest band (I) in ID+ lane of Figure 3.11 as template DNA.

3.4. Plasmid curing for selection of *gap1*-disrupted mutants

Homologous recombination is a rare event which occurs between two similar DNA strands and results in exchange of these strands. With the introduction of *gap1::aphII* into *S. clavuligerus* DEPA strain, the last part of this study should involve the selection of *gap1*-disrupted mutants which exchanged the original copy of *gap1* in their genome with the disrupted copy on pL21. After plasmid curing, some cells would lose pL21 and return to their previous state while some of them will contain *gap1::aphII* in their genome and lose original copy on pL21. Therefore, the desired mutant would show kan^R , apr^S phenotype whereas the others will become kan^S , apr^S .

Prior to plasmid curing experiments, DEPA strain was inoculated into minimal medium (Appendix A) to test its growth in the presence of glycerol as the sole carbon source. After five days of incubation, highly scattered small colonies were observed on the plate. Although the growth was slow, it proved the existence of functional glycerol utilization pathway in DEPA strain. Therefore, if there would be a *gap1::aphII* mutant, it could survive through utilizing glycerol as the sole carbon source which is added to the selection medium. Up to 10% (v/w) glycerol was added into TSA medium in order

to ensure the survival of disrupted mutants. It was recorded that this concentration was not interfering with the growth of DEPA strain on TSA plates whereas higher concentrations of glycerol caused loss of integrity of agar medium.

There are several ways of curing plasmids in bacterial cells including subsequent sporulation cycles, using chemical agents (acrydine dyes, ethidium bromide etc.), exposure to UV light, introduction of incompatible plasmid, protoplast regeneration, and subculturing in an antibiotic free environment. These methods generally depend on halting plasmid segregation and replication (Grinsted and Bennett, 1988; Liu *et. al.*, 2012).

The use of chemical agents, or UV has proven its success of plasmid curing, it is also a fact that such treatments can cause random mutations on other parts of the DNA which is not desired for an extensively mutated strain like DEPA. In addition, there are no standard protocols applicable to all plasmids and bacterial strains since the effectiveness of curing agents may vary (Trevors, 1986; Grinsted and Bennett, 1988; Liu *et. al.*, 2012).

When a plasmid carrying both a lethal gene and the desired construct is introduced into a target organism, the lethal gene is only activated when a certain condition is met like a certain temperature or sensitivity to high sucrose. Then, a second plasmid in the same incompatibility group of the first one is introduced to the same organism. Since they cannot coexist in the same organism due to shared mechanisms for replication and partitioning, one of them will be eliminated. When the condition for lethality gene is applied, only the cells with second plasmid can survive (Liu *et. al.*, 2012). This method is not feasible since the introduction of DNA into DEPA strain is difficult.

Subculturing in an antibiotic free environment might cause curing of plasmids. The absence of antibiotic stress will allow survival of plasmidless cells. These cells arise from plasmid containing cells during proliferation by failure of plasmid replication. For this reason, ID+ cells were grown in liquid TSB medium and subcultured seven times. After last growth, the cells were spread on TSA plates with kanamycin (50 µg/ml), and then with apramycin (50 µg/ml). No singular colony was detected, and the growth on both plates was high showing that this method was ineffective.

Plasmid curing through subsequent rounds of sporulation is a widely used method to remove plasmids without damaging cells (Kieser *et. al.*, 2000). As the next step of the experiment, the cells were subjected to five subsequent rounds of sporulation via replica plating in order to remove the plasmid. After last round of sporulation, replica plating was employed in order to find kan^R, apr^S clone(s) on TSA plates with kanamycin (50 µg/ml) and then with apramycin (50 µg/ml). From these replicas, a few numbers of viable colonies were obtained, but all grew on both plates, and in common, there were many hyphae structures on plate surface rather than single colonies. Therefore, several conditions and parameters were thought to be optimized.

It was observed that each round of replica plating decreases the number of spores transferred to a new plate, and each sporulation cycle takes 7 to 10 days. In order to optimize the method for a limited time frame, the number of rounds was gradually decreased to 4, 3, and finally to 2. It was observed that after second round of sporulation, the number of viable colonies obtained decreased significantly in that the total number was no different than after fourth and fifth rounds. The number of colonies varied, but as an average, it was seen that 4 to 5 colonies appeared on each plate. In addition, these colonies showed no sign of plasmid curing.

According to a study based on sporulation levels and conditions of several *Streptomyces* species, several alterations in protocol were tested for plasmid curing (Daza *et al.*, 1989). First of all, to produce high amounts of spores and decrease the time of sporulation, liquid medium was used. In replica plating, vegetative cells are also transferred from plate to plate which interferes with the goal of obtaining plasmid free cells. Since spore formation on agar plates requires aerial hyphae consisting of several cells, obtaining a colony of homogeneous plasmid-free cells would be problematic. It was assumed that liquid medium would also help to obtain high number of spores well-separated from each other and independent of hyphae structure. Separating spores from vegetative cells via passing the cell suspension through cotton plugged tube would provide hyphae free spores which could be inoculated into fresh liquid sporulation medium. In this way, after a few rounds, a highly dense spore suspension ready for selection could be obtained. Nevertheless, at liquid YEME medium, no growth or sporulation could be observed in this study. According to the findings reported by Daza *et al.* (1989), not all *Streptomyces* spp. sporulated in liquid media, but some species shown to have a higher rate of sporulation in certain conditions. It was reported that decreasing concentrations of KH_2PO_4 and increasing concentrations of CaCl_2 facilitates sporulation of *Streptomyces*. For this purpose, liquid R2YE medium with no KH_2PO_4 was used, but only vegetative cell growth occurred. In addition to the removal of KH_2PO_4 from medium, the amount of CaCl_2 was doubled, but still no sporulation occurred in liquid R2YE.

3.4.1. Application of a newly developed plasmid curing method for selection of *gap1::aphII* mutants of *S. clavuligerus* DEPA

A new method was designed which is based on sporulation on agar plates and the knowledge reported so far (Daza *et al.*, 1989) in order to obtain single colony spores. First, a 100 ml culture in TSB medium with antibiotics was grown for 3 days at 28 °C in an orbital shaker. Cells were harvested at 8000xg for 10-15 minutes, washed twice with TSB to remove antibiotics, and homogenized in a Dounce homogenizer tube. Homogenization step is required to break hyphae structures as much as possible. This homogenate was spread on 20 different YEME sporulation plates and left to incubation at 26 °C for 10 days. In this way, it was aimed at increasing the amount of spores.

Spores were harvested by scratching via micropipette tip easily owing to thick layer of spores. Then, these spores (some attached to unwanted vegetative hyphae) were mixed with dH₂O. dH₂O creates osmotic pressure to disrupt vegetative cells, but not spores. This suspension was incubated at 55 °C for 30 minutes to kill vegetative cells. Spores are resistant to this temperature, still some of them might be lost. Since the number of spores was high enough because of high inoculation volume, the loss would not affect the final result significantly. Then, heat-treated suspension was homogenized for 2 min to break the bonds between spores and hyphae. Spores are relatively resistant to mechanical stress, but vegetative cells would be easily destroyed in their weakened state after water pressure and heat. The suspension was washed twice with dH₂O at 8000xg for 5-10 minutes to remove unwanted elements from spores. The pellet was dissolved in 200 ml of dH₂O, and spread on YEME sporulation plates in 1 ml aliquots. This high dilution was necessary to separate spores from each other to obtain colonies from single spores. After 5-7 days of incubation, individual spore colonies appeared. These colonies were grown on TSA plates containing 25 mg/ml kanamycin. At least 50 colonies were counted on each plate. Viable colonies were also tested if they grew on TSA including 50 mg/ml apramycin. Colonies giving positive results for kan^R apr^S were tested via PCR amplification by using *gap1* primers (Figure 3.14). Although about 50% of tested colonies lost their plasmids (lane 1-11), no colonies were detected as positive for *gap1::aphII* integration to their genome.

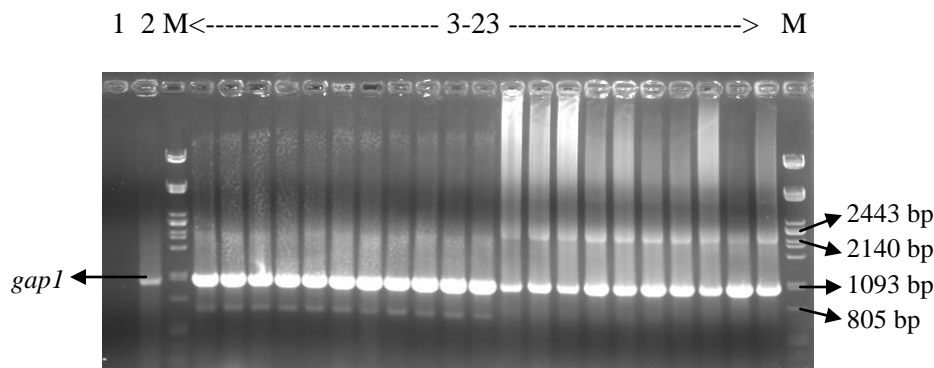


Figure 3.14. Screening of colonies by colony PCR with *gap1* primers; M: λ /*Pst*I DNA ladder, 1: Negative control with no template, 2: Positive control (*gap1* PCR amplicons obtained by using *S. clavuligerus* DEPA colony as template DNA), 3-23: PCR amplicons from DNAs of ID+ colonies subjected to plasmid curing

CHAPTER 4

CONCLUSION

- In this study, the *gap1* of *S. clavuligerus* DEPA strain was disrupted by kanamycin resistance gene (*aphII*) cassette. The construct was cloned into the vector aac(3)IVKS+ to form suicide plasmid, pL21. Among three different methods used to introduce DNA into *Streptomyces* cells, a single recombinant colony of *S. clavuligerus* DEPA strain (ID+) carrying pL21 was obtained via conjugation.
- Subsequent rounds of sporulation were employed for plasmid curing, so *gap1*-disrupted mutant could be selected via its kan^R, apr^S phenotype. This method was ineffective, so a new plasmid curing method was innovated. With this method, over 3000 single spore colonies were screened through antibiotic tests, and around 150 of them were selected as kan^R apr^S. From PCR tests with *gap1* primers, about 30-50% of them showed successful plasmid curing whereas none of them showed any sign of homologous recombination. They should have either kept the plasmid or been cured before homologous recombination occurred.
- *S. clavuligerus* DEPA strain is sensitive to kanamycin and apramycin whereas resistant to nalidixic acid, like the wild type strain.
- *S. clavuligerus* DEPA strain has a well-working glycerol utilization system for its survival, but survival through this system on minimal medium decreases growth rate at least twice.
- Many trials for the transfer of pL21 into *S. clavuligerus* with all three different methods failed. For this, plasmid incompatibility is not thought to play a role.

Future Prospects

- Higher number of spore colonies could be screened to find the disrupted mutant, since the rate of homologous recombination is low, as reported by Li and Townsend (2006). After confirmation of disruption by PCR and Southern blotting, CA production will be measured by bioassay and HPLC analysis of samples taken from batch culture at 24 h intervals for a week, and DNA quantification will give the cell density when it comes to fermentation. From these data, specific production will be calculated and compared for original DEPA strain and *gap1*-deleted mutant to show the effect of this disruption on CA yield.

- Several characteristics of the industrial DEPA strain should be determined via proteomics to show which parts of the cell function have been affected throughout random and continuous mutagenesis/selection processes conducted at the laboratories of the relevant industry.
- Also remains to be analyzed will be as to whether a homologous recombination system still exists with full functionality in the DEPA strain.
- There might be more than one copy of *gap1* existing in the genome of *S. clavuligerus* DEPA strain. In this case, it would be more difficult to obtain a disrupted mutant since all copies of this gene should be replaced with *gap1::aphII* construct. In addition, even if one copy was replaced with disrupted one, it could not be detected due to amplification of both original and disrupted copies by PCR. Therefore, *gap1* copy number should be determined through Southern blotting and/or qPCR.
- The fate of other glycolysis intermediates which cannot be produced due to *gap1* disruption should be determined, and checked as if glycolysis was the sole pathway to produce these metabolites. It is possible that random mutagenesis may render alternative pathways nonfunctional.

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APPENDIX A

COMPOSITION AND PREPERATION OF CULTURE MEDIA

Luria Broth (LB)

25 g/L Luria Broth

Autoclave sterilization at 121 °C for 15 min

Tryptic Soy Broth (TSB)

30 g/L Tryptic Soy Broth

Autoclave sterilization at 121 °C for 15 min

LB Agar (LA)

25 g/L Luria Broth

15 g/L Agar

Autoclave sterilization at 121 °C for 15 min

TSA

30 g/L Tryptic Soy Broth

15 g/L Agar

Autoclave sterilization at 121 °C for 15 min

Mannitol-Soybean Flour (MS) Agar

20 g/L Mannitol

20 g/L Soybean Flour

20 g/L Agar

Autoclave sterilization at 121 °C for 15 min

Sterile MgCl₂ (10mM final concentration) addition before use

CRM Medium

10 g/L Glucose

103 g/L Sucrose

10.12 g/L MgCl₂

15 g/L TSB

5 g/L Yeast Extract

Autoclave sterilization at 121 °C for 15 min

R2YE Regeneration Medium

103 g Sucrose

0.25 g K₂SO₄

10.12 g MgCl₂.6H₂O

10 g Glucose

5 g Yeast Extract

0.1 g Casamioacids

22 g Agar

800 ml dH₂O

Autoclave sterilization at 121 °C for 15 min

Addition of following sterile solutions;

10 ml 0.5% KH_2PO_4

80 ml 3.68% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

15 ml 20% L-proline

100 ml 5.73% TES Buffer (pH 7.2)

5 ml 1N NaOH

2 ml Trace Elements Solution

Yeast Extract-Malt Extract (YEME) Medium

4 g Yeast Extract

10 g Malt Extract

1 L dH_2O

pH adjustment to 7.3 with KOH

20 g Agar

Autoclave sterilization at 121 °C for 15 min

Addition of following sterile solutions;

8 ml 50% Glucose

0.2 ml $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (100 mg/ml)

Minimal Medium Agar for *S. clavuligerus* (Kieser *et al.*, 2000; modified)

2.1 g $(\text{NH}_4)_2\text{SO}_4$

0.5 g K_2HPO_4

0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

20 g Agar

980 ml dH₂O

Autoclave sterilization at 121°C for 15 min

Addition of following sterile solutions;

20 ml 50% Glycerol

APPENDIX B

BUFFERS AND SOLUTIONS

SET Buffer

75 mM NaCl

25 mM EDTA (pH 8.0)

20 mM Tris-HCl (pH 7.5)

TE Buffer

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

STE Solution

10.3% sucrose

25 mM EDTA (pH 8.0)

25 mM Tris-HCl (pH 8.0)

2 mg/ml lysozyme

Lysis Solution

0.3 M NaOH

2% SDS

TTX Solution

0.5 g/L Triton-X-100

9 g/L NaCl

Phenol-Chloroform Solution (Water Saturated) (Hintermann, 1981)

500 g Phenol

500 mL Chloroform

400 mL dH₂O

Storage at RT in dark

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

242 g Tris base

57.1 mL Glacial acetic acid

125 mL EDTA (0.4 M, pH 8.0)

Complete volume to 1 L with dH₂O

Loading Buffer (6X)

0.25% Bromophenol Blue

0.25% Xylene Cyanol FF

40% Sucrose in water

Ethidium Bromide Solution

100 µL/L Ethidium Bromide (10 mg/mL)

1 L TAE buffer (1X)

Competence Buffer 1

0.145 g $\text{C}_2\text{H}_3\text{KO}_2$ (30 mM)

0.605 g RuCl_2 (100 mM)

0.075 g CaCl_2 (10 mM)

8.6 ml Glycerol (87%)

Before filter sterilization volume is completed to 50 ml with dH_2O .

Competence Buffer 2

0.105 g MOPS (10 mM)

0.060 g RuCl_2 (10 mM)

0.350 g CaCl_2 (75 mM)

8.6 ml Glycerol (87%)

Volume is completed to 50 ml with dH_2O and pH is adjusted to 6.5 with KOH before filter sterilization.

PE Buffer

171 g Sucrose

0.1 g K_2SO_4

784 ml dH_2O

Autoclave sterilization at 121 °C for 15 min

Add following sterile solutions before use;

1 ml Trace Elements Solution

25 ml 5.72% TES Buffer (pH:7.2)

190 ml 3.68% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

TES Buffer

25 mM Tris-HCl

25 mM EDTA

10.3% Sucrose

Adjust pH to 7.2

Trace Elements Solution

40 mg/L ZnCl₃

200 mg/L FeCl₃.6H₂O

10 mg/L CuCl₂.2H₂O

10 mg/L MnCl₂.4H₂O

10 mg/L Na₂B₄O₇.10H₂O

10 mg/L (NH₄)₆Mo₇O₂₄.4H₂O

Antibiotic Stocks

	Stock concentration	Final concentration
Ampicillin	100 mg/mL dH ₂ O	100 µg/mL
Kanamycin	50 mg/mL dH ₂ O	25 or 50 µg/mL
Apramycin	50 mg/mL dH ₂ O	50 µg/mL
Chloramphenicol	25 mg/mL ethanol	25 µg/mL
Nalidixic Acid	25 mg/mL dH ₂ O	25 µg/mL
Thiostrepton	50 mg/mL dH ₂ O	50 µg/mL

APPENDIX C

CHEMICAL, ENZYME AND KIT SUPPLIERS

C.1. Chemicals

Agar	Sigma
Agarose	Sigma
Ampicillin	Sigma
Apramycin	Sigma
Bromophenol Blue	Sigma
$C_2H_3KO_2$	Merck
$CaCl_2 \cdot 2H_2O$	Merck
Casaminoacids	Becton Dickinson
Chlorofom	AppliChem
Chloramphenicol	Sigma
$CoCl \cdot 6H_2O$	Merck
$CuCl_2 \cdot 2H_2O$	Merck
DMSO	Sigma
EDTA	Sigma
Ethanol	Botafarma
Ethidium Bromide	Sigma
$FeCl_3 \cdot 6H_2O$	Merck
$FeSO_4 \cdot 7H_2O$	Merck
Glacial Acetic Acid	Merck
Glucose	Merck

Glycerol	Merck
HCl	Sigma
Isopropanol	Merck
Kanamycin	Sigma
K_2HPO_4	Merck
K_2SO_4	Ventron
KH_2PO_4	Merck
L-Proline	Merck
Luria Broth	Sigma
Malt Extract	International Diagnostics Group
Mannitol	Merck
$MgCl_2 \cdot 6H_2O$	Merck
$MgSO_4 \cdot 7H_2O$	Merck
$MnCl_2 \cdot 4H_2O$	Merck
MOPS	Sigma
$Na_2B_4O_7 \cdot 10H_2O$	Merck
NaCl	Sigma
Nalidixic Acid	Sigma
NaOH	Merck
$(NH_4)_2SO_4$	Merck
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Sigma
PEG1000	Merck
Phenol (Water-Saturated)	AppliChem
$RuCl_2$	Merck
Sodium Dodecyl Sulfate (SDS)	Merck

Soybean Flour	Commercial
Sucrose	Merck
Tris-HCl	Merck
Tris Base	Sigma
Triton-X-100	Sigma
Tryptic Soy Broth	Oxoid
Xylene Cyanol FF	Sigma
Yeast Extract	Becton Dickinson
ZnCl ₃	Merck

C.2. Enzymes

Lysozyme	Sigma
Proteinase K	Sigma
<i>Apa</i> I	Roche
<i>Bsp</i> I	Roche
<i>Eco</i> RI	Roche
<i>Hind</i> III	Roche
<i>Spe</i> I	Roche
Taq DNA polymerase	Fermentas
Phusion DNA polymerase (Pfu)	NEB
T4 DNA polymerase	Fermentas
Klenow Fragment	Fermentas
T4 DNA ligase	Fermentas
Alkaline Phosphatase	Roche

C.3. Kits

Plasmid Isolation Kit (Mini)

Fermentas

Plasmid Isolation Kit (Midi)

Qiagen

Gel Elution Kit

GeneMark

Gel Extraction Kit

Qiagen

APPENDIX D

VECTOR MAPS AND DNA LADDERS

D.1. Vector maps

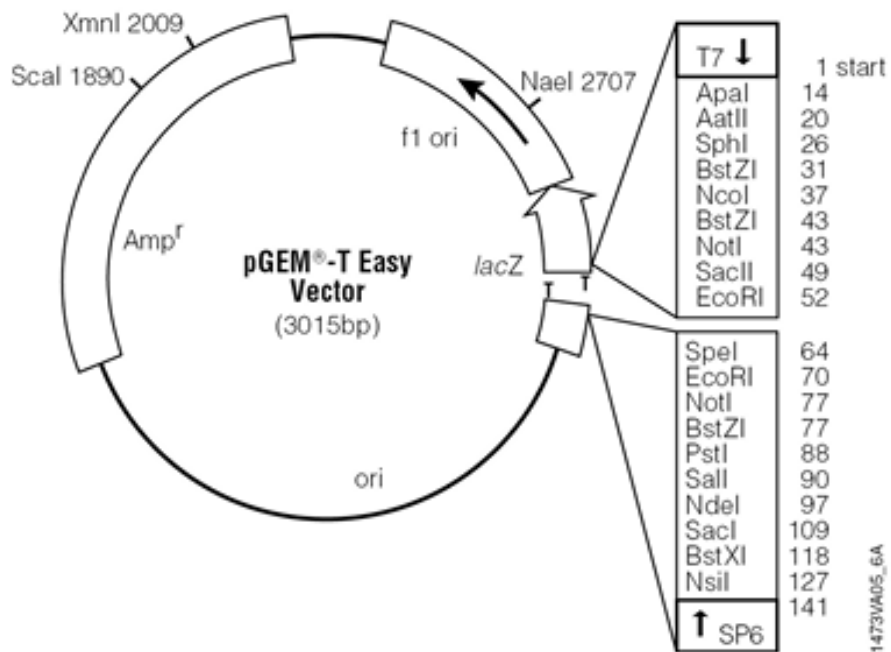


Figure D.1. pGEM-T Easy Vector (Promega)

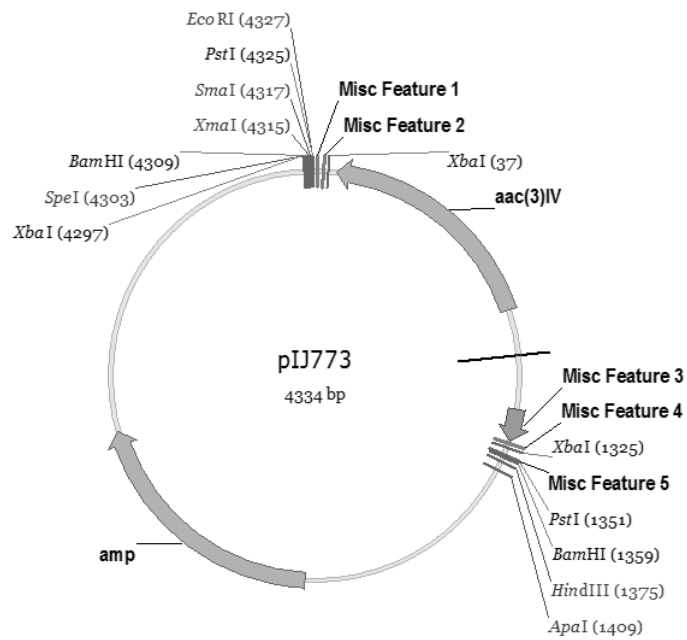


Figure D.2. pIJ773

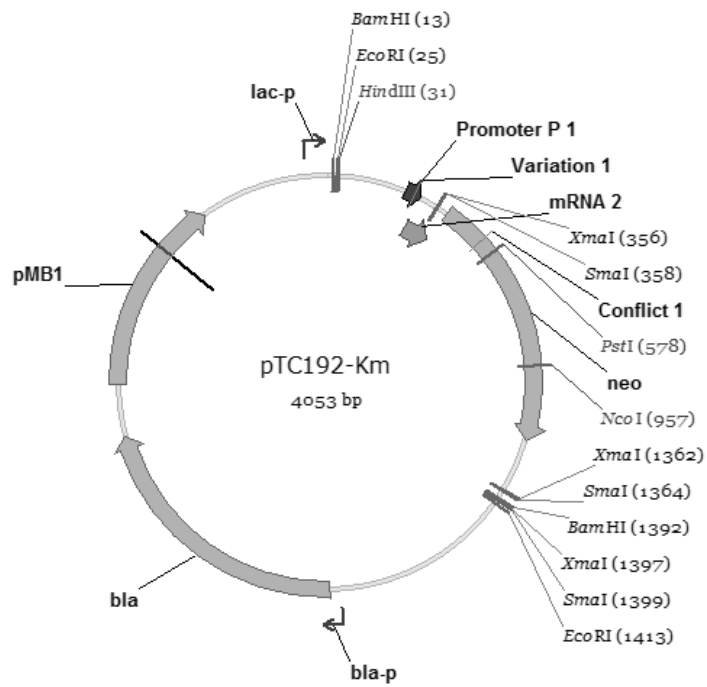


Figure D.3. pTC192-Km

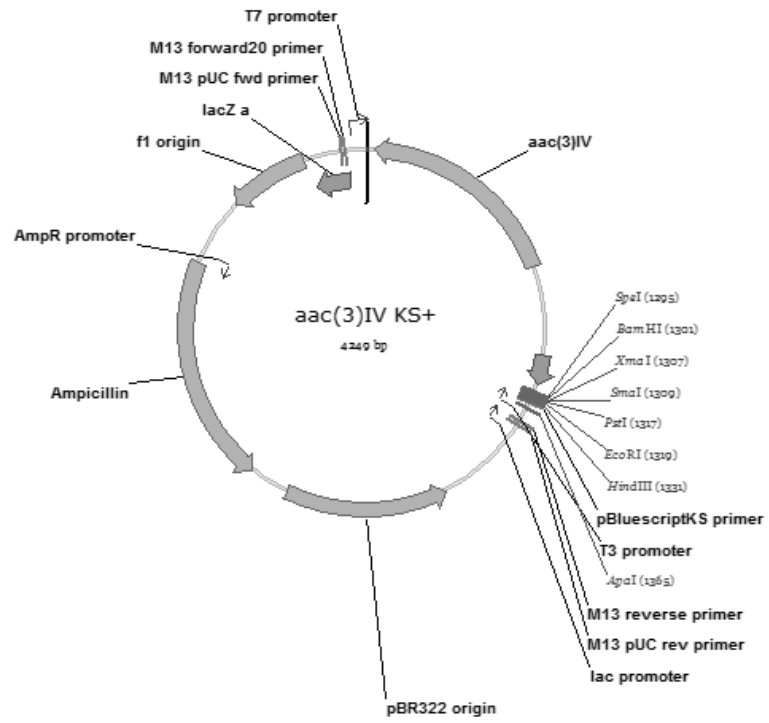


Figure D.4. *aac(3)IVKS(+)*

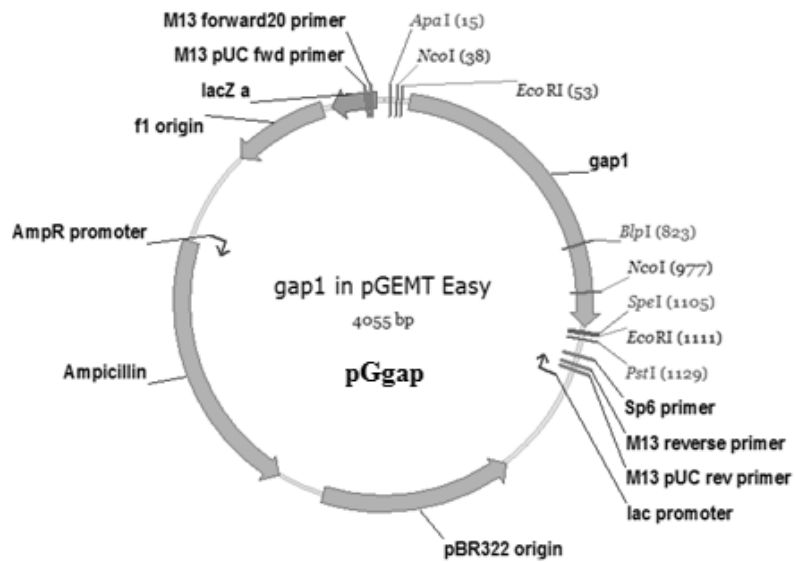


Figure D.5. *pGgap*

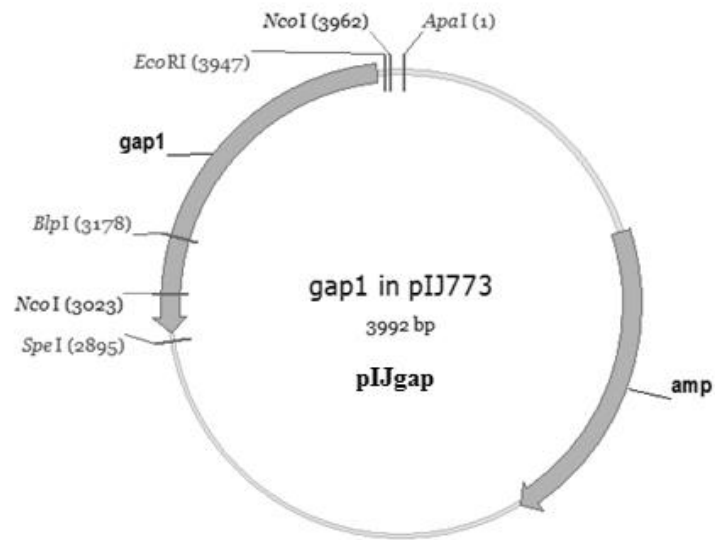


Figure D.6. pIJgap

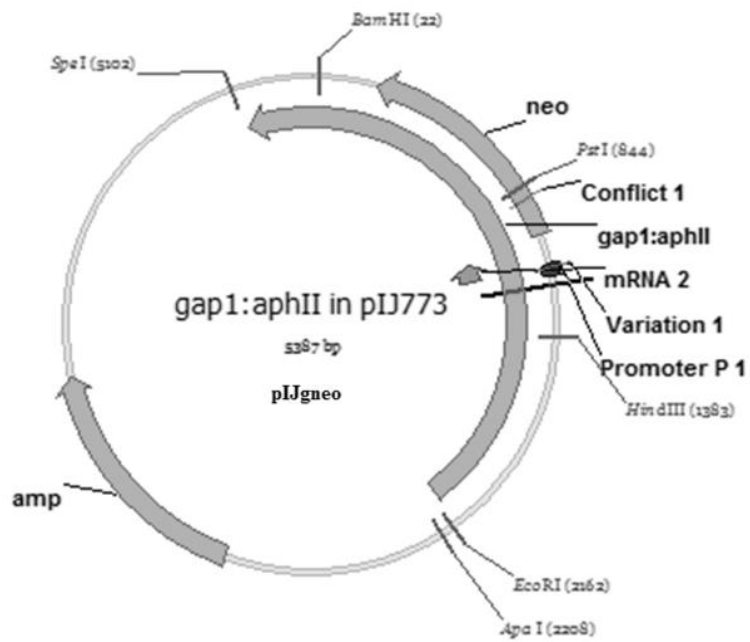


Figure D.7. pIJgneo

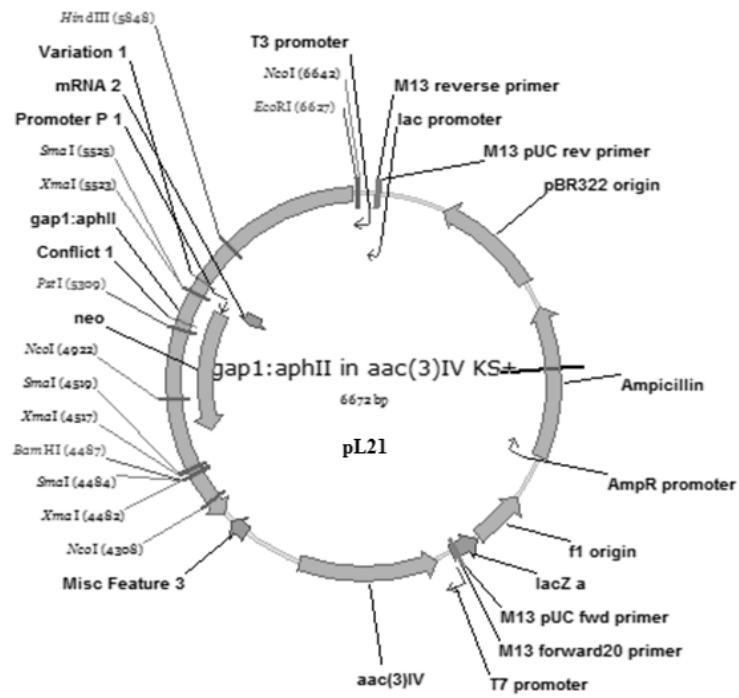


Figure D.8. pL21

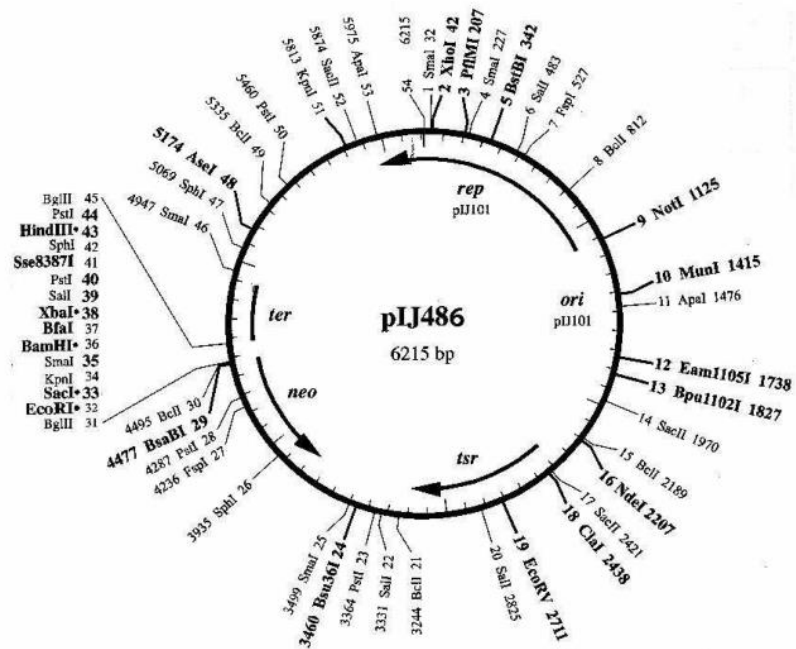


Figure D.9. pIJ486

D.2. DNA ladders

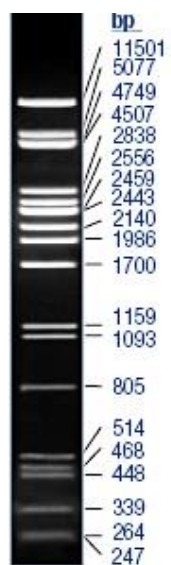


Figure D.10. Fermentas λ DNA/*Pst*I Ladder

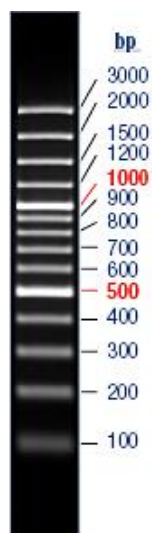


Figure D.11. Fermentas O'GeneRuler 100 bp Plus DNA Ladder