THE REGULATORY EFFECT OF CCAR ACTIVATOR ON THE CEPHAMYCIN C GENE CLUSTER OF *STREPTOMYCES CLAVULIGERUS*

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ASLIHAN KURT

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOLOGICAL SCIENCES

DECEMBER 2011

Approval of the thesis:

THE REGULATORY EFFECT OF CCAR ACTIVATOR ON THE CEPHAMYCIN C GENE CLUSTER OF *STREPTOMYCES CLAVULIGERUS*

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ABSTRACT

THE REGULATORY EFFECT OF CCAR ACTIVATOR ON THE CEPHAMYCIN C GENE CLUSTER OF *STREPTOMYCES CLAVULIGERUS*

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December 2011, 162 pages

Streptomyces clavuligerus produces industrially important secondary metabolites such as cephamycin C (a β-lactam antibiotic) and clavulanic acid (a potent β-lactamase inhibitor). Cephamycin C is active against penicillin-resistant bacteria due to presence of methoxyl group in C-7 position of cephalosporin nucleus. Clavulanic acid is prescribed in combination with β-lactams for treatment of various bacterial infections. Cephamycin C and clavulanic acid gene clusters form β-lactam supercluster in *S. clavuligerus* genome. CcaR (**C**ephamycin C-**C**lavulanic **A**cid **R**egulator), encoded by *ccaR*, located in cephamycin C gene cluster, is a positive regulator of β-lactam supercluster. Previous studies on cephamycin C gene cluster have used different techniques, such as S1 nuclease (Paradkar *et al*., 1994), Northern blot (Perez-Llarena *et al*., 1997), and Western blot (Alexander and Jensen, 1998) to determine expression of cephamycin C

genes at mRNA level and to identify their functions at protein level, and they have studied on different parts of the cluster. Hence, a comprehensive study is needed to understand molecular mechanisms of pathway-specific regulation of cephamycin C production by *S. clavuligerus*.

In this study, time-dependent expression levels of cephamycin C gene cluster in a *ccaR*-disrupted mutant and *ccaR*-overexpressed recombinant strain of *S. clavuligerus* as compared to those in the wild strain were analysed by RT-PCR and qRT-PCR. In addition, DNA-binding sequences of CcaR on cephamycin C gene cluster were examined by EMSA. The effect of *ccaR* disruption and overexpression on cephamycin C and clavulanic acid yields were determined by bioassay and HPLC.

Three polycistronic and two monocistronic transcripts were obtained by RT-PCR. CcaR regulation showed its effect on mostly *ccaR, lat*, *cmcI*, *cefD*, *blp* and *cefF* expression levels. qRT-PCR data was supported by EMSA showing CcaR binding to *lat*, *cefD–cmcI* and *ccaR* promoters. *ccaR* overexpression from multi-copy recombinant plasmid resulted in significant increase in cephamycin C and clavulanic acid yields, making the respective recombinant strain as an attractive industrial strain. qRT-PCR data presented herein constitute the first that reveal the effect of CcaR activator on the expression of cephamycin C genes in a time-dependent manner.

Keywords: *Streptomyces clavuligerus*, cephamycin C gene cluster, *ccaR*, regulation, qRT-PCR

ÖZ

CCAR AKTİVATÖRÜNÜN *STREPTOMYCES CLAVULİGERUS*'A AİT SEFAMİSİN C GEN KÜMESİ ÜZERİNDEKİ DÜZENLEYİCİ ETKİSİ

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Aralık 2011, 162 sayfa

Streptomyces clavuligerus, bir β-laktam antibiotiği olan sefamisin C ve güçlü bir β-laktamaz inhibitörü olan klavulanik asit gibi endüstriyel açıdan önemli sekonder metabolitleri üretmektedir. Sefamisin C, sefalosporin çekirdeğinin C-7. pozisyonunda bir metoksil grubunun varlığı dolayısıyla penisiline dirençli bakterilere karşı etkilidir. Klavulanik asit β-laktam antibiyotikleri ile birlikte bakteri nedenli enfeksiyonların tedavisinde kullanılmaktadır. Sefamisin C ve klavulanik asit gen kümeleri *S. clavuligerus*' un genomunda β-laktam süperkümesini oluştururlar. Sefamisin C gen kümesinde yeralan *ccaR* geni tarafından kodlanan CcaR (Sefamisin C-Klavulanik asit Regülatörü) β-laktam süperkümesinin pozitif bir regülatörüdür. Sefamisin gen kümesi ile ilgili daha onceki çalışmalar sefamisin C genlerin ekspresyonlarını ve protein düzeyinde fonksiyonlarını belirlemek üzere S1 nükleaz (Paradkar *ve ark.,* 1994), Northern (Perez-Llarena *ve ark.,* 1997) ve

Western blot (Alexander ve Jensen, 1998) gibi farklı teknikleri kullanmış olup gen kümesinin farklı kısımları üzerinde çalışmışlardır. Bu yüzden, *S. clavuligerus* tarafından antibiyotik üretimini anlamaya yönelik geniş kapsamlı bir çalışmaya gereksinim vardır.

Bu çalımanın amacı, hücre içerisinde sefamisin C biyosentezinin yolak-özgü CcaR proteini tarafından regülasyonunun moleküler mekanizmalarını detaylı bir şekilde incelemektir. Bu amaçla, RT-PCR ve qRT-PCR çalışmalarıyla, hücrede pozitif regülatör gen *ccaR*' nin yokluğunda ve bu genin çoklu ifadesi durumunda tip suşa kıyasla sefamisin C biyosentetik gen kümesinin zamana bağlı ekspresyon profili analiz edilmiştir. Ayrıca, CcaR proteininin sefamisin gen kümesindeki DNA-bağlanma bölgeleri EMSA deneyleri ile incelenmiştir. Yine *ccaR* geninin olmadığı ya da çok kopya olarak bulunduğu rekombinant suşlarda sefamisin C ve klavulanik asit üretimi biyoassay ve HPLC analizleri ile tespit edilmiştir.

Sefamisin C gen kümesindeki genlerin RT-PCR analizleriyle, üç farklı polisistronik ve iki monosistronik transkript belirlenmiştir. Sefamisin C gen kümesinin CcaR tarafından kontrolü, en çok *ccaR*, *lat*, *cmcI*, *cefD*, *blp* ve *cefF* genlerinin ekspresyon seviyelerinde gözlenmiştir. qRT-PCR ile elde edilen bu veriler, CcaR'nin *lat*, *cefD–cmcI*, *cefF*, *blp* ve *ccaR* promotor bölgelerine bağlandığının gösterildiği EMSA sonuçlarıyla desteklenmiştir. *ccaR*' nin hücrede çoklu ifadesi her iki sekonder metabolitin üretiminde önemli bir artış sağlayarak biyoteknolojik açıdan etkin bir üretici suş geliştirilmesini sağlamıştır. Bu çalışmanın sonuçları, CcaR aktivatörünün sefamisin C kümesindeki genlerin ekspresyonları üzerindeki zaman bağlı etkisini ortaya koyan ilk raporu oluşturmaktadır.

Anahtar kelimeler: *Streptomyces clavuligerus*, sefamisin C gen kümesi, *ccaR* geni, regülasyon, qRT-PCR

To My Family

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude and sincerest appreciation to my supervisor Prof. Dr. Gülay Özcengiz. Her supervision, constructive criticism, guidance, understanding and insights throughout the study and also the years that I spent in her laboratory have triggered invaluable contributions to my scientific progress and intellectual maturity. I owe a deep indebtedness to her more than she knows.

I am also grateful to Prof. Dr. Ufuk Gündüz and Assist. Prof. Dr. Servet Özcan for their guidance and advices throughout the research.

I would like to acknowledge Prof. Dr. Paloma Liras and Prof. Dr. Juan Francisco Martín for their hospitality and support during my research in their laboratory in Inbiotec for one year. I also thank to Dr. Irene Santamarta and Dr. Maria Lopez-Garcia for their invaluable help on *Streptomyces* genetics and their great friendship during my stay over there.

It is a pleasure to thank my labmates Volkan Yıldırım, Orhan Özcan, Burcu Tefon, Elif Tekin, Alper Mutlu, İbrahim Sertdemir, Çiğdem Yılmaz, Eser Ünsaldı, Aycan Apak, İsmail Cem Yılmaz, Mustafa Demir, Mustafa Çiçek and Ayça Çırçır who made this thesis possible with their cooperation, understanding, patience and friendship. Very special thanks also go to my previous labmates İsmail Öğülür and Güliz Vanlı for their friendships, understanding and support during my studies.

I am thankful to Sezer Okay for his support and patience as well as understanding, encouragment and great friendship that made easier for me to overcome diffuculties in all hard times.

I dedicate this thesis to my mother Saime Kurt, my father Mustafa Kurt, my sister Türkan Ertan and my brother Refik Alper Kurt for their unconditional love, endless trust and support at any moment of my life. I also owe a very big thank to my sweetheart nephew Recep Kağan Ertan for giving me a joy of living with his participation to my life.

This thesis was accomplished with the continuous help, encouragment, support and unconditional kindness of numerous precious people. I would like to thank all of them for staying with me.

This study was supported by The Scientific and Technological Organization of Turkey (TÜBİTAK) Grant No: TBAG-109T962.

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LIST OF ABBREVIATIONS

ATTC	American Type Culture Collection
NRRL	Agricultural Research Service Collection
bp	Base pair
DepC	Dietylpyrocarbonate
dH_2O	Distilled water
dNTP	Deoxy Nucleotide Triphosphate
EtBr	Ethidium Bromide
ORF	Open Reading Frame
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
rpm	Revolution per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standard Error of the Means
aa	Aminoacid
kb	Kilo base
kDa	Kilo Dalton
EMSA	Electrophoretic Mobility Shift Assay
tsp	Transcription start point
Mb	Megabase
o/n	Overnight
RT	Room temperature

CHAPTER 1

INTRODUCTION

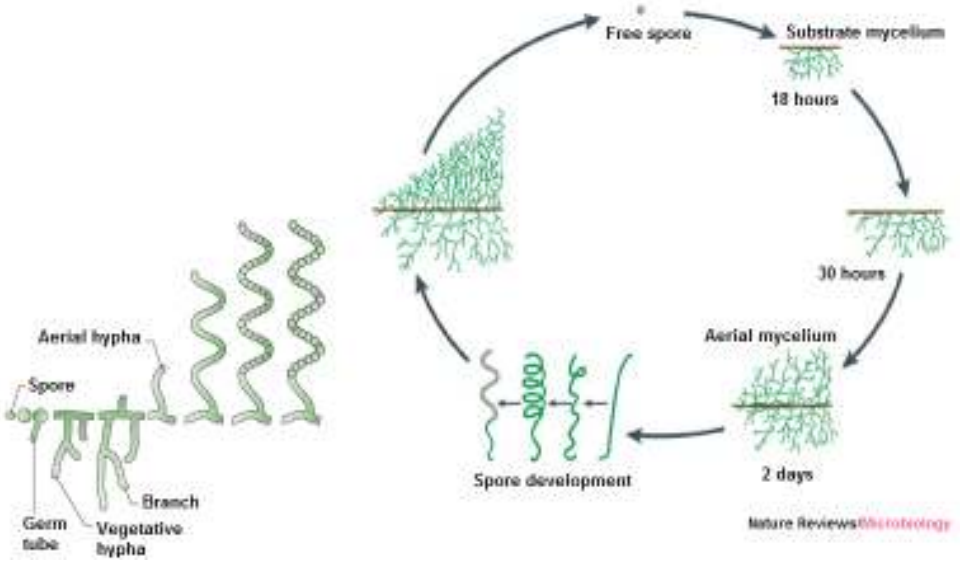
1.1. The genus *Streptomyces*

Streptomyces, are fasnicating genus as being a member of Actinomycetes. They are gram-positive, filamentous, spore forming and strictly aerobic bacteria having linear chromosome with a very high G/C content (often quoted as 70-74 mole %). Although most streptomycetes are saprophytic bacteria in soil, they also have adapted to live in different niches such as marine, freshwater and other terrestrial habitats as well as on plant (*S. scabies*) and animals (i.e. *S. griseus* causing the symptoms of a disease of fish lungs) (Hopwood, 2007; Chater *et al*., 2010). The classification of *Streptomyces*, according to the Bergey's manual of Sistematic Biology, was presented in below (Garrity *et al*., 2004, II. edition) (Table 1.1).

Table 1. 1. The classification of *Streptomyces* according to the Bergey's manual of Sistematic Biology

Domain	*Bacteria*	Order I	*Actinomycetales*
Phylum BXIV	*Actinobacteria*	Suborder XIV	*Streptomycineae*
Class I	*Actinobacteria*	Family I	*Streptomycetaceae*
Subclass V	*Actinobacteridae*	Genus I	*Streptomyces*

Streptomycetes have a strikingly different growth and developmental biology when compared to the other bacteria, rather resembling to filamentous fungi in their apical growth, branching, and morphogenesis. Its complex life cycle is divided into four steps: germination of free spore (i), vegetative mycelial growth (ii), aerial mycelial growth-acquired ability to produce abundant secondary metabolites (iii) and spore morphogenesis (iv) (Flärdh 2003; Goriely and Tabor, 2003; Chater, 2006) (Figure 1.1).



(a) **(b)**

Figure 1. 1. Life cycle of *S. coelicolor.* (a) Flärdh and Buttner, 2009. (b) http://twistedbacteria.blogspot.com/2007/08/streptomyces-theyre-twisted.html.

Spores are germinated via tip extension by forming long filamentary hyphae which grow in and on the nutrient surface and then undergo branching to give a substrate mycelium (the vegetative growth phase). This phase is followed by aerial mycelial growth. Subsequently, the microorganism produces secondary metabolites on solid media at this developmental stage depending on the nutrient

depletion and other signals. In liquid-grown cultures, the production of secondary metabolites is generally confined to stationary phase, and it is frequently assumed as a manifestation of nutrient limitation. The life cycle ends up with extensive cellular division of the aerial hyphae, uni-nucleoidal prespore compartments' formation, and maturing into free spores which have characteristic grey pigment formation (Goriely and Tabor, 2003; Bibb, 2005; Flärdh and Buttner, 2009; Chater *et al*., 2010) (Figure 1.2).

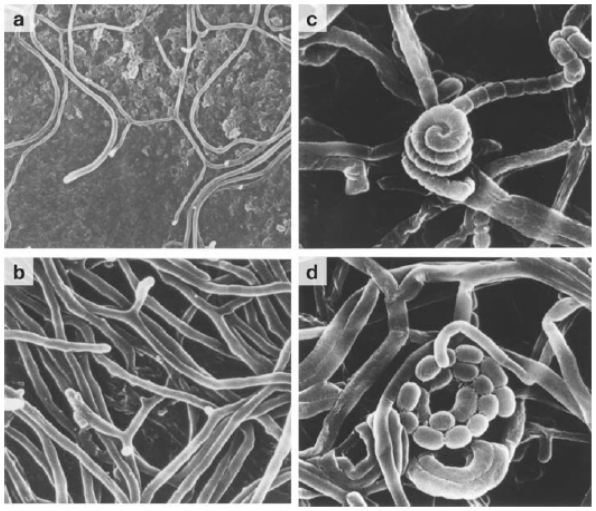


Figure 1. 2. Scanning electron microscopic examination of four stages in colony development in *S. lividans* (a) Young vegetative mycelium, (b) mature vegetative mycelium producing aerial branches, (c) aerial hypha developing into prespore, (d) chain of mature spores (Hopwood, 2006).

Streptomyces spp. are very well known for their production of a large variety of biologically active secondary metabolites including antibacterial and antifungal antibiotics, siderophores, antitumour agents, plant growth promoting factors, herbicides and immunosuppressors, and also degradative enzymes and enzyme inhibitors. 66 % of all the known secondary metabolites discovered up to now, are produced by actinomycetes, and 70 to 80 % of them are produced by the genus *Streptomyces* (Challis and Hopwood, 2003). The characteristic mycelial growth of streptomycetes have a very important impact on their performance and

productivity in large-scale fermentations thus improving their value as secondary metabolite producers in industry (Fladth, 2003).

The genome of actinomycetes are very large, being in a range of 5 to 9 megabases (Mb) and 5-10% of their DNA has been used for the production of secondary metabolites (Baltz, 2008). The presence of large linear chromosome is another striking feature of streptomycetes. These chromosomes consist of a “core” region and two “arms,” including essential housekeeping genes, and conditionally adaptive genes (acquired via horizontal gene transfer), respectively. Besides, both circular and linear plasmids, from 12 to several hundreds of kilobases (kb), have been found in *Streptomyces*, and among them, linear plasmids are involved in secondary metabolite production (Chater and Hopwood, 1993; Chang and Cohen, 1994; Kieser *et al*., 2000). In *Streptomyces*, replication occurs bidirectionally from a typical central origin, *oriC*. The end problem following replication in linear plasmids and chromosomes was solved by the presence of a terminal protein at the 5' ends to prime DNA synthesis on the lagging strand. In addition, both ends of the linear chromosomes and plasmids of *Streptomyces* contain terminal inverted repeats, (TIRs) varying from 1 to 550 kb in size and are composed of some palindromic sequences (Huang *et al*., 1998; Hopwood, 2006). It was determined that the *Streptomyces* linear chromosomes display dynamic rearrangements by the help of TIRs resulting with circular chromosomes (as a consequence of recombination between the sequences near the ends) as well as the large terminal deletions and amplifications via unequal crossing over take place (Volf and Altenbuchmer, 1998).

Streptomyces spp. are hardly transformable bacteria due to the presence of a very strong restriction barrier. This restriction modification system makes diffucult the transfer of DNA from non streptomycetes origins to *Streptomyces* hosts (Matsushima *et al*., 1987).

The complete genome sequences of *Streptomyces coelicolor* (A3) (Bentley *et al.*, 2002), *S. avermitilis* (Ikeda *et al.*, 2003), *S. clavuligerus* ATCC 27064 (Medema *et al*., 2010) and *S. clavuligerus* NRRL 3585 (Song *et al*., 2010) have been

published. Their genome sizes were found as 8.8, 9, 6.8 and 6.7 Mbs, respectively.

1.2. *Streptomyces clavuligerus* (*S. clavuligerus*) being as a biotechnologically important β-lactam producer

S. clavuligerus was first isolated from soil samples in South America and found as a cephalosporin producer among 1852 isolated strains. Due to the presence of club-like side chains, its name "*clavuligerus*" refers to "bearing little clubs". It was recorded as NRRL 3585 and ATCC 27064 in Agricultural Research Service Collection and American Type Culture Collection, respectively. The type strain is NRRL 3585 (= 27064) (Higgens and Kastley, 1971).

Since 30 years of antibiotic research, *S. clavuligerus* has become a vital source organism for the biotecnological approaches because of its invaluable secondary metabolite production capability of more than 20, such as β-lactam antibiotics, antitumor agents, antifungals and β-lactamase-inhibitors. *S. clavuligerus* produces the sulphur containing β-lactam antibiotics; cephamycin C, deacetoxycephalosporin C, penicillin N (an intermediate in cephamycin C pathway) and the oxygen containing β-lactamase inhibitor; clavulanic acid, and several other clavams (Thai *et al*., 2001) (Figure 1.3).

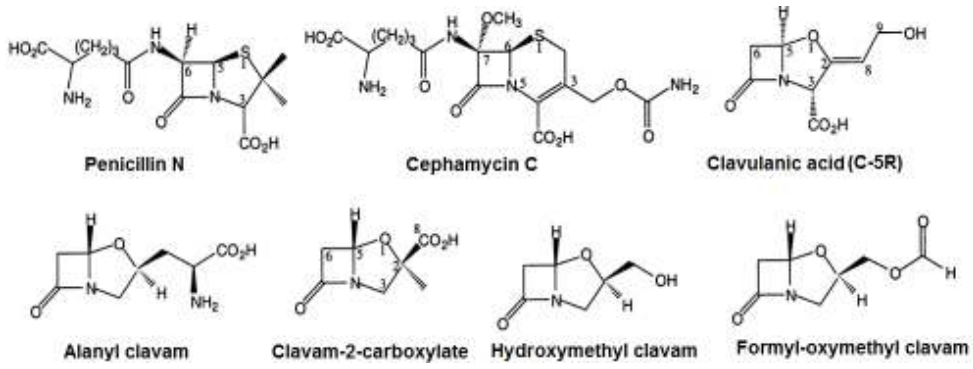


Figure 1. 3. β-lactam compounds produced by *S. clavuligerus* (Thai *et al*., 2001).

Another β-lactamase-inhibitory protein (BLIP) and a BLIP-homologous protein (BLP) were also present in the list of secondary metabolites produced by *S. clavuligerus* (Pérez-Llarena *et al*., 1997b; Thai *et al*., 2001; Santamarta *et al*., 2002). The production of these kinds of compounds is advantegous for bacterial survival against harsh conditions in their natural habitats (Demain, 1989).

The 5S-clavam compounds, such as clavam-2-carboxylate, 2-formyloxymethylclavam, 2-hydroxymethylclavam, hydroxyethylclavam and alanylclavam, lack β-lactamase-inhibitory activity, which is apparently linked to the (3R, 5R) stereochemistry of clavulanic acid, but possess antifungal or antibacterial activities. In addition, *S. clavuligerus* produces a non-β-lactam antibiotic holomycin which is an antitumor agent with pyrrothine structure, and tunicamycin, a glucosamine-containing antibiotic (Fuente *et al.,* 2002; Liras *et al*., 2008; Huang *et al*., 2011).

1.3. β-lactams; attractive antibiotics in biotechnology

Antibiotics (anti means “against”, bios means “life” in Greek) are small molecular weighed (<1000 Da) secondary metabolites mostly synthesized by bacteria (gram positive and gram negative), filamentous fungi and plants (Fabbretti *et al*., 2011). Among all known antibiotics, β-lactams are crucially important for human health due to their high antibacterial capability plus low toxicity. β-lactam antibiotics constitute a broad class of antibiotics and they have a characteristic β-lactam ring in the molecular core structure. They are grouped into four main categories: (i) penicillin derivatives (penams), (ii) cephalosporins (cephems), (iii) monobactams and (iv) carbapenems. In 2003, Elander reported that the β-lactam antibiotics, particularly penicillins and cephalosporins, are the golden products of biotechnology being as the world’s major industry constituting of the ~65 % of the total world market for antibiotics. The synthesis of penicillin antibiotic is specific to fungi (i.e. *Penicillium chrysogenum* and *Aspergillus nidulans*) whereas cephalosporin production is a common process for both fungi (i.e. *Acremonium chrysogenum*) and bacteria (i.e. *S. clavuligerus*).

Bacterial cell wall is composed of covalently crosslinked peptide and glycan strands structure named as peptidoglycan layer. The crosslink between the adjacent peptides is accomplished by amide bond formation via the action of transpeptidase enzyme. Besides, transglycosylation reaction occurs on the glycan strands to add N–acetylglucosamine β–1,4-N-acetylmuramyl-pentapeptide- pyrophosphoryl- undecaprenol units to newly forming peptidolycan layer. β-lactam antibiotics target the substrate of transglycosylase and transpeptidase enzyme (D-alanyl-D-alanine residue of the stem peptide), by acting as pseudosubtrates, thus, they acylate the active sites of the enzyme, preventing the normal crosslinking reaction. As a consequence, the structural integrity of the cell wall could not be provided, causing the osmotic lysis of the bacteria (Walsh, 2000) (Figure 1.4).

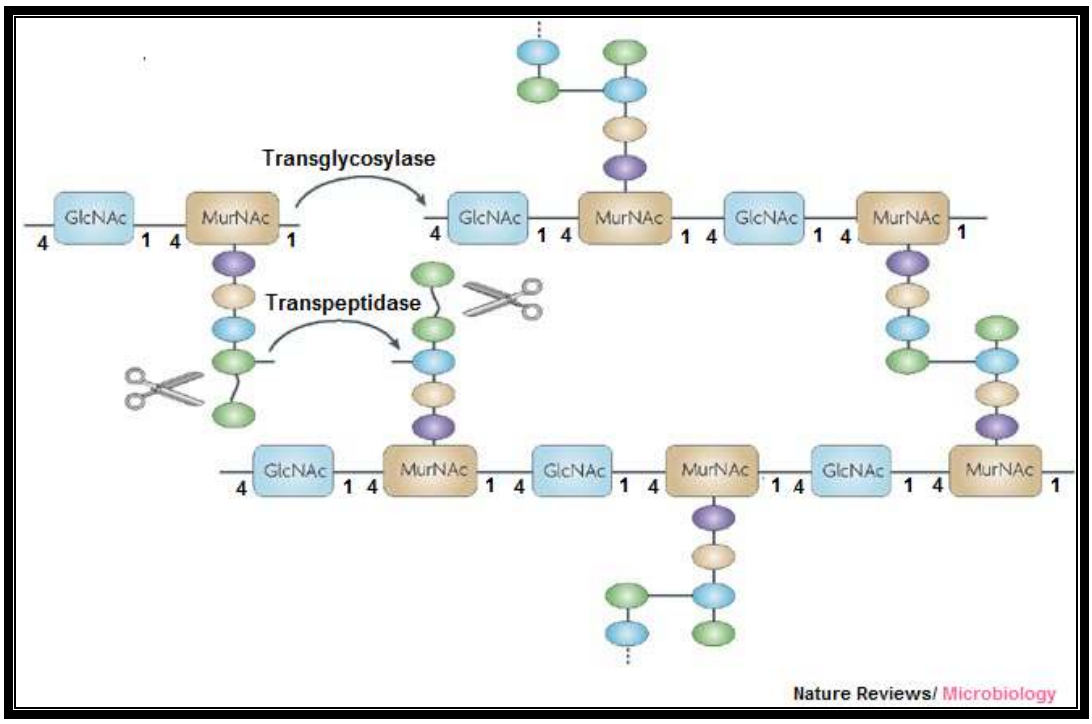


Figure 1. 4. Cell wall structure adapted from Thanbichler and Lucy Shapiro (2008).

The genes involved in the biosynthesis of β-lactams are clustered in the producer organisms. In general, the genes and enzymes for β-lactam biosynthesis are remarkably well conserved in prokaryotic and eukaryotic producers. Surprisingly, the percentages of amino acid identity among the enzymes for β-lactam biosynthesis are higher than those for primary metabolism. In recent years, many researchers have focused on this question: are β-lactam gene clusters originated from a fungal or bacterial ancestor? Two hyposeses have been developed upon following new informations acquired by genome sequencing: (i) horizontal gene transfer [HGT] (ii) vertical transfer of genes. Therefore, the absence of introns in some biosynthetic genes as well as the clustering of genes involved in antibiotic biosynthesis (assembly of genes as a cluster) have favored HGT from bacteria to fungi option. However, the regulators on fungi genome are not cluster-specific, in contrast to cluster-specific regulators found in bacterial genome, by making possible the expression of genes in eukaryotic system. In addition, a new discovery of paralogues of some genes such as *penDE* (a biosynthetic gene involved in penicillin biosynthesis) in the genomes of penicillin non-producer filamentous fungi via gene duplication supports the hypothesis that some biosynthetic genes in β-lactam cluster might be transferred to some fungi without their regulatory genes (Brakhage *et al*, 2005; Brakhage *et al*., 2009; Garcia-Estrada *et al*., 2010). The β-lactam gene clusters on both fungi and bacteria are shown in Figure 1.5.

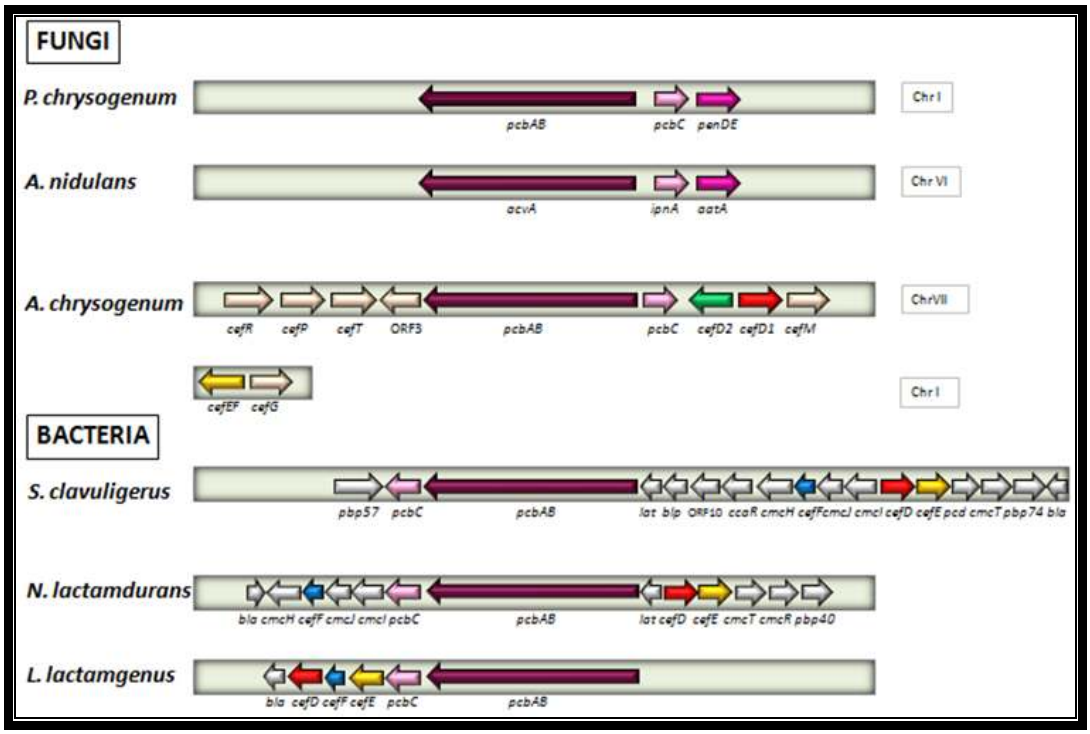


Figure 1. 5. β-lactam gene clusters and their location on both fungi and bacteria.

The indiscriminate usage of antibiotics for a long time brings with it emergence of penicillin resistant strains either by producing new β-lactamases or modifying the existing ones. Therefore, new investments on discovery of novel antibiotics as well as the development of new generation synthetic or semisynthetic antibiotics has been ongoing worldwide although the number of newly discovered ones decreases markedly (Baltz, 2008; Gomez-Escribano and Bibb, 2011). Meanwhile, β-lactams are still the most frequently used antibiotics in the treatment of infectious diseases as they specificly target peptidoglycan synthesis of prokaryotes by not affecting eukaryotic cells (Brakhage, 1998; Glazer and Nikaido, 1998).

β-lactam resistant bacteria have the ability to synthesize β-lactamase enzyme that hydrolyse amide bond of β-lactam ring found in structures of all β-lactam antibiotics (Matagne *et al*., 1999; Öster *et al*., 2006). Cephamycin C is an

extracellular and second-generation 7-methoxy-cephalosporin. The presence of methoxyl group in the 7th position of cephalosporin nucleus increases its inhibitory effect on transpeptidases involved in the cell wall synthesis, also reduces inactivation by β-lactamases and increases their activity against gram-negative and anaerobic pathogens, thus making cephamycin C an important clinical antibiotic. Cephamycin C is effective on many bacteria resistant to penicillin and cephalosporin (Stapley *et al*., 1979; Glazer and Nikaido, 1998). Clavulanic acid, on the other hand, has a weak antibacterial activity but is a very potent β-lactamase inhibitor produced by *S. clavuligerus*. Dual presence of β-lactam antibiotics with β-lactamase inhibitors in drug composition provides a superior effect to combat with infectious diseases when compared to the usage of drugs composed of β-lactams alone. There are other examples of actinomycetes that produce cephamycin alone (i.e. *Nocardia lactamdurans*, *S. griseus* NRRL 3581), however there is no record of only clavulanic acid producer bacteria in the literature. Thus, the production of cephamycin C and clavulanic acid by *S. clavuligerus* succeeds the competence of this bacterium against the β-lactam-resistant ones inhabiting the same environment (Challis and Hopwood, 2003). Clavulanic acid is a member of serine type (or classes A, C, and D) β-lactamase family. Its potassium clavunanate form is involved in formulation of Augmentin™ and Timentin™ along with amoxycillin (co-amoxiclav) and with ticarcillin, respectively (Watve *et al*., 2000).

By the facility of newly published genome sequence of *S. clavuligerus* ATCC27064, Medema *et al*. (2011) have performed microarray studies of an industrial strain which have 100 times higher level of clavulanic acid production when compared to clavulanic acid yield of the wild type strain. They have found that some of the genes important for primary and secondary metabolism were expressed differently in this industrial strain. These data supported the findings of previously published reports; the deletion of *gap1* gene (Li and Townsend, 2006), overexpression of *claR* and *ccaR* regulatory genes (Hung *et al*., 2007) and the combination of both strategies in a single strain (Jnawali *et. al*., 2010), in which genetic manipulations were performed to increase clavulanic acid production capacity of the wild type *S. clavuligerus*. In addition, increased expression levels of glutamine and glutamate synthetase genes as well as ammonium and

phosphate transporter genes were determined in this clavulanic acid overproducer strain. The data obtained from the transcriptome studies might lead to creation of new molecular approaches to generate more productive industrial *S. clavuligerus* strains.

1.4. Cephamycin C Biosyntesis

Biosynthetic pathway of cephamycin C can be examined mainly in three parts: (i) the two steps (early steps) shared with penicillin biosynthesis, (ii) the following three steps (intermediate steps) associated with cephalosporin C biosynthesis and (iii) the final three ones (late steps) unique for cephamycin biosynthesis (Aharonowitz *et al*., 1992; Liras, 1999) (Figure 1.6). The early steps of cephamycin C biosynthesis starts with formation of the core structure of *β*-lactam nucleus by three aminoacids, namely, L-*α*-aminoadipic acid, L-cysteine and L-valine. L-*α*-aminoadipic acid (α-AAA) is an intermediary product in lysine pathway of fungi. However, in *S. clavuligerus*, this precursor is synthesized from lysine (from aspartate pathway) at the end of two enzymatic reactions catalyzed by lysine-6-aminotransferase (LAT encoded by *lat* gene) and piperideine-6-carboxylate dehydrogenase (PCD, encoded by *pcd* gene), respectively. The lysine is coverted to α-AAA semialdehyde by a deamination reaction catalysed by lysine-6-aminotransferase. α-AAA semialdehyde is spontaneously cyclized to compose piperideine-6-carboxylic acid (P6C). As a result of oxidation reaction, P6C is converted to aminoadipic acid by (P6C)-dehydrogenase (PCD) enzyme (Kern *et al*.,1980; Madduri *et al*., 1989, 1991; Tobin *et al*., 1991, Coque *et al*., 1991; Perez-Llarena *et al*., 1997b; Fuente *et al*., 1997; Perez-Llarena *et al*., 1998; Alexander and Jensen, 1998). Therefore, LAT and PCD are involved in the initial two steps of cephamycin C pathway (Alexander *et al*., 2007). As LAT enzyme and its encoding gene (*lat*) are unique for β-lactam-producing actinomycetes, they might be crucial indicators for detection of novel β-lactam producers (Liras and Martin, 2006). Significantly, chromosomal integration of *lat* gene in the wild type *S. clavuligerus* resulted two to five fold increase in the cephamycin C biosynthesis of the recombinant strain, indicating the rate-limiting property of LAT in secondary metabolite production (Malmberg *et al*., 1993; Khetan *et al*., 1999).

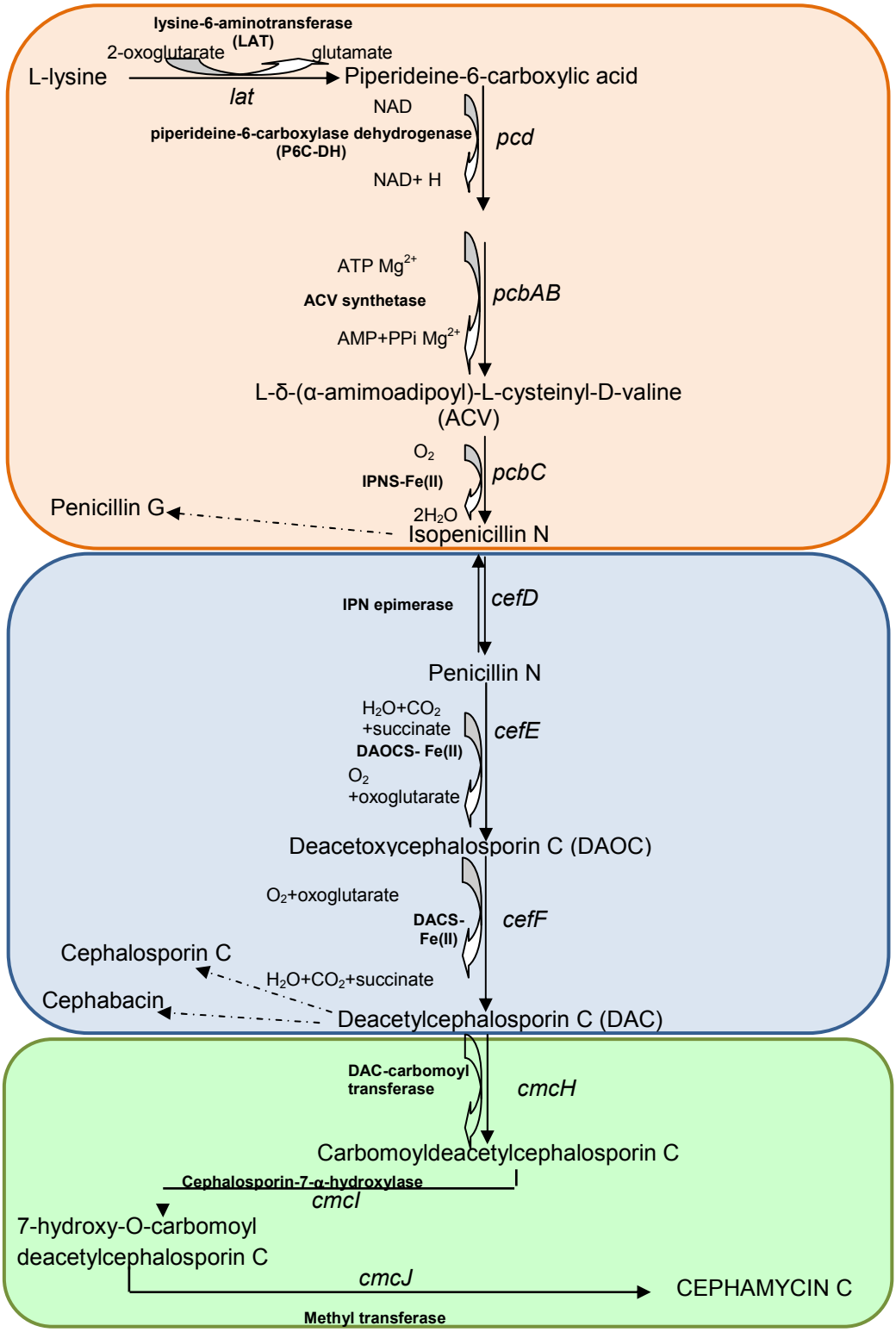


Figure 1. 6. Biosynthetic pathway of cephamycin C in *S. clavuligerus* (adapted from Tyker and Nielsen, 2003 and Öster, 2006).

As previously mentioned, L-α-aminoadipic acid, L-cysteine and L-valine are condensed to form δ-(L-α-aminoadipyl-L-cysteinyl-D-valine) (ACV) tripeptide, a non-ribosomal peptide synthetase, by ACV synthetase enzyme encoded by the *pcbAB* gene (Tobin *et al*., 1991; Coque *et al*., 1991; Coque *et al*., 1996). In the following step, β-lactam and thiazolidine rings are formed by the activity of ACV cyclase (isopenicillinN synthase) encoded by *pcbC* gene. The ACV tripeptide is cyclized to isopenicillin N, a β-lactam with a weak antibiotic activity and the first intermediary compound of the cephamycin C pathway (Jensen *et al*., 1986; Leskiw *et al*., 1988; Coque *et al*., 1991).

Afterwards, isomerization of the L-α-aminoadipyl lateral chain of isopenicillin N to its D configuration takes place in the presence of isopenicillin N epimerase encoded by *cefD* gene to give rise to penicilin N (Usui and Yu, 1989). Five-membered thiazolidine ring in the structure of penicillin N is then expanded to a six-membered dihydrothiazinic ring by penicillin N expandase (deacetoxycephalosporin C synthase, DAOCS) (Jensen *et al*., 1985) encoded by *cefE* gene (Kovacevic *et al.* 1989, 1990; Coque *et al*., 1991). The first intermediate product of cephalosporin, deacetoxycephalosporin (DAOC) is formed. The last shared step of cephamycin C biosynthesis with cephalosporin pathway is the hydroxylation of C-3´ group of DAOC by DAOC hydroxylase (encoded by *cefF*) to form deacetylcephalosporin C (DAC), the primary cephalosporin C precursor (Kovacevic and Miller, 1991).

Unique steps in cephamycin C biosynthesis include carbomoylation and methoxylation reactions. The C-3´ hydroxyl group of DAC is carbamoylated by the deacetylcephalosporin C-O-carbamoyltransferase encoded by the *cmcH* gene and O-carbomoyl-deacetyl-cephalosporin C (OCDAC) is formed (Brewer *et al*., 1980; Coque *et al*., 1995b; Alexander and Jensen, 1998). The metoxylation of β-lactam ring at C-7 position is very crucial for the synthesis of cephamycin C as it supplies β-lactamase resistance to the cephamycin C producer. Thus, C-7´ position of β-lactam ring has to be hydroxylated by cephalosporin-7-α-hydroxylase encoded by *cmcI* gene before this process. After, metoxyl group is transferred to the C-7´ position by *cmcJ* encoded enzyme and finally cephamycin

C is formed (Xiao *et al*., 1993; Coque *et al*., 1995a; Enguita *et al*., 1996; Alexander and Jensen, 1998).

1.5. Cephamycin C gene cluster

Cephamycin C gene cluster is approximately 38 kb in size (Fig. 1.7) and 1.4 Mb away from the clavulanic acid gene cluster on the chromosome. Both clusters constitute a β-lactam supercluster of about 60 kb (Ward and Hodgson, 1993).

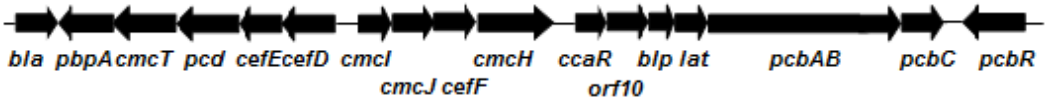


Figure 1. 7. Cephamycin C gene cluster in *S. clavuligerus* adapted from Liras, 1999.

The cephamycin C gene cluster is composed of sixteen genes encoding enzymes with several functions. These enzyme-encoding genes are responsible for biosynthesis of cephamycin C as well as its regulation, transportation and self-resistance of organism to the β-lactam antibiotic. The genes in the cephamycin C gene cluster, their functions with the respective references plus GenBank accession numbers are tabulated in Table 1.2.

bla, the furthest gene in cephamycin C cluster from clavulanic acid gene cluster, encodes a 35 kDa class A type-low activity β-lactamase composed of 332 aminoacids (aa) and it has capability of binding to penicillin (Pérez-Llarena *et al*., 1997a). In ∆*bla* mutants of *S. clavuligerus* grown on solid media, no differences were observed both in resistance to penicillin G and cephalosporin C. Indeed, the cephamycin C and clavulanic acid production of the mutant strain were the same with that of the wild type. The changes were detected only at the onset of sporulation and at its morphology. All these findings led to propose that BLA

might have a role on cell wall morphogenesis like penicillin binding proteins (PBPs) (Thai *et al*., 2001).

Table 1. 2. The genes and their functions in cephamycin C gene cluster

Name	Function	Reference/Accession Number in NCBI (http://www.ncbi.nlm.nih.gov)
bla	β-lactamase class A	Perez-Llarena *et al.,* 1997/ Z54190 Alexander&Jensen1998/ AF073895
pbpA(pbp74) /pbp2	Penicillin binding protein	Perez-Llarena *et al.,* 1997/AJ001743
cmcT	Putative cephamycin C transport	Perez-Llarena *et al.,* 1997/AJ001743 Alexander&Jensen 1998/AF073895
pcd	Piperideine 6-carboxylase dehydrogenase	Perez-Llarena *et al.,* 1997/AJ001743 Alexander&Jensen 1998/AF073895
cefE	Penicillin N expandase	Perez-Llarena *et al.,* 1997/AJ001743
cefD	Isopenicillin N epimerase	Kovacevic *et al.,* 1990/M32324
cmcI	OCDAC hydroxylase	Alexander&Jensen,1998/AF073896 Alexander&Jensen,1998/AF073896
cmcJ	OCDAC methyl transferase	Kovacevic and Miller,1991/M63809
cefF	DAOC hydroxylase	Alexander&Jensen, 1998/AF73897
cmcH	DAC carbomoyl transferase	Perez-Llarena *et al.,* 1997/Z81324; Alexander&Jensen, 1998/AF73897
ccaR	Transcriptional activator of cephamycin C & clavulanic acid gene cluster	Perez-Llarena *et al.,* 1997/Z81324; Alexander&Jensen, 1998/AF73897
orf10	Unknown	Perez-Llarena *et al*., 1997/Z81324
blp	β-lactamase inhibitory protein	Wu *et al.,* 2004/AY742798; Tobin *et al* .,1991/M64834; Yu *et al*., 1994/U12015
lat	Lysine 6 εα aminotransferase	Tobin *et al*., 1991/M64834; Yu *et al.,* 1994/U12015
pcbAB	ACVS [δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine- synthethase]	Yu *et al*., 1994/U12015; Tobin *et al*., 1991/M64834
pcbC	Isopenicillin N synthethase	Leskiw *et al*.,1998/A01132
pcbR(pbp57)	Penicillin binding protein	Jensen *et al.,* 2000/ U87786

On the other end of the cluster*, pcbR* (*pbp57*) gene, which is located next to the clavulanic acid cluster, encodes a penicillin-binding protein (57.3 kDa) involved in β-lactam resistance and it belongs to the high-molecular-weight group B class PBPs (Paradkar *et al*., 1996). PcbR is anchored to the membrane through 26 aa of N-terminal region. In addition, the presence of a conserved sequnence belong to penicillin binding motifs that is characteristic for high molecular weight PBPs in the structure of this protein save resistance of *S. clavuligerus* against penicillin G and cephalosporin C. Disruption of *pcbR* gene is lethal for the organism itself (Liras, 1999).

A putative low-molecular-weight PBP encoding *pbp74* gene (*pbpA*/*pbp2*) is located upstream of *bla* and transcribed in reverse direction. The *pbpA* encoded PBP is composed of 696 aa with a molecular weight of 74 kDa. Most probably, *pcbR* and *pbpA* have complementary functions in autoresistance against β-lactam antibiotics (Alexander and Jensen, 1998).

cmcT gene is located at the upstream of *pbpA* and it is responsible for transport of cephamycin C outside of the cell. 53 kDa CmcT protein composed of 532 aa is a multidrup efflux pump class transporter settled in the cell membrane (Coque *et al*., 1993; Alexander and Jensen, 1998). It is the only gene found in the cluster responsible for the antibiotic transport. *cmcT* probe did not give any hibridization signals with the DNAs of other cephamycin C producer actinomycetes indicating that the genes involved in cephamycin export were conserved among β-lactam producers (Liras, 1999).

A 56.2 kDa P6C dehydrogenize enzyme encoded by *pcd* gene is located in upstream of *cmcT* gene. As previously described, this enzyme has a role in the formation of α-AAA from piperideine-6-carboxylate (P6C), an intermediary product generated from lysine by the activity of LAT (Alexander and Jensen, 1998). In this respect, it was expected that *pcd* has to be an important structural gene for cephamycin C biosynthesis. However, Alexander *et al*. (2007) reported that cephamycin C production was reduced to 30-70% of its normal level in *pcd*-disrupted mutant of *S. clavuligerus*. The *pcd*-disrupted mutant restored its

cephamycin C production capacity after complemented with an intact *pcd* gene. Indeed, the presence of only one *pcd* gene in the genome of *S. clavuligerus* has been proved by Southern analyses. These findings might be interpreted in these manner; (i) partial role of *pcd* in the formation of α-AAA, (ii) the negative effect of *pcd* mutation on other genes of the cluster leading a reduction in cephamycin C biosynthesis, (iii) stimulatory effect of PCD on self-formation of α-AAA from P6C.

Upstream region of *pcd* is occupied by the biosynthesis genes, such an order of *cefE, cefD, cmcI, cmcJ, cefF* and *cmcH*, respectively. The other biosynthetic genes, *lat*, *pcbAB* and *pcbC*, are located between *cmcH* and *pcbR* genes in the cluster.

cefE and *cefD* genes are located at the upstream of *pcd* and at the same direction. However, *cefF* gene is oriented in reverse direction to those genes and is located at upstream of *cmcJ.* They are penicillin N expandase (*cefE*), isopenicillin N epimerase (*cefD*) and DAOC hydroxylase (*cefF*) enzymes encoding genes of cephamycin C gene cluster. *cefE* and *cefF* genes are found to be very similar in sequence. They were cloned, expressed in *E. coli* and in other *Streptomyces* species as well as related proteins (35-38 kDa) were purified by different groups (Jensen *et al*., 1985; Kovacevic *et al*., 1989; Kovacevic and Miller, 1991; Baker *et al*., 1991; Coque *et al*., 1993, 1996). *cefD* gene that encodes a 44 kDa of IPN epimerase was cloned and expressed in *E.coli* and *S. lividans* (Kovacevic *et al*., 1990; Coque *et al*., 1993; Kimura *et al*., 1996). *cefF* has capability of expanding penicillin G ring formation, a more stable and industrially important antibiotic, by a modification reaction (Cho *et al*., 1998). In addition, random mutagenesis approaches were performed to enhance its penicillin G formation (Wei *et al*., 2003; Wei *et al*., 2005).

cmcI, *cmcJ* and *cmcH* genes are biosynthetic genes involved in the late steps of cephamycin C biosynthesis. *cmcH* encodes a 57 kDa DAC carbamoyl transferase enzyme responsible for catalysis of the carbamoylation reaction in the cephamycin biosynthesis in which a carbamoyl group is transferred to the C-3 hydroxyl group of the deacetylcephalosporin C (Coque *et al*., 1995b; Alexander and Jensen, 1998). CmcI and CmcJ, a molecular weight of 28 kDa and 32 kDa, respectively, act as a protein complex in formation of 7α-cephem-methoxylation

reaction. The methoxlation complex is found in Cmcl. *cmcI* encoded cephalosporin-7-α-hydroxylase enzyme hydroxylases the C-7 position of the β-lactam ring of the OCDAC and *cmcJ* encoded methyltransferase enzyme adds methoxyl group to the C-7 position of 7-hydroxy-O-carbomoyl deacetylcephalosporin C to give cephamycin C (Enguita *et al*., 1996; Öster *et al*., 2006).

In the cluster, positive regulatory gene *ccaR, orf10, blp* and *lat* genes remain in between the cephalosporin-specific and the penicillin biosynthesis genes (Pérez-Llarena *et al*., 1997b). *ccaR* (*dclX*) encodes a 28 kDa kDa ActII-ORF4-like SARP regulatory protein (***S****treptomyces*-**A**ctivator **R**egulatory **P**rotein) (Liras, 1999; Liras *et al*., 2008). *blp* encodes BLP protein (**B**LIP **l**ike **p**rotein) (33 kDa) of which sequence resembles to *bli* encoded β-lactamase-inhibitory protein, BLIP (Doran *et al*., 1990). However, BLP has no β-lactamase-inhibitory activity (Thai *et al*., 2001). The function of ORF10 has not been clarified yet (Perez-Llarena *et al*., 1997a,b). Gene disruption studies showed that cephamycin C production titer did not change in *blp* or *orf10* null mutants of *S. clavuligerus* (Alexander and Jensen, 1998).

LAT enzyme encoded by *lat* gene is a 51.3 kDa protein and is a rate limiting enzyme for cephamycin C biosynthesis. Supplementation of *S. clavuligerus* cultures with high amount of L-lysine increased cephamycin C production via in two ways; (i) being as a substrate of LAT promotes α-AAA formation, (ii) being as an inducer of LAT yields even more α-AAA. But, LAT is not induced by α-AAA (Rius *et al*., 1996). LAT was characterized and partially purified by Romero *et al*. (1997). Integration of *lat* gene into the chromosome of *S. clavuligerus* resulted with a 2 to 4 fold increase in cephamycin C production, whereas its deletion caused a 2 to 5 fold increase in clavulanic acid yiled of the mutant strain of *S. clavuligerus* (Malmberg *et al*., 1993; Paradkar *et al*., 2001). Besides, the addition of α-AAA to the cultures of *lat::apr* mutants provided a low titer of cephamycin C production (Alexander *et al*., 2000). The temporal and spatial distribution of LAT during cephamycin C biosynthesis in *S. clavuligerus* grown on solid media was investigated by using GFP protein as reporter. It was found that LAT expression was at the highest level at the stage of substrate mycelium while there was no

expression in aerial mycelia indicating that it is an early enzyme involved in cephamycin C biosynthesis (Khetan *et al*., 2000).

ACV synthetase enzyme encoded by *pcbAB* gene of *S. clavuligerus* was partially purified and characterized (Jensen *et al*., 1990; Scwecke *et al*., 1992). The completion of genome sequence of *S. clavuligerus* reveals a detailed examination of *pcbAB* gene. The 11 kbp *pcbAB* gene of *N. lactamdurans* was cloned to *S. lividans* and a 430 kDa protein was purified by Coque *et al*. (1995). ACV synthetase binds 3 phosphopantetheines covalently to sythesize LLD-ACV.

pcbC gene encodes ACV cyclase (Isopenicillin N synthetase) enzyme of 329 aa with a deduced MW of 37 kDa. It is a kind of oxygenase activated by molecular oxygen and iron, and cyclize the ACV tripeptide to isopenicillin N (IPN) (Liras, 1999). *ccaR*-disrupted mutants of *S. clavuligerus* do not exert ACV synthetase and ACV cyclase activities (Alexander and Jensen, 1998).

1.6. The sequences differences in the cephamycin C genes of *S. clavuligerus* NRRL3585 and ATTC27064

Two different groups, Perez-Llarena *et al* (1997) and Alexander and Jensen (1998), conducted their studies on cephamycin C regulation by specific/global regulatoty systems in *S. clavuligerus* ATTC27064 (i) and NRRL3585 (ii) strains, respectively. They have found some nucleotide differences in the upstream region of *ccaR* and *orf10* as well as in the downstream region of *blp* gene (Table 1.3). Besides, the length, start and/or stop codons of *pcd*, *cmcT* and *pbpA* genes were found to be different. According to their reports, *pcd* gene starts from approximately 15 nt downstream of *cefE* gene while its length was recorded as 1491(i) and 1539 (ii) bp, respectively. It should be also noted that the 5' N-terminal regions of the both sequences were found to be the same, however, the aa sequence in C-terminal site of them was completely different from each other. *cmcT* gene which have nucleotide sizes of 1572 (i) and 1471 (ii) bp, is located at 69 and 179 bp downstream of *pcd*. Indeed, there are nucleotide differences at

nine different positions between the two sequences reported by Perez-Llarena *et al*., (1997) and Alexander and Jensen (1998). The following gene that named as *pbp2* (i) and *pbpA* (ii) was also reported in different sizes as 2091(i) and 1230 (ii) bp, respectively. According to the sequence analysis of Alexander and Jensen (1998), the ORF of *pbpA* starts from an ATG codon located in 862 nt downstream of *pbp2*. Correspondingly, the intergenic region between *pcd* and *pbpA* (*pbp2*) genes alters to 332 (i) and 1537 (ii) bp in size (Table 1.4).

Table 1. 3. Sequence comparison of *ccaR*, *blp* and *orf10* genes in *S. clavuligerus* NRRL3585 and ATCC27064 strains

Sequence	Location	Comparison	
		Perez-Llarena *et al*. (1997) Accession # Z81324	**Alexander and Jensen (1998) Accession # AF73897**
		ATCC27064	NRRL3585
Upstream of *ccaR*	2278–2280	cc	ccc
	2356–2358	cc	ccc
	2450–2451	a	aa
Upstream of *orf10*	3502-3503	c	cc
	3513	gg	g
	3643-3645	ccg	cgc
	3729-3730	gg	gc
Downstream of *blp*	5754-5756	cc	ccc
	5761-5764	ggg	gggg
	5790-5791	g	gg
	5803-5804	c	cc

Table 1. 4. The differences between the size of intergenic regions of the cephamycin C gene cluster in *S. clavuligerus* NRRL3585 and ATCC27064

Intergenic region	Size (bp)	Reference
cmcH-ccaR	915	Santamarta *et al*., 2002
ccaR-orf10	321	Alexander and Jensen, 1998; Perez-Llarena *et al*., 1997
orf10-blp	346	Alexander and Jensen, 1998; Perez-Llarena *et al*., 1997
blp-lat	~612	Alexander and Jensen, 1998
lat-pcbAB	152-153	Tobin *et al*., 1991; Yu *et al*., 1994
pcbAB-pcbC	17-32-116	Petrich *et al*., 1992
cefD-cmcI		
cmcI-cmcJ	48	Alexander and Jensen, 1998
cmcJ-cefF	96	Alexander and Jensen, 1998
cefF-cmcH	~84	Alexander and Jensen, 1998
cefD-cefE	78	Kovacevic *et al*., 1989
cefE-pcd	15	Perez-Llarena *et al.,* 1997
pcd-cmcT	69-429	Perez-Llarena *et al*., 1997; Alexander and Jensen, 1998
cmcT-pbpA(2)	332-1205	Perez-Llarena *et al*., 1997; Alexander and Jensen, 1998

Alexander and Jensen (1998) proposed that these discrepancies in the published sequences belong to the same genes might be resulted from sequencing errors, allelic differences or probably due to the genuine differences of two separate wild type strains (NRRL 3585 and ATCC 27064) despite the fact that they have evolved from a common origin.

1.7. Transcriptional analysis of cephamycin C gene cluster

Several studies were performed to determine transcriptional organization of the cephamycin C cluster on the basis of Northern and Western blot analyses, 5' primer extension and S1 nuclease mapping studies (Petrich *et al*., 1994; Perez-Llarena *et al*., 1997b; Alexander and Jensen, 1998; Liras, 1999; Alexander *et al.,* 2000). In the cephamycin C cluster of *S. clavuligerus*, broad distances between successive genes enable the presence of promoters in these intergenic regions

(Liras, 1999). However, the same orientation of early genes of cephamycin C biosynthesis, *lat-pcbAB-pcbC*, as well as the presence of short intergenic regions between them make possible their coordinated transcription. Transcription start point (tsp) of *lat* gene is located at 88 bp upstream of translation initiation codon. Although a 155 bp distance is present in between *lat* and *pcbAB* genes, no promoter or tsp for *pcbAB* but a stem-loop structure between the two genes have been found. A 1.2 kb of monocistronic transcript initiating form 92 bp upstream of *pcbC* and 60 bp downstream of the *pcbAB* gene was detected by Northern and 5' primer extension analyses. Indeed there was no evidence for monocistronic transcription of *lat* and *pcbAB* genes (Petrich *et al*., 1994; Yu *et al*., 1994). Besides, S1 nuclease analysis showed a polycistronic transcript of 14 kb, directed by *lat* promoter, extending across all the three genes. In addition, Western Blot analysis of the Δ*lat*::*tsr*/term mutants (having no promoter of *lat* gene and a terminator sequence inside the gene) confirmed the presence this large polycistronic transcript (*lat-pcbAB-pcbC*) and the monocistronic transcript of *pcbC* gene from its own promoter. The stem-loop between *lat* and *pcbAB* genes might have a function for protection of proceeding transcription from RNase activity or might have a role of antiterminator regulation in normal growth conditions (Alexander and Jensen, 1998; Alexander *et al*., 2000).

cefD and *cmcI* genes in the cephamycin C cluster are oppositely oriented and their expressions are under the control of a strong bidirectional promoter located between them. The transcription initiation of *cefD* gene was shown as 130 bp distant from the translation start codon via primer extension analysis. The short distances between *cefD-cefE* (78 bp) and *cefE-pcd* (15 bp) may not hinder their cotranscriptions. *cefD* together with *cefE*, *pcd*, *cmcT* and *pbpA* genes are located at the same direction in the cluster and are transcribed together from this strong promoter by giving a large mRNA transcript of 10 kb (Kovacevic *et al*., 1990). In 1997, two different hybridization signals, 4.1 and 2.6 kb in length were obtained by Northern blot analyses of TBS cultures. These signals corresponded to a large transcript of *cefD-cefE-pcd* and a small one of *cefD-cefE* (Perez-Llarena *et al*., 1997). In addition, Pérez-Llarena *et al.* (1998) reported that *cmcT* and *pbpA* genes are transcribed monocistronically by giving 1.9 kb transcripts. However, the formation of polycistronic transcripts of *cefD-cefE-pcd-cmcT* and posibbly

pbpA genes under the control of *cefD-cmcI* promoter is well accepted (Alexander and Jensen, 1998). The transcriptional organization of cephamycin C-specific genes was provided via two separate polycistronic mRNAs. *cmcI* and *cmcJ* genes were co-transcribed as a 1.6 kb mRNA transcript, whereas, *cefF* and *cmcH* genes were transcribed in two bicistronic transcripts of 2.8 and 4.1 kb, respectively. Probably, the 4.1 kb transcript covered the sequences located downstream of *cmcH* (Liras, 1999).

Monocistronicaly transcribed *ccaR* and *blp* showed 0.9 and 1.2 kb hybridization signals in Northern analysis and the signal of *ccaR* was apparent prior to cephamycin C and clavulanic acid productions while no *ccaR* transcripts were detected once secondary metabolite production had begun (Pérez-Llarena *et al*. 1997b). However, Bignell *et al.* (2005) reported that in *S. clavuligerus*, *ccaR* is expressed as two transcripts of ~1 and ~1.4 kb. Although the intensity of the transcript bands was faint in RNA samples from the later time points, both were detectable during the cultivation. The natural start codon of *ccaR* was found as an ATG codon located at 18 bp upstream of GTG which was previously reported as the start codon (Wang *et al*., 2004). In addition, S1 nuclease protection and primer extension analyses were performed by the same group to localize the *ccaR* tsp. Two tsps which are located at 74 and 173 bp upstream of the ATG codon of *ccaR* were found (Wang *et al*., 2004).

It was previously proposed that transcription model of the genes differs in size, quailty and abundancy according to medium type used for cultivation (Perez-Redondo *et al*., 1998). However, Bignell *et al*., (2005) repealed this conclusion by showing the presence of the same transcripts of a specific gene in different culture conditions.

1.8. Regulation of antibiotic production in *S. clavuligerus*: Global pleiotropic factors and pathway-specific regulators

Several regulatory mechanisms control antibiotic biosynthesis in *Streptomyces*. These regulatory networks are either global pleiotropic factors or pathway-specific regulators. During the life cycle of *S. clavuligerus*, secondary metabolites are produced at the stage of morphological differentiation. *bld* genes are one of

the most characteristic pleitropic factors' genes studied in *S. coelicolor*. ***bldA*** encodes the $tRNA^{Leu}$ for the translation of UUA codon. Due to the high G/C content of *Streptomyces* genome, this codon is confined to the ORFs of secondary metabolite genes and development, such as actinorhodin pathway-specific activator, *actII-ORF4* (Fernández-Moreno *et al*., 1991). The disruption of *bldA* in *S. coelicolor* has resulted in a bald phenotype and cease of actinorhodin production. Although *ccaR* regulatory gene has two TTA codon, *bldA*-null mutants of *S. clavuligerus* was unable to form aerial mycelium but still produced related antibiotics. The antibiotic production capability of *bldA* mutants might be explained by mistranslation of TTA codon (Trepanier *et al*., 2002; Santamarta *et al*., 2002). However, cephamycin C and clavulanic acid production in *S. clavuligerus* were effected by *bldG* gene as the expression of *ccaR* gene is *bldG*-dependent. ***bldG*** encodes an anti anti-sigma factor that plays role in posttranslational regulation. In the absence of *bldG* gene, morphologically defective and antibiotic non-producer mutants of *S. clavuligerus* were obtained (Bignell *et al*., 2005).

Another *bld* gene, a TTA codon containing *adpA*, encodes **AdpA** positive modulator of antibiotic production in *S. clavuligerus*. qRT-PCR analysis showed that transcriptions of *ccaR* and *claR* genes were reduced seven- to four-fold, respectively, in an *ΔadpA* mutant strain, resulting a decrease in cephamycin C and clavulanic acid production as well as a medium dependent sparse aerial mycelium formation and lack of sporulation. However, multiple copies of *adpA* gene in *S. clavuligerus* cells enhanced antibiotic production. It was proposed that AdpA exerts its regulatory effect by binding to the conserved sequences located at *cmcH-ccaR* intergenic region and between ARE box and tsp of *ccaR* gene: (i) 406 bp from the ATG start codon, a single (type II) sequence, 59-TGGCCGGATT-39; and (ii) 565 nt upstream from the ATG, two direct sequences, 39-TGGCCCTTTT-14-TGGCCGCTGT-59 (Lopez *et al*., 2010).

Pathway-specific regulators are mostly found in the clusters together with the biosynthetic genes, however, sometimes they might also control other biosynthetic pathways (Huang *et al*., 2005). ***ccaR*** encodes CcaR (**C**ephamycin C and **C**lavulanic **A**cid **R**egulator) that is a SARP type positive regulator of β-lactam

supercluster composed of cephamycin C and clavulanic acid gene clusters and located in the cephamycin C gene cluster. There are several patway-specific regulatory proteins that belong to SARP family (i.e. AfsR, DnrI, RedD and the *act*II-ORF4 encoding protein) in *Streptomyces* (Horinouchi *et al*., 1990; Narva and Feitelson, 1990; Fernandez-Moreno *et al*., 1991; Stutzman-Engwall *et al*., 1992). CcaR is a 28 kDa regulatory protein resembling to positive actII-ORF4-like transcriptional regulator. EMSA experiments proved that CcaR protein is an autoregulatory protein that binds to its own promoter as well as to the *cefD-cmcI* bidirectional promoter and *lat* promoter (Kyung *et al*., 2001; Santamarta *et al*., 2002). Thus, CcaR positively controls the early (*pcd* is transcribed from *cefD*), middle and late steps of cephamycin C biosynthesis by regulating the *cefD-cmcI* promoter. In this promoter region, SARP boxes were identified (Liras *et al*., 2008). It was previously shown that disruption of *ccaR* gene represses all three secondary metabolite productions (cephamycin C, clavulanic acid and clavams) and high copy number of this gene in the cell enhances cephamycin C and clavulanic acid production (Pérez-Llarena *et al*., 1997b; Alexander and Jensen, 1998).

claR, on the other hand, is a LysR-type regulatory gene, encoding a regulatory protein of 47 kDa, located in clavulanic acid gene cluster. It was shown that *claR* disrupted mutant of *S. clavuligerus* is capable of producing cephamycin C but is unable to produce clavulanic acid. qRT-PCR analysis showed that the expression of genes involved in late steps of clavulanic acid biosynthesis has ceased, whereas *ccaR* expression has rised in this mutant strain (Martin and Liras, 2010). The absence of an intact *ccaR* gene in the cell drastically effects transcription of *ceaS2* (carboxyethyl-arginine-synthase) gene in the clavulanic acid gene cluster (Tahlan *et al*., 2004a). CcaR regulates clavulanic acid gene cluster by binding to both *ceaS* and *claR* promoters. However, DNA-binding experiments are still needed to determine CcaR effect on this cluster completely (Liras *et al*., 2008; Martin and Liras, 2010).

In actinomycetes, γ-butyrolactone type autoregulators function as microbial hormones for the onset of antibiotic regulation (Horinouchi, 2007). *brp* encoded **Brp,** butyrolactone receptor, was well characterized by two groups (Kim *et al*.,

2004; Santamarta *et al.*, 2005). The repressory role of Brp on cephamycin C and clavulanic acid production was verified by indicating a 1.5-fold overproduction of these antibiotics in a *Δbrp* null mutant of *S. clavuligerus*. γ-butyrolactone type autoregulators recognize and bind to specific palindromic inverted sequences, namely **ARE** (**A**uto **R**egulatory **E**lements) boxes. ARE boxes (consensus sequence TNANAWACNNACYNNNCGGTTTKTTT) consist of 26 nt in sequence and are present in the upstream of or close to promoter of SARP encoding genes (Santamarta *et al.,* 2005). Brp binds to two diferent ARE boxes located at (i) its own promoter region and (ii) 890 nt upstream of *ccaR* ATG codon (Liras *et al*., 2008). Another ARE box (59-TCTCATGGAGACATAGCGGGGCATGC-39) located upstream of *adpA* gene was determined, as well. The binding of *S. clavuligerus* Brp to this ARE box was shown by EMSA analysis. Similarly, the binding of Brp represses the transcription of *adpA.* In the *Δbrp* mutant, it was estimated that *adpA* expression has increased up to 2.5-fold. It was concluded that *ccaR* expression is controlled directly by Brp while indirectly controlled through Brp-dependent AdpA regulator (Lopez *et al*., 2010).

An IclR-like regulatory protein, **AreB**, is another modulator of antibiotic production by effecting *ccaR* expression, was isolated and characterized via DNA-affinity chromatography (Santamarta *et al*., 2007). AreB controls its own expression and leuCD cluster due to the presence of a divergent promoter between *areB* and leuCD gene cluster thereby connecting primary and secondary metabolism. It has also pleiotropic effects on leucine assimilation and fatty acid utilization. It was shown that *ΔareB* mutant of *S. clavuligerus* strain is able to produce a slightly higher amount of cephamycin C and CA. In addition, the expression of *ccaR* has increased mildly while a drastic decrease in the transcription of *brp* was observed. Interestingly, AreB requires an additional small protein like structure in a critical level for binding to ARE (Liras *et al*., 2008).

As mentioned in earlier studies, there is no correlation between antibiotic production and ppGpp level in the cell (Bascaran *et al*., 1991; Jones *et al*., 1996; Gomez-Gomez-Escribano *et al*., 2006). However, a higher level of cephamycin C (four fold) and clavulanic acid (six fold) yields were obtained in *ΔrelA* mutant

strains of *S. clavuligerus* than that of the wild strain depending on the increase in *cefD* and *ceaS2* transcript amounts (Gomez-Escribano *et al*., 2008).

1.9. Nutritional regulation of cephamycin C biosynthesis in *S. clavuligerus*

As *S. clavuligerus* is an industrially important β-lactam producer, nutritional regulation of the antibiotic production was also studied in detail by many researchers.

Although other actinomycetes have the ability to utilize all carbon sources, *S. clavuligerus* cells are unable to utilize simple sugars such as glucose and other hexose, pentose, disaccharides, sugaralcohols. Because, they do not have an active transport system required for glucose uptake inside the cell. Besides, the sucrose utilization was undetectable (García-Domínguez *et al*., 1989). The presence of a defective glucose permease gene might explain this phenotype in *S. clavuligerus*. The absence of an active glucose permease gene is most probably due to the adaption of *S. clavuligerus* to glucose-deficient habitats (Perez-Redondo *et al*., 2010). On the other hand, starch and glycerol, favorable carbon sources for this microorganism, have different effects on clavulanic acid (glycerol) and cephamycin C (starch) biosynthesis (Efthitoimu *et al*., 2008). High concentrations of glycerol and maltose inhibit cephamycin C production, but its production might be induced in the presence of low amounts of glycerol (Romero *et al*., 1984). In addition, starch, α-ketoglutarate and succinate stimulate cephamycin C prodution in the cell (Aharonowitz and Demain, 1978).

Inorganic phosphate in high concentration drastically represses cephamycin C and especially clavulanic acid production in *S. clavuligerus*. Sulphur is a crucial source for cephamycin C production, but its high level can also inhibit the antibiotic production (Aharonowitz and Demain, 1977; Romero *et al*., 1984). Aminoacids such as asparagine support both growth and antibiotic production, however, ammonium seriously depresses cephamycin C and clavulanic acid yields in *S. clavuligerus* (Aharonowitz and Demain, 1979; Romero *et al*., 1997).

The nitrogen sources such as glutamine and arginine enhance cephamycin C production by the cell (Aharonowitz and Demain, 1979).

1.10. Draft genome sequence of *S. clavuligerus*

Song *et al* (2010) has published a draft genome sequence of *S. clavuligerus* NRRL 3585, the type strain. The genomic data was obtained from high throughput techniques; a combination of Sanger sequencing, Roche/454 pyrosequencing, optical mapping and partial finishing. Following the analyses, it was reported that NRRL3585 strain has one linear chromosome (6,736,475 bp in size having a G/C content of 72.69 %) and four linear plasmids, namely; pSCL1 (10,266 bp in size with a G/C content of 71.96 %), pSCL2 (149,326 bp in size with a G/C content of 70.07 %), pSCL3 (442,792 bp in size with a G/C content of 70.77 %) and pSCL4 (1,796,117 bp in size with a G/C content of 71.85 %). 7898 protein-coding genes were reported, as well. Afterwards, a genomic data belonging to *S. clavuligerus* ATCC 27064 has been published by another group (Medema *et al.* 2010). They have sequenced and assembled ATCC 27064 genome by random shotgun sequencing. Both group reported quite similar sequences of the linear chromosome and the mega plasmid pSCL4, however, the remaining three plasmids were unique findings of Song *et al.* (2010). pSCL4 is very attractive megaplasmid, because of being as one of the largest plasmid that have determined and sequenced so far. It has 25 putative gene clusters responsible for secondary metabolite biosynthesis, especially antibiotics such as staurosporine, moenomycin, β-lactams, and enediy. Cephamycin C- clavulanic acid gene cluster lie on the main chromosome whereas, the alanyl clavam cluster-paralogous cluster of clavulanic acid - is located at pSCL4. The distance between the cephamycin C and clavulanic acid clusters was found to be approximately 1.4 Mb. The genome consists of a totaly 48 gene clusters of secondary metabolites like terpenes, pentalenenes, phytoenes, siderophores, antitumor compounds and lantibiotics (Medema *et al*., 2010; Song *et al*., 2010).

1.11. Contribution of genome sequencing in discovery of new secondary metabolite gene clusters

Genome sequencing of actinomycetes has revealed the presence of numerous cryptic secondary metabolite gene clusters of unknown function that could provide future clinically useful antibiotics (Zerikly and Challis, 2009). Identification of these novel cryptic pathways necessiates the use of bioinformatics together with cloning studies to express these genes in a well characterized secondary metabolite non-producer host (Baltz, 2010). The genome-minimized strains were used to express heterologous secondary metabolite pathways, including the streptomycin gene cluster from *S. griseus*, and the cephamycin C gene cluster from *S. clavuligerus*, and the pladienolide pathway from *S. platensis*. With the availibility of complete genome sequence of *S. coelicolor*, the model organism of actinomycetes, Gomez-Escribano and Bibb (2011) has created a novel host for heterologous expression of biosynthetic gene clusters. They removed four antibiotic clusters of the organism itself to eliminate competence for precursors, to increase production capacity of hererologously expressed antibiotics and to simplify characterization of expected products. By this way, high level production of heterologously expressed antibiotics was achieved in engineered *S. coelicolor* strains. The reduction of metabolic profile in such kind of manipulated strains might play a key role in the discovery of novel secondary metabolites as well as their high capacity production by combinatorial biosynyhesis (Gomez-Escribano and Bibb, 2011).

1.12. Quantitative RT-PCR

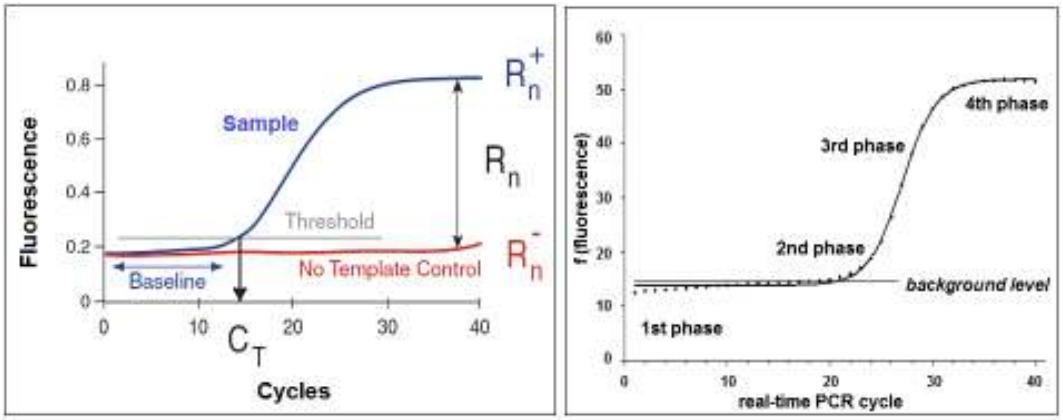
Quantitative RT-PCR (qRT-PCR) is a postmodern PCR technology that allows simultaneous amplification and detection of DNA templates on the basis of flourescence emmission. As opposed to the conventional PCR, qRT-PCR detects the initial amount of DNA sample accurately even if a trace amount of DNA is present in the reaction. qRT-PCR has many application areas in diagnostics, clinical studies, pathogen detection, forensics, food technology, cancer quantification and functional genomics (Bustin, 2005, Yuan *et al*., 2008). qRT-PCR is used for quantification of temporal and spatial patterns of gene

expression and has many advantages over traditional techniques (i.e. Northen Blot, nuclease protection assays) with its simplicity, high sensitivity, accuracy and rapidity. The uniqueness of this technology is also depends on the necessity of low amount of starting template while considerably high amount of starting template is needed for Northen Blot and nuclease protection assays because of their low sensitivity. Its major disadvantage is the requirement of expensive equipment and reagents. The accuracy and reliability of the data obtained from a qRT-PCR is primarily based on following factors: (i) RNA quality and quantity, (ii) RT-PCR efficiency, (iii) detection chemistry, (iv) quantification strategy, (v) amplification efficiency evaluation, (vi) data normalization and (vii) statistical comparison (Bustin, 2000; Pfaffl and Hageleit, 2001; Pfaffl, 2005; Derveaux, 2010).

The first principle in qRT-PCR is to standardize an isolation method to obtain high quality and intact RNA free from DNA and protein contamination (Swift *et al*., 2000; Mannhalter *et al*., 2000). The quality of RNA and primers used in cDNA synthesis are important factors effecting RT efficiency. Besides target specific primers, target gene unspecific primers (such as random hexamers) and poly-T oligonucleotides can be used for cDNA synthesis. However, obtained RT efficiency can be ranked in an order of random hexamer primers, poly-dT primer and gene-specific primer, respectively (Pfaffl, 2004).

qRT-PCR has mainly four phases; the first 3-15 cycles correspond to the initial phase (linear ground phase) and the increase in fluorescence is above the baseline indicating the detection of accumulated PCR product (Pfaffl, 2004) (Figure 1.8). Subsequently, the initial copy number in the original template and the cycle number used to calculate experimental results are detected in the exponential phase. The first significant increase in the flourescence emmission above the background is named as threshold cycle “Ct” (ABI Prism® literature, Applied Biosystems, Foster City, CA, USA) or crossing point “Cp” (LightCycler® literature, Roche Applied Science, Indianapolis, IN, USA) value. This first significant increase in the amount of PCR product is proportional to the initial amount of target template. If the initial number of the target DNA is high, it needs shorter time to get a considerable increase in the fluorescence emisson. In optimal conditions, it is assumed that PCR product will double during each cycle

if efficiency is 100 %. The efficiency of amplification depends on the primer and template quality, as well as suitable PCR conditions and amplicon lengths (Valesek and Repa, 2005; Dorak, 2006; Yuan *et al*., 2006). In the third phase of qRT-PCR a linear fluorescence emission occurs. The plateau phase is the last phase of kinetic PCR where reaction components are exhausted and no fluorescence intensity was obtained for data calculation (Wong and Medrano, 2005).



(a) **(b)**

Figure 1. 8. A typical amplification plot in qRT-PCR http://www.nursa.org/qpcr_tutorials/qPCR_A&E_08.pdf (a) and four characteristic phases of qRT-PCR (Kain, 2000) (b).

There are several fluorescent probes used in detection chemistry of Real time PCR, such as SYBR green I, Taqman-hydrolysis probes, hybridization probes, molecular beacons, sunrise and scorpion primers, LUX primers, and peptide nucleic acid (PNA) light-up probes. All of them have distict properties and different strategies have been used, but they lead change in the fluorescence correlating to amplification of DNA. Taqman and SYBR green I chemistries are comparable dynamic range and sensitivity among all fluorescent probes and the latter one detects more precisely and produces a more linear decay plot than the

TaqMan probe detection (Schimittgen *et al*., 2000). However, nonspecific binding is a disadvantage in SYBR green I chemistry. This disadvantage is eliminated by melting point curve determination in amplicon detection by applying extensive optimization studies. SYBR green I binds to minor groove of DNA and the fluorescence highly increases upon binding to double-stranded DNA. Maximum excitation and emission wavelengths are 494 ve 521 nm, respectively (Pfaffl, 2004).

qRT-PCR is conducted as one-step or two-step reactions. In one-step qRT-PCR, cDNA synthesis and PCR amplification is performed in a single tube in order to minimize experimental variation while reverse transcription and PCR amplification occur in separately in two-step qRT-PCR. Two-step qRT-PCR is preferred when SYBR green I chemistry is used for detection as it reduces primer dimer formation and is reproducible. The use of labile RNA as a template and the tendency of contamination are drawbacks of one-step and two-step qRT-PCR, respectively (Wong and Medrano, 2005).

There are two ways to quantitate gene expression: The relative and the absolute quantitation of gene expression. Both methods use Ct value for quantification. In absolute quantification strategy, the quantification of an unknown sample is based on a standard curve of a sample with known quantity by assuming that all standards and samples have quite similar amplification efficiencies (Souzae *et al*., 1996). The principle of relative quantitation approach is determination of the changes in the steady-state levels of a gene of interest relative to an invariate control gene, being either an external standard or a reference gene (calibrator/endogenous control/normalizer) (Livak and Schmittgen, 2001). Generally, this reference gene is a continuously expressed housekeeping gene and the results are given as target/reference ratio. As there is no need for time consuming and labour intensive calibration curve generation, the relative quantification is used more frequently than the absolute quantification. Physiological changes in gene expression levels are measured by relative quantification strategy. In this method, the absolute quantity of internal standard is not known but its sequence is known. The mean normalized expression level of a target gene (target/reference ratio) is calculated by using different mathematical models. Comparative Ct ($2^{-\Delta\Delta Ct}$) method and the efficiency-corrected Ct model of

Pfaffl are most frequently preferred mathematical models but differs from each other in simplificity and efficiency correction (Fleige *et al*., 2006). The comparative Ct method calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample by assuming the amplification efficiencies of the target and reference genes are equal (the normalizer is the reference) (Livak and Schmittgen, 2001). In this case, the expression differences between Ct values of the control and sample can be formulated:

R (Relative expression ratio) = $2^{-(\Delta\Delta Ct)}$ where;

$(\Delta\Delta Ct) = (\Delta Ct \text{ target} - \Delta Ct \text{ reference})_{sample} - (\Delta Ct \text{ target} - \Delta Ct \text{ reference})_{control}$

ΔCt = Ct (target) - Ct (normalizer/calibrator/reference)

However, most of the time, the efficiencies of target and control sample are unequal. In such conditions, efficiency corrected Ct model have to be used. Here, amplification efficiencies of both target and reference gene are incorporated to the formula (Pfaffl, 2001).

$$R = \frac{(E_{target})^{\Delta C_T \text{ target (MEAN control – MEAN sample)}}}{(E_{reference})^{\Delta C_T \text{ reference (MEAN control – MEAN sample)}}}$$

The amplification efficiency is obtained from the slope of a standard curve plotted Ct versus log of nucleic acid concentration and is based on the following formula (Wong and Medrano; 2005).

$$\text{Exponential amplification} = 10^{(-1/slope)}$$

$$\text{Efficiency} = [10^{(-1/slope)}]^{-1}$$

Data evaluation and statistical comparison in qRT-PCR are other imporant parameters for meaningful and reliable conclusions. The use of independent

biological replicates and adequate technical replicates as well as the right reference gene selection strenghts the results by reducing intra- and inter-assay variations. Several software tools have been developed to accomplish relative quantification results, i.e. REST, Q-Gene, SoFAR,.DART-PCR. The relative expression software tool (REST©) provides an automated data analysis based on the same principle described by Pfaffl (2002). In this software, significance of results based on Pairwise Fixed Reallocation Randomization Test© is calculated and the availability of the reference gene for normalization is determined (Pfaffl, 2002; Wong and Medrano, 2005).

1.13. Electrophoretic Mobility Shift Assay (EMSA)

EMSA is a rapid and very sensitive technique to detect DNA binding properties of a transcription factor thereby, allowing to identification and characterization of transcriptional regulation of gene expression. In EMSA assay, affinity of a transcription factor to a specific nucleotide sequence (radio labeled or flourescence labeled oligonucleotide probe) leads the formation of protein-nucleic acid complex and this complex migrates slower than free DNA through a nondenaturing polyacrylamide gel by giving an additional retarded band on the gel (Hellman and Fried, 2007).

Mobility-shift assays (also named as gel retardation and DNA binding assay) can be used for both qualitative and quantitative manner under appropriate conditions for the determination of binding stoichiometries, affinities, thermodynamics and kinetics. In addition, the availibility of the use of crude or whole cell extracts in EMSA is a big advantage. The radioisotope-labeled probes enhance sensitivity of the assay and make possible to study with low concentrations of protein and nucleic acids (0.1 nM or less). On the other hand, detection step is long and labor intensive indeed, the radiolabeled probes are hazardous and quite unstable. Due to these kind of limitations, new detection types were developed such as fluorescence, chemiluminescence and immunohistochemical detections which are not hazardous and stable for a long time as well as the detection is very fast (Jason *et al*., 2000; Steiner and Pfannschimidt, 2009).

Nitrocellulose filter-binding and footprinting assays are alternative techniques of EMSA for detection and characterization of protein-nucleic acid complexes and they have superorities and drawbacks that are different from those of EMSA. In nitrocellulose filter-binding assay, nucleic acid fragments bound by DNA-binding proteins filtered through nitrocellulose while unbound nucleic acids were washed from the filter. Protein bound DNA is eluted by denaturing the enzyme, and analysed by gel electrophoresis. A quantitative measurement about the binding activity of a specific nucleic acid is achieved by this method. It is non-equilibrium like EMSA and there is no limitation of salt concentration (Woodbury *et al*., 1983; Oehler *et al*., 1999).

DNA footprinting is a powerful technique used to examine binding of proteins to specific DNA sequences. The protein-nucleic acid mixture is exposed to DnaseI digestion. Protein bound DNA is protected from clevage by nucleases. When the digested DNA mixture was run through polyacrylamide gel, a gap is formed in the ladder named as “footprint” of protein. Comparison of the protected pattern with sequencing reaction products makes possible the identification of protected sequences. In this technique, two optimization studies are required at the same time: optimization of binding by the related protein and optimization of the nucleic acid modification reaction to produce the footprint signal. In this manner, it is more diffucult technique than EMSA and filter binding assay. However, DNA footprinting and gel shift assays altogether give comprehensive knowledge about physical interactions between transcription factors and DNA (Ralston, 2008).

1.14. Present study

Cephamycin C is a clinicaly important β-lactam antibiotic which is active on penicillin resistant bacteria while clavulanic acid is prescribed clinicaly in combination with β-lactam antibiotics to treat bacterial infections. Due to its importance in medicine, cephamycin C biosynthetic pathway is a very important research era for understanding the regulation of the antibiotic biosynthesis by *S. clavuligerus.* Both global and pathway-specific regulation on cephamycin C biosynthesis was reported by different groups. In this study, the expression of genes of the cephamycin C gene cluster in a *ccaR*-disrupted *S. clavuligerus*

strain and in a *ccaR* overexpressed strain of *S. clavuligerus* were compared with that of the wild type strain in a time-dependent manner by using qRT-PCR technique to examine the effect of pathway-specific CcaR activator on cephamycin C biosynthesis. The possible DNA-binding sites of CcaR within the upstream regions of cephamycin C genes were identified by EMSA analyses. In addition, the effects of *ccaR* disruption and overexpression on cephamycin C and clavulanic acid yields were determined by bioassay and HPLC analyses. All these findings revealed the molecular mechanism of pathway-specific CcaR regulation of cephamycin C production in the cell to contribute improvement strategies of cephamycin C biosynthesis by the producer strain.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, media and culture conditions

The microorganisms and plasmid vectors used during this study are listed in Table 2.1. *Escherichia coli* (*E. coli*) was grown in either Luria broth (LB) or on agar plates (LA) (Appendix C) with orbital agitation at 37 °C. *E. coli* was maintained on agar plates up to 4 weeks at 4°C. For long term preservation, 3 ml of *E. coli* cultures incubated for o/n at 37°C was dissolved in 1 ml of 25 % glycerol and stored at -80 °C until use. *Streptomyces clavuligerus* was grown in Trypticase soy broth (TSB) (Appendix C) by incubating on a rotary shaker (220 rpm) in baffled flasks at 28 °C. Tripticase soy agar (TSA) (Appendix C) was used to check any contamination in liquid cultures of this strain. *S. clavuligerus* was stored as mycelium stocks. The mycelium stocks were prepared from the cultures in which OD_{600} reached to 5-7. 800 µl of this culture was mixed with 800 µl of 87 % glycerol in 2 ml of Eppendorf tubes and stored at -80 °C.

TSB and Starch-Asparagine (SA) media were used for antibiotic production studies (TSB for cephamycin C and SA for clavulanic acid production) in *S. clavuligerus*. Seed culture of *S. clavuligerus* was required for fermentation studies. 100 ml of TSB was inoculated with 500-750 µl of glycerol stocks of *S. clavuligerus* and incubated at 28 °C and at 220 rpm till OD_{600} becomes 5. Then, 5 ml of this seed culture was centrifuged at 3500 rpm for 10 min. The supernatant

was discarded completely and the pellet was inoculated into 100 ml of TSB or SA medium according to the purpose.

E. coli vectors were pGEM-T easy (Promega) and pBluescript II KS (+) (Stratagene). *E. coli*/*Streptomyces* shuttle vector pSET152, and pSPG expression vector with its strong glycerol promoter were used for genetic manipulation of *S. clavuligerus* strain. pET28a(+) was the His-Tag expression vector used for purification of CcaR protein (Figure A.1, Appendix A).

Table 2. 1. List of bacterial strains and plasmids used in this study

Strains and plasmids	Description	Source or reference
Strains		
S. clavuligerus		
ATCC27064	Wild type, cephamycin C and clavulanic acid producer	Prof. P. Liras, INBIOTEC, Leon, Spain
ccaR-	*aphII* disrupted *ccaR* containing strain	Prof. P. Liras, INBIOTEC, Leon, Spain (Perez-Llarena *et al*., 1997)
NRRL3585	Wild type, cephamycin C and clavulanic acid producer	Prof. J. Piret, Northeastern University, USA
pGV	pSPG carrying recombinant *S. clavuligerus* NRRL3585	This study
C11	pAK23 carrying recombinant Indicator organism *S. clavuligerus* NRRL3585	This study
pTV	Recombinant *S. clavuligerus* NRRL3585 in which pSET152 was integrated to its chromosome	This study
PC	Recombinant *S. clavuligerus* NRRL3585 in which pSET-PC was integrated to its chromosome	This study
Klebsiella pneumoniae		
ATCC 29665	Indicator organism	Prof. P. Liras, INBIOTEC, Leon, Spain

Table 2.1 (cont'd).

E. coli		
DH5α	F` ϕd*lac*ZM15 (*lac*ZYA *arg*F), U169, *sup*E44λ⁻, *thi*-1, *gyr*A, *rec*A1, *rel*A1, *end*A1, *hsd*R17	*E. coli* Genetic Stock Center
BL21(DE3)	F⁻ *ompT gal dcm lon hsd*S_B(r_B- m_B-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 ind1 sam7 nin5])	Novagen, Merck (Germany)
ESS	β-lactam supersensitive strain	Prof. J. Piret, Northeastern University, USA
ET12567	F⁻ dam 13::Tn9 dcm-6 hsdM hsdR, lacYI	Prof. Keith Chater, John Innes Centre, Colney, Norwich, UK
Plasmids		
pGEM-T®-Easy	Amp^R, *lacZ*'	Promega
pSET152	*lacZ, rep*^{puc}, *att*$^{\Phi C31}$, *oriT*	Prof. Paloma Liras, INBIOTEC, Leon, Spain (Bierman *et al*., 1992)
pSPG	Amp^R, Ap^R,Pglp	Prof. Paloma Liras, INBIOTEC, Leon, Spain
pBluescript II KS (+)	Phagemid, Amp^R, *lacZ*'	Stratagene
pET28a(+)	T7 Promoter, His Tag, *lacI*	Novagen
pG23	*ccaR* carrying recombinant pGEM-T easy	This study
pG15	p*ccaR* carrying recombinant pGEM-T easy	This study
pKS15	p*ccaR* carrying recombinant pBluescript II KS (+)	This study
pAK23	*ccaR* carrying recombinant pSPG	This study
pSET-PC	p*ccaR* carrying recombinant pSET152	This study
pET-C23	*ccaR* carrying recombinant pET28a(+)	This study

2.2. Culture media

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

2.3. Buffers and solutions

Buffers, solutions, their compositions and preparations are listed in Appendix D.

2.4. Chemicals and enzymes

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

2.5. Isolation of genomic DNA from *S. clavuligerus*

Genomic DNA isolation from *S. clavuligerus* was carried out by using salting out method described by Pospiech and Neumann (1995). 50 µl of mycelium stock of *S. clavuligerus* was inoculated into 50 ml of TSB in 250 ml of baffled flask and incubated at 220 rpm and at 28 °C for 48-60 h. 30 ml of this seed culture was harvested by centrifugation at 2000 rpm for 15 min, and the pellet was resuspended in 5 ml of SET buffer (Appendix D). After addition of 100 µl of lysozyme (Appendix D) to the resuspension and incubation at 37 °C for 1 h, 140 µl of proteinase K (Appendix E) was also included to the mixture. Following this, 600 µl of 10 % SDS (Appendix E) was added and the solution was mixed by inversion. Next, 2 ml of 5 M NaCl (Appendix E) was mixed thoroughly. The procedure continued by addition of 5 ml of chloroform (Appendix E) to the mixture. After mixing of the solution by inversion for 30 min at 20 °C, it was centrifuged at 6000 rpm and at 20 °C for 15 min. The supernatant was transferred to a falcon tube and 0.6 vol. isopropanol was added and mixed by inversion again. After 3 min, DNA was spooled onto a sealed Pasteur pipette, and rinsed in 5 ml of 70 % ethanol, air dried and finally dissolved in 1 ml TE (Appendix D) at 55 °C (Kieser *et al*., 2000).

2.6. Manual plasmid isolation from *S. clavuligerus* and *E. coli*

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were used for isolation of *E. coli* plasmid DNA as specified by the manufacturers. In addition, plasmid isolation form *Streptomyces* and *E. coli* was performed by using the small scale plasmid DNA isolation method (Hopwood *et al*., 1985). 1 square cm of bacterial patches on selective LA (*E. coli*) and TSA (*S. clavuligerus*) (Appendix C) was put into an Eppendorf tube containing 50 µL STE solution (Appendix D). Lysozyme as a final concentration of 2 mg/ml (Appendix E) was added to STE solution containing *S. clavuligerus* cells and mixed. *E. coli* tubes were incubated on ice for 20 min while *S. clavuligerus* cells in STE were

incubated at 37 °C for 30 min. Then, 3/5 volume of the lysis solution (Appendix D) was added to the mixtures and vortexed immediately. For lysis of the cells, the mixtures were incubated at RT for 10 min and then at 70 °C for 10 min for denaturation of DNA. The tubes were cooled rapidly in cold water immediately. An equal amount of phenol-chloroform solution was added (water-saturated, Appendix D), vortexed hard until a homogeneous and milky white mixture was obtained. Finally, the samples were centrifuged at 13000 rpm for 5 min to separate phases. 10 µl of supernatant was loaded on an agarose gel for electrophoresis.

2.7. Preparation of *E. coli* competent cells

E. coli competent cells were prepared by using $RbCl_2$ method described by Hanahan *et al*. (1982). One *E. coli* DH5α/ *E.coli* BL21/ *E.coli* ET12567/pUZ8002 colony from fresh LA culture was inoculated into 3 ml of LB (related antibiotics were added if necessary) and incubated by shaking at 37 °C for o/n. 3 ml of this culture was transferred to 200 ml of LB in 500 ml Erlenmayer flask and incubated on an orbital shaker at 37 °C until OD_{600} reaches to 0.4-0.7. The culture was then incubated on ice for 15 min. and centrifuged at 3500 rpm and at 4 °C for 5 min. After decanting the supernatant, the pellet was dissolved in 20 ml of ice cold Buffer 1 (Appendix D). The cells were kept on ice throughout the procedure. The resuspended cells were centrifuged in the same conditions and the supernatant was discarded again. The cells were resuspended in 8 ml of ice cold Buffer 2 (Appendix D) and this cell suspension was aliquoted in 100 µl volumes to the 1.5 ml Eppendorf tubes. They were incubated on ice for 15-30 min. Finally, the tubes were frozen in liquid nitrogen and stored at -80 °C until use.

2.8. Transformation of *E. coli* competent cells

Competent *E. coli* cells were transformed as described by Sambrook *et al*. (1989) with slight modifications. *E. coli* competent cells kept at -80 °C were slowly thawed on ice or directly thawed on hand. Plasmid DNA 1-10 µl (1- 50 ng) or ligation product (in a maximum volume of 10 µl) was added to 100 µl of *E. coli* competent cells and mixed gently by pipetting. The tubes were kept on ice for 30 min. Then, the cell-DNA mixture was heat-shocked at 42 °C for 1 min. in a water

bath. Next, the tubes were immediately put on ice and incubated for 5 min. 0.9 ml of LB was added to each tube and the cultures were incubated for 1 h at 37 °C with a gentle shaking (100 rpm). The cells were centrifuged at 3400 rpm for 10 min., the supernatant was removed and the pellet was resuspended in 100-200 µl LB. Finally, the cell suspension was spread onto LA plate containing a selective antibiotic and incubated at 37 °C for o/n. Single colonies of transformants were selected and plasmids from these colonies were isolated for further analysis.

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

Intergeneric conjugation between *Streptomyces* and *E. coli* was carried out by using modified procedure of Flett *et al*. (1997) which was first described by Mazodier *et al*. (1989). Competent cells of *E. coli* ET12567/pUZ8002 were prepared by using $RbCl_2$ method. The competent cells were grown in the presence of kanamycin (Km) (25 µg/ml) and chloramphenicol (Cm) (25 µg/ml) (Appendix D) to maintain selection for pUZ8002 and the *dam* mutation, respectively. The competent cells were transformed with the *ori*T-containing vector, and were selected with the appropriate antibiotics. A colony from recombinant *E.coli* ET12567/pUZ8002 containing the desired plasmid was inoculated into 10 ml of LB plus required antibiotics (Cm, km, and apramycin to select *ori*T-integration vector) and it was grown at 37 °C and 200 rpm for o/n. The culture was then diluted 1:100 in fresh LB containing three antibiotics and grown at 37 °C to reach an OD_{600} value of 0.4-0.6. The cells were washed twice with an equal volume of LB and were resuspended in 0.1 volume of LB. 24 h old solid cultures of *S. clavuligerus* grown on TSA plates were scraped and this mixture was dissolved in 5 ml of 20 % glycerol by pipetting or vortexing until a homogenous *Streptomyces* cell suspension was obtained. 0.5 ml of this suspension was added to 500 µl of *E. coli* cells and mixed thoroughly. The *E.coli*-mycelium suspension was centrifuged at 3000 rpm for 10 min and three quarters of the supernatant was poured off and the pellet was resuspended in the residual liquid. The cells were plated out on Mannitol-Soybean flour (MS) agar containing 10 mM $MgCl_2$ and incubated at 30 °C for 16-20 h. Next, the MS agar plates were overlaid with 1 ml of dH_2O containing nalidixic acid and apramycin as final

concentrations of 0.5 mg/ml and 1 mg/ml, respectively (Appendix D) by using a spreader. The incubation was continued at 30 °C up to 4 days.

2.10. Manipulation of DNA

2.10.1. Restriction endonuclease digestions

Restriction endonucleases (Roche and Fermentas) were used for cloning purposes and verification of recombinations, as well. Restriction enzyme digestions were performed under the conditions specified by the manufacturers.

2.10.2. Agarose gel electrophoresis

DNAs/RNAs to be analysed were run in 1% agarose gels by applying 90 V for 1 h. 1X TAE was used as electrophoresis buffer. RT-PCR products ranged between 100-200 bp in size were run in 1.5 % agarose gel. The runned gels were stained with 1X TAE buffer containing ethidium bromide (Appendix D) as a final concentration of 1 mg/ml for 15 min at RT. The DNA bands on the gel were visualized on a UV transilluminator (UVP) and photographed by using Vilber Lourmat Gel Imaging System. 1 kb DNA Ladder (Invitrogen), Lambda (λ)/*Pst*I DNA (Fermentas) and/or O'GeneRuler 100 bp DNA ladder plus (Fermentas) (Appendix A) were used to as the size markers.

2.10.3. Extraction of DNA fragments from agarose gels

After electrophoresis, desired DNA bands were excised from the gel and weighed in Eppendorf tubes. Purification of DNA slices were performed by using Genemark Gel Extraction kit (http//www.genemark.com.tv). The purified DNA concentrations were measured by using NanoDrop® ND-2000 (ThermoScientific).

2.10.4. Ligations

Ligation reaction of PCR products with pGEM-T easy vector was performed as follows: 1 µl T4 ligase (Promega), 5 µl of 2X ligation buffer (supplied by the manufacturer), 1 µl (55 ng/µl) pGEM-T easy vector, 3 µl insert DNA were mixed

and volume was completed to 10 µl with dH_2O. Ligation reaction was proceded at 4°C for 16 h.

For other ligation reactions, insert: vector molar ratios were used. Generally, 3:1 and 5:1 insert: vector molar ratios were applied for sticky and blunt end ligation reactions, respectively. The reaction volume was set to 10 µl and incubation was performed at 4 °C for 16 h.

2.11. Primer design

ccaR gene, alone and with its own promoter, was amplified from genomic DNA of *S. clavuligerus* NRRL3585 by using forward ccaR_F containing *NdeI* restriction site/pccaR_F and ccaR_R reverse primers (Figure B.1, Appendix B). Verification of recombination was also performed via PCR by using specific primers (Table B.1, Appendix B).

The sequences of *bla*, *pbpA*, *cmcT*, *pcd*, *cefD*, *cmcI*, *cmcJ*, *ccaR*, *orf10*, *blp*, *lat*, *pcbAB*, *pcbC, pcbR*, *cefE, cefF* and *cmcH*, all the genes in the cephamycin C gene cluster, having GenBank Accession number recorded in NCBI webpage (http://www.ncbi.nlm.nih.gov/) were used for primer design. Besides, the primers for intergenic regions between *cefD-cefE*, *cefE-pcd*, *pcd-cmcT*, *cmcT-pbpA*, *cmcI-cmcJ*, *cmcJ-cefF*,*cefF-cmcH*, *ccaR-orf10*, *orf10-blp*, *blp-lat*, *lat-pcbAB*, *pcbAB-pcbC*, *cmcH-ccaR* genes were also designed. PrimerSelect programme of DNAStar software was used to design primers for RT-PCR experiments (Table B.2 and B.3, Appendix B). For Q-RT-PCR experiments, both PrimerSelect and Primer3 plus (www.bioinformatics.nl/cgi-bin/**primer3plus**/**primer3plus**.cgi) programmes were used. The amplicons sizes designed for RT-PCR and Q-RT-PCR analyses were 120-200 bp (Table B.2 and 3.2, Appendix B). However, the sizes of amplicons for analysis of co-transcription of successive genes were ranged between 421 and 1416 bp. Furthermore, primers incuding upstream of the genes were also designed for use in EMSA experiments (Table B.4, Appendix B). All primers designed were synthesized by Alpha DNA (Montreal, Canada).

2.12. Polymerase chain reaction

2.12.1. Standard PCR

PCR reactions were performed by using Go-Taq polymerase (Promega). The ingredients used in a standard PCR reaction and the optimized conditions were given in Table 2.2.

Table 2. 2. A standard PCR reaction and applied conditions

Ingredients	Final concentration	Volume (µl)	PCR conditions	Temperature/Time °C/min or sec	
Go-Taq Buffer (5X)	1X	5	Initial denaturation	94 °C	5-10 min
dNTP mixture (10 mM)	200 nM	1	Denaturation	95 °C	30-40 sec
Forward primer (10 µM)	0.2 µM	1.25	Annealing	(Tm -10)	30 sec
Reverse primer (10 µM)	0.2 µM	1.25	Extension	72 °C	1 min/kb
Template DNA	50 ng	2	Cycle #	30-35	
$MgCl_2$ (25 mM)	2 mM	2	Final extension	72 °C	10 min
DMSO 100%	5 %	1.25			
GoTaq DNA Pol (5 u/µl)	2.5 u	0.25			
dH_2O		11			

2.12.2. Colony PCR

In order to perform PCR reaction from solid cultures, a small amount of colonies was dissolved in 50 µl dH_2O and incubated at 94 °C for 15 min. 2-3 µl of this suspension was used as template DNA in a standard PCR reaction.

2.13. Sequencing reactions

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

2.14. Fermentation studies: Bioassay and HPLC analyses

2.14.1. Growth determination in terms of DNA quantification

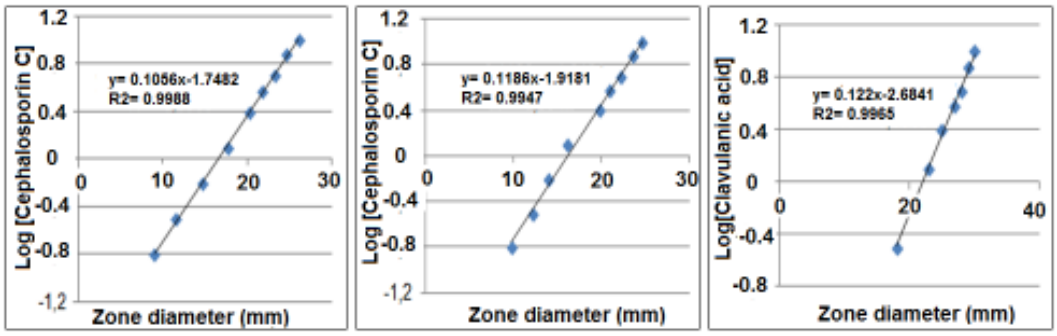
Growth measurement of cultures in terms of DNA quantification was performed according to Burton (1968). The pellets stored at -20 °C were thawed on ice, resuspended in 1 ml of 0.85 % NaCl solution and a homogenous solution was obtained by vortexing. 0.3 mg/ml stock solution of DNA from Herring Sperm (Sigma) (dissolved in 5 mM NaOH) was used for standard preparation. Standard DNA concentrations used were as follows: 6, 10, 20, 30, 50, 60 75 and 100 µg/ml. 400 µl of 5 mM NaOH (for blank sample), 400 µl of standards and 400 µl of diluted samples were transferred into 2 ml Eppendorf tubes. 400 µl of 1 N $HClO_4$ was added to each sample and standard tubes. The tubes were mixed by inversion and incubated at 70°C for 20 min. 800 µl of diphenylamine reagent (Appendix D) was added into each tube. The samples were mixed by inversion and incubated at 30 °C for 15-17 h. Samples taken out of the incubator were mixed and the mycelia were precipitated by centrifugation at 13200 rpm for 10 min. The absorbance of the supernatant was measured at 600 nm. The amount of DNA samples were calculated according to a standard curve drawn using the data obtained by Herring sperm and expressed as µg of DNA per ml of culture.

2.14.2. Bioassays experiments of cephamycin C and clavulanic acid

E. coli ESS 22-35 supersensitive strain to β-lactam antibiotics (Aharonowitz and Demain, 1978) was used as an indicator organism in cephamycin C bioassay while clavulanic acid bioassay was performed by using *K. pneumoniae* ATCC 29665 as the indicator organism. Clavulanic acid in the samples inhibits β-lactamase produced by *K. penumoniae* and allows penicillin G in TSA to produce inhibition zone (Foulstone and Reading, 1982; Romero *et al*., 1984). Supernatant

of 1 ml culture, collected at 24 h intervals during the fermentation process, was used for the determination of cephamycin C and clavulanic acid production in the cultures. As cephamycin C is a quite stable antibiotic, the supernatants can be stored at -80 for one week. However, bioassay studies for determination of clavulanic acid in the supernatants were performed just after harvesting the samples because of its low stability. Potassium clavulanate and cephalosporin C were used as standards in the clavulanic acid and cephamycin C bioassays, respectively.

Samples kept at -80 °C was thawed on the ice on the assay day. *E. coli* ESS and *K. pneumoniae* cells were grown in TSB up to an OD_{600} value of 1.0. Sterile glass-made petri plates, 17 cm in diameter, were used for the bioassay experiments. 100 ml TSA (2 % agar) was cooled to 45 °C-47 °C for each petri plate before *E. coli* ESS and *K. pneumoniae* inoculations into the agar, respectively. 100 µl of penicillin G (25 mg/ml) (Appendix D) was added to 100 ml TSA to which *K. pneumoniae* would be added. Then, 3.3 ml of the culture was mixed with 100 ml of melted TSA and mixed immediately and gently. TSA-culture mixtures were poured into each petri plate and left for solidification. 5 mm diameter holes were made on TSA plates and 60 µl of sample supernatant and also the standards were added into the holes. 100 mg/ml of potassium clavulanate and cephalosporin C stock solutions were used for dilution. 10-7.5-5-3.5-2.5-1.25-0.625-0.3125 and 0.156 mg/ml concentrations of the standards diluted with 1 M MOPS (pH: 6.8) (Appendix D) were loaded to the holes. Each sample was loaded in two different plates. After adding the samples and the standards, the plates were first kept at 4 °C for 2 h and then incubated at 30 °C for 12-15 h. Inhibiton zones larger than 27 cm for cephamycin C and 30 cm for CA bioassays were accepted as unreliable. Cephamycin C and clavulanic acid concentrations in the samples were calculated according to linear regression equations obtained from the standard curves of cephalosporin C and clavulanic acid (Figure 2.1).



(a) **(b)** **(c)**

Figure 2. 1. Cephalosporin C and clavulanic acid calibration curves, obtained from SA (b, c) and TSB (a) grown cultures, to be used in bioassay experiments.

2.14.3. Fermentation for HPLC analyses of the strains for comparison of their cephamycin C and CA productions

0.5 ml of the mycelium stocks of *S. clavuligerus* was inoculated into 500 ml baffled flask containing 100 ml of TSB in an orbital shaker at 28 °C and 220 rpm. When optical density (OD_{600}) of the 10X diluted suspension of mycelia reached to 0.5, 5 ml of this seed culture was centrifuged at 3500 rpm for 10 min. The supernatant was decanted completely and the remaining pellet was added to 500 ml baffled flask containing 100 ml TSB or 100 ml of SA for the determination of cephamycin C and clavulanic acid productions by the strains, respectively. Incubation was continued at 28 °C and at 220 rpm for 120 h. Triplicate flasks were fermented for each strain. 1 ml sample was taken from each flask for cephamycin C and clavulanic acid bioassays, and DNA quantification. An additional 0.5 ml of culture sample was taken from the cultures for HPLC analysis of clavulanic acid production by these strains.

2.14.4. Sample collection and conditions of HPLC

0.5 ml of sample from each flask was taken from cultures at 24 h intervals. The samples in Eppendorf tubes were poured into 50 ml falcon tubes containing 25 ml of sodium acetate buffer (Appendix D) and the mixtures were shaken vigorously to avoid any clumps. The suspensions were filtered through Millipore membrane (pore diameter 0.4 µm) to remove any mycelia and other insoluble ingredients. The filtered samples were transferred to HPLC vials. The vials filled with filtered samples were placed to carrousels of HPLC and kept at 4 °C prior to the HPLC analysis.

HPLC was performed by using Waters: Alliance 2695 separations module and Bondapak C18 (300×3.9 mm, 5-10 m) column with a flow rate of 1.0 ml/min. The samples were eluted with a mobile phase which consists of sodium dihydrogen phosphate buffer, pH: 4 plus HPLC grade methanol (Appendix E) in 95:5 ratio. The column eluant was monitored at 220 nm by Waters 2487 Dual λ Absorbance detector. As CA peak of the standard sample was obtained at 4-5 th minutes of run, 7 min was chosen as the total run time for each injection. All injections were performed at RT. The data was processed by Waters Empower™ software. CA concentration in the cultivation media was assayed by using HPLC analysis based on the method from the European Pharmacopoeia (1993).

2.14.5. Determination of clavulanic acid calibration curve for HPLC analysis

Potassium clavulanate salt, kindly provided from DEPA Pharmaceuticals, İzmit, Turkey, was used as the standard to obtain clavulanic acid calibration curve in HPLC analysis. 50 mg clavulanic acid was dissolved in 1 ml of sodium acetate buffer. The 50 mg/ml stock solution of clavulanic acid was diluted to 0.4, 0.8, 1, 1.5, 2, 3.5, 5, 7.5, 10, 15 mg/ml standard clavulanic acid solutions to obtain a standard curve as for clavulanic acid calculations from the samples (Figure 2.2). 10 µl of standard samples was taken in each injection and two injections were performed for each sample. The standard solutions were freshly prepared for each run day. The injections were done according to the method from European Pharmacopoeia (1993).

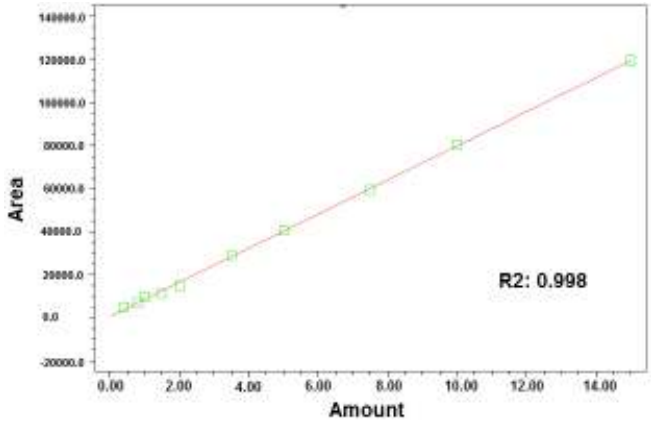


Figure 2. 2. Clavulanic acid calibration curve for HPLC analysis of clavulanic acid production capacities of the strains.

2.15. Preparation of RNA samples

50 µl of mycelial stocks stored at -80°C were used for inoculation of 100 ml of TSB in 500 ml baffled flasks. The seed cultures were incubated at 220 rpm and 28 °C. When OD_{600} reached 0.5 value, 5 ml of seed cultures were transfered to 100 ml of sterile TSB and fermented for 30 h at the same conditions. 600 µl of samples were taken from the cultures at 15 h, 24 h and 30 h points of incubation. 2 volumes of RNA protect solution (Qiagen) was added to 1 volume of culture sample. The solution was mixed by inversion and incubated at RT for 5 min. After centrifugation at 3000 rpm for 5 min, the supernatant was decanted completely and the pellets were stored at -80 °C until use. Slightly modified procedure of Qiagen RNeasy mini isolation kit (procedure for yeast and fungi) was used for RNA isolation. These modifications in the recommended procedure were as follows: (i) addition of lysosyme to RLT buffer as a final concentration of 30 mg/ml, (ii) sonication of samples at middle power (4.5) for 20 sec for 2 min intervals on ice (7 times), (iii) application of phenol-chloroform treatment and usage of phase lock gel heavy tubes to minimize sample lost during the phenol extraction process, (iv) addition of DNase (Qiagen) treatment step adapted to remove DNAs bound to the column together with RNA samples, (v) application of DNA-free™ kit (Ambion) to remove any remaining DNA from the solution eluted

from the column at the last step of the isolation of RNA. The integrity, clarity and amount of RNA (as 1/10 diluted sample) was determined by using NanoDrop® ND-2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A_{260}/A_{280} ratio in a range of 1.8-2.0±0.1 is required for having pure and high quality RNA samples. Furthermore, a A_{260}/A_{230} ratio of about 2.2 ($1.8< A_{260}/A_{230}<2.2$) implies that RNA samples are free from reagent contaminants like salt, phenol, carbohydrates, peptides, or aromatic compounds, reflecting a pure RNA sample. The concentration of RNA samples were given as ng/µl. Stock RNA samples were stored in aliquots at -80°C until used.

2.16. Reverse Transription PCR (RT-PCR)

Superscript™ One-Step RT-PCR kit (Invitrogen) was used for RT-PCR experiments. It includes (2X) Reaction mixture buffer (0.4 mM of each dNTP, 2.4 mM $MgSO_4$) and RT/platinum® Taq mix. In RT minus (RT-) control, only Platinum® Taq polimerase, instead of RT/platinum® Taq mix, was used to detect any possible DNA contamination in the RNA samples used as template in RT-PCR reactions. 20 µl of reaction was carried out as shown below (Table 2.3):

Table 2. 3. Standard RT-PCR reaction conditions

Ingredients		Volume (µl)	Final concentration	
2X reaction mix		10	1X (0.2 mM dNTP+1.2 mM $MgSO_4$)	
Total RNA (100 ng/µl)		2	10 ng	
Forward primer (10 µM)		0.5	250 nM	
Reverse primer (10 µM)		0.5	250 nM	
DMSO		1	5 %	
$MgSO_4$		if required	-	
dH_2O		to 20 µl	-	
RT/Platinum® Taq mix (RT+)		0.3		
Platinum® Taq (RT-) (5 u/µl)		0.16	0.04 u/µl	
RT-PCR conditions				
cDNA synthesis		50 °C	30 min.	1 cycle
Pre-denaturation		94 °C	2 min.	1 cycle
PCR amplification	I- Denaturation	94 °C	30 sec.	
	II- Annealing	Tm-10 °C	30 sec.	15-30 cycles
	III- Extension	72 °C	1 min/kb	
Final extension		72 °C	10 min	1 cycle

2.17. Quantitative RT-PCR (qRT-PCR)

SuperScript™ III Reverse Transcriptase (Invitrogen) was used for qRT-PCR in the present study. 250 ng of random primers (Invitrogen), 2 µg of total RNA, 2 mM dNTP mixture and 14 µl dH_2O were used to synthesize first strand cDNA. This mixture was incubated at 70 ^oC for 5 min and then 4 µl of 5X first strand buffer, 1 µl 0.1 M DTT and 1 µl SuperScript™ III RT (200 u/µl) were added to solution and incubated at 25 ^oC for 5 min and at 55 ^oC for 60 min, respectively. The reaction was inactivated by heating it at 70 ^oC for 15 min. cDNA was diluted 2 times with RNase-free dH_2O and 2 µl of this was used in real time PCR reaction. SYBR Green I (Takara) was used as the flourescent dye in the experiment. A standard kinetic PCR reaction was proceded as follows: 2X SYBR mix, 10 µl; forward primer (10 µM), 0.3 µl; reverse primer (10 µM), 0.3 µl; cDNA, 2 µl; dH_2O, 5.4 µl. Roche LightCycler 1.5 robotics was used in kinetic PCR. Reaction conditions were as follows (Table 2.4):

Table 2. 4. Real time PCR conditions in LightCycler 1.5 instrument

Denaturation-Holding stage	95 °C	30 sec.
Amplification-Cycling stage	95 °C 60 °C	10 sec. 1 min.
Cycle #	40	
Melting curve stage	95 °C 60 °C 95 °C	15 sec. 1 min. 15 sec.
Cooling stage	40 °C	30 sec.

Two independent qRT-PCR runs were performed and each run included two biological replicates and three technical replicates for each sample. Two negative controls, NTC (no template control) having dH_2O instead of cDNA and RTC (RNA

template control), were run simultaneously in each time for each sample to detect the background signal and unwanted primer dimer formation, respectively. The amplification of both reference gene and the target gene was performed by application of sample maximization set-up in each qRT-PCR run in order to minimize run to run variations between the samples as previously proposed by Hellemans *et al*. (2007) and Dervaux *et al*. (2010).

Melting curve analysis (Dissociation curve analysis) is crucial for SYBR Green I based amplicon detection as it detects any double stranded DNA such as primer dimers, contaminating DNA, and PCR products resulted from misannealed primers. Dissociation curves were obtained at melting curve stage of qRT-PCR experiments. The curves were shown in a graph, in which –d/dT Fluorescence (530) vs temperature (^{o}C) was plotted, to be sure that only the desired amplicon was amplified.

2.17.1. Relative quantification of qRT-PCR data based on efficiecy correction

Relative quantification is most commonly used to analyse physiological changes in the level of gene expression in a given sample relative to untreated one. During the qRT-PCR reaction, theoretically, the amount of amplicon increases by doubling in each cycle. In this case, the expression differences between C_T values of the control and sample (expressed as relative expression ratio) can be formulated as follows:

$$R = 2^{-[\Delta C_T \text{ sample} - \Delta C_T \text{ control}]}$$

$$R = 2^{-\Delta\Delta C_T}$$

However, most frequently, the efficiencies of target and control sample may not be equal. In such conditions, efficiency correction based quantification model (shown below) have to be used for calculation of the relative expression ratio. The relative expression of the target gene is normalized by at least one non-regulated reference gene (REF) expression, which is mostly housekeeping gene (Bustin, 2000; Vandesompele *et al*., 2002; Pfaffl *et al*., 2005). The C_T values (Cp)

were determined by 'Second Derivative Maximum Method' of LightCycler software 4.01 (Roche Diagnostics) (Pfaffl *et al*., 2002).

$$R = \frac{(E_{target})^{\Delta C_T \text{ target (MEAN control – MEAN sample)}}}{(E_{reference})^{\Delta C_T \text{ reference (MEAN control – MEAN sample)}}}$$

2.17.2. Statistical analysis of qRT-PCR data

The qRT-PCR data was statistically evaluated by carrying out one-way ANOVA with Tukey post-hoc test (for comparison of gene expression levels in *ccaR* minus strain at T_{15}, T_{24} and T_{30} that in the wild type) and repeated measures of ANOVA test with Bonferroni post-hoc test (the data was analyzed with bacterial groups, namely, the wild type, pGV and C11 strains as independent factor and time as repeated measures) in SPSS v. 17 and Graphpad Prism Software (Graphpad Software. San Diego, CA). The level of significance was stated as * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) in the graphs.

2.18. Purification of CcaR protein

The *ccaR* gene was PCR-amplified and ligated to pGEM-T easy vector. *Not*I digested *ccaR* gene, rescued from pGEM-T easy, was ligated to linearized pET28a (+) with the same enzyme to obtain the expression vector pET-CCAR which was transformed in *E. coli* DH5α and finally in *E. coli* BL21 (Novagen). Overnight grown culture of *E.coli* [pET-CCAR] in 100 ml of LB was used to inoculate 2 % (v/v) of fresh sterile LB and incubated at 37 °C by shaking at 200 rpm. When OD_{600} value of the cultures reached to 0.6, 1 mM IPTG was added to one of the flask containing the culture while remaining the other as control. Both were incubated at 37 °C and 200 rpm for 5 h more. All the cultures were harvested at 6000 X g at 4 °C for 15 min. Supernatants and pellets were kept at -80 °C. If the procedure would be continued, the pellet of IPTG induced culture was stored at -80 °C for 30 min. and then thawed at RT. Following the thawing

step, the pellet was resuspended in 5 ml of LEW buffer (Appendix D) either by pipetting or vortexing. The sample was sonicated for 6 X 10 sec with 5 sec intervals at amplitude of 8. The sonicated samples were pelletted by centrifugation at 15000 rpm and at 4 °C for 15 min. The supernatant taken might be stored at -80 °C or might be used for His Tag purification. The column (Protino® Ni-TED 2000) was equilibrated with 4 ml of LEW buffer (plus 10 mM imidazole, pH: 8) and the supernatant was applied to the column gradually. The flow-through was stored at -80 °C. After washing of the column 3 times with LEW buffer (supplemented with 20 mM imidazole) (the first wash solution was also kept at -80 °C), the proteins were eluted from the column with 3 ml of Elution buffer (Appendix D). 10 µl of this sample was taken for SDS-PAGE analysis of the expression control. The proteins (5 ml) were renatured by dialysis (1 l, 200X volume of protein solution) at 4 °C for o/n in a buffer containing low concentration of denaturants (Appendix D). The renatured proteins were stored at -80°C in small aliquots.

2.19. Determination of protein concentration

Protein concentrations were measured by the Bradford quantification method (1976). The principle of the assay is based on the observation of a visible color change due to the absorbance shift of Coomassie Brilliant Blue G-250 acidic solution from 465 to 595 nm when it binds to protein. Bovine serum albumin (BSA) was used as the standard to prepare protein calibration curve. 2, 4, 6, 8 and 10 µl of 1 mg/ml BSA were added to tubes and volumes were adjusted to 100 µl with dH_2O. For blank reaction, 100 µl dH_2O was added into the tube. 900 µl of assay reagent (Appendix D) was added to each tube and mixed by vortexing. After 5 min. incubation at RT, the protein concentrations were determined by the help of a standard curve of OD_{600} vs µg protein.

2.20. Gel mobility shift assay

Promoter/ intergenic regions between the genes on the cephamycin C gene cluster, ranging from 109 to 1209 bp in length, were amplified via high fidelity

PCR. *S. clavuligerus* NRRL 3585 genomic DNA was used as template and the PCR reaction for each target region was proceeded with specific primers.

100 ng of specific DNA was incubated with partialy purified CcaR protein at RT for 30 min in binding buffer (80 mM HEPES, 200 mM KCl, 20 mM $MgCl_2$, 0.5 mM $MnCl_2$, 40 % glycerol, 16 mM Tris-HCl, pH 7.5, 1 µg poly[dI-dC], or Salmon Sperm DNA (1µg/µl)). Following the addition of loading dye buffer [0,25XTBE, 60 %; glycerol, 40 %; bromophenol blue, 0,1 %(w/v)] to each reaction, obtained mixtures were load on pre-run (4°C, 80V, 30 min) 6 % native polyacrylamide gel at 4 °C in 0.5X TBE running buffer (90 mM Tris borate, 2 mM EDTA, pH 8.3) for complexes resolution using a Mini-PROTEAN® 3 Cell system (Bio-Rad) at 90 V for 1 h. Following the PAGE, in order to monitor the migrated DNA fragments, gel was treated with SYBR Green I Nucleic Acid Gel Stain (1/10000, v/v) (Invitrogen) and incubated at RT for 30 min with gentle agitation and visualized with UV-transulliminator.

2.21. Polyacrylamide gel electrophoresis (PAGE)

2.21.1. Native PAGE

CcaR protein was run through 6 % polyacrylamide gels (Table 2.5) under nondenatured (native) conditions.

Table 2. 5. Preparation of native polyacrylamide gels

	Gel composition
Monomer concentration	6 %
Acrylamide/bis (30:1) dH_2O 1.5 M Tris-HCl, pH 8.8 43 % Glycerol 10 % Ammonium persulphate (fresh) TEMED	1.670 ml 5.13 ml 2.5 ml 600 µl 90 µl 5 µl
Total monomer	10 ml

One lane was generally loaded with sample buffer including 0.1 % bromophenol blue to monitor the progress of the electrophoresis. The DNA-protein complexes were stained with SYBR Green I Nucleic Acid Gel Stain and visualized with UV-transulliminator.

2.21.2. SDS-PAGE

Proteins were separated on 4.5 % stacking and 12 % seperating polyacrylamide gels under denatured conditions. 16 mA current/gel was applied during the electrophoresis.

The SDS-polyacrylamide gels were prepared as described below (Laemmli, 1970) (Table 2.6):

Table 2. 6. Preparation of denatured polyacrylamide gels

	Stacking gel	Separating gel
Monomer concentration	4.5 %	12 %
Acrylamide/bis	1.3 ml	4ml
dH_2O	6.1 ml	3.35 ml
1.5 M Tris-HCl, pH 8.8	-	2.5ml
0.5 M Tris-HCl, pH 6.8	2.5 ml	-
10 % (w/v) SDS	100 µl	100 µl
10 % Ammonium persulphate (fresh)	50 µl	50 µl
TEMED	10 µl	5 µl
Total monomer	10 ml	10 ml

2.22. Staining of polyacrylamide gels

2.22.1. SYBR Green I nucleic acid staining of the Native-Polyacrylamide gels

In EMSA, the DNA-protein complexes were stained with SYBR Green I Nucleic Acid Gel Stain (1/10000, v/v) that was diluted in 0.5X TBE buffer.

2.22.2. Coomassie Blue R-250 staining of the SDS-Polyacrylamide gels

Proteins were visualized by Coomassie Blue R-250 staining of the gels. After electrophoresis, the gel was soaked in 50 ml of freshly prepared Coomassie blue stain (Appendix D) for 1 h at RT. The gel was fixed in fixation solution (Appendix C) for 1 h, then rinsed with dH_2O. Followingly, the gel was kept in destaining solution (Appendix D) for 2-3 h.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Expression of *ccaR* gene under a strong glycerol promoter in pSPG expression vector or with its own promoter in the integrative expression pSET152 vector

3.1.1. Cloning of *ccaR* gene into pSPG expression vector having a strong glycerol promoter (PglP)

ccaR gene sequence with a GenBank accession number of AF073897 was used to design related primers (ccaR_F-ccaR_R forward and reverse primers) for PCR amplification of the gene by using genomic DNA of *S. clavuligerus* as template (Figure 3.1). Gel eluted PCR product of *ccaR* was ligated to pGEM-T easy vector and transferred into *E. coli* DH5α cells via transformation. Putative recombinant colonies were grown on ampicillin (100 µg/ml) containing agar plates overlaid with X-Gal-IPTG as a final concentration of 20 µg/µl and 0.5 mM. The recombinant colony which was cloned into the vector in the correct orientation was verified by PCR, restriction enzyme digestion (Figure 3.2) and sequencing reactions. The recombinant *E. coli* strain was designated as *E. coli* pG23 (Figure 3.3).

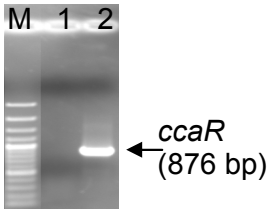


Figure 3. 1. PCR amplification of *ccaR* gene. M: λ *Pst*I DNA ladder, 1: *ccaR* PCR product obtained with primers ccaR_F-ccaR_R by using genomic DNA of *S. clavuligerus* as template, 2: Negative control of PCR with no template DNA.

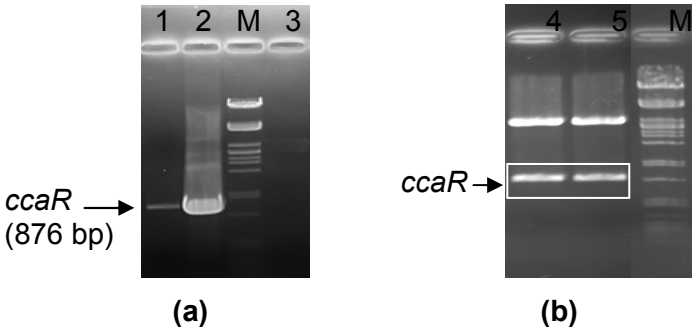


Figure 3. 2. Verification of recombination via PCR **(a)** and double digestion **(b)**. M: λ *Pst*I DNA ladder, 1: *ccaR* PCR product obtained by using pG23 as template DNA 2: Positive control of PCR (template DNA was genomic DNA of *S. clavuligerus*), 3: Negative control of PCR with no template DNA, 4: *Eco*RI digested *ccaR* containing pG23, 4: *Nde*I-*Spe*I digestion of pG23 that releases *ccaR*.

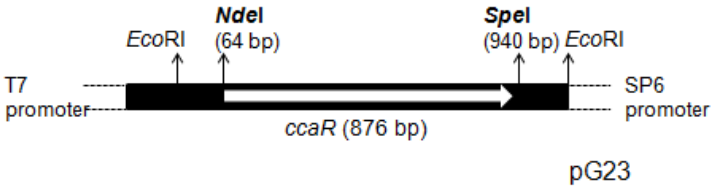


Figure 3. 3. The orientation of *ccaR* gene in MCS of pG23 plasmid.

Afterwards, *ccaR* gene was rescued from pG23 by *Nde*I-*Spe*I digestion and ligated to *Nde*I-*Spe*I site of linearized pSPG vector that is located just behind downstream of the glycerol promoter (Figure 3.4). The ligation product was introduced into *E. coli* DH5α cells via transformation. Apramycin as a final concentration of 50 µg/ml was added to the LA for selection of recombinant colonies after transformation process. One of the transformants grown on the selective media was chosen and the verification of the recombination was performed by PCR and restriction enzyme analyses (Figure 3.5). This recombinant was designated as pAK23 (Figure 3.6).

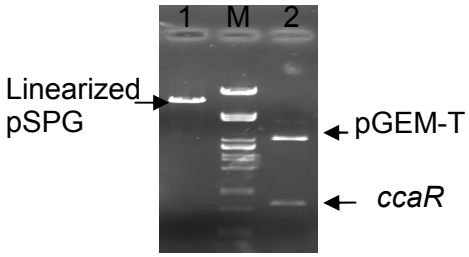


Figure 3. 4. Digestion of pSPG and pG23 with *Spe*I and *Nde*I restriction endonucleases. 1: Linearized pSPG, M: λ *Pst*I DNA ladder, 2: Linearized pGEM-T easy and *ccaR* rescued from the vector as a result of double digestion with the related enzymes.

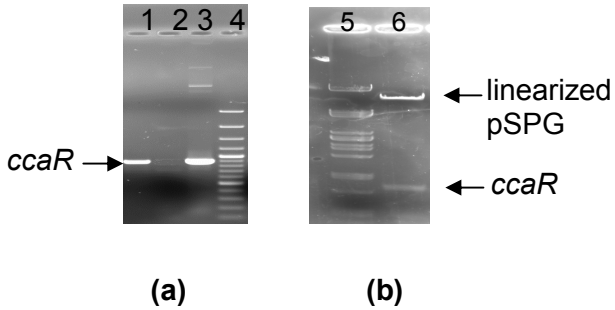


Figure 3. 5. Verification of cloning of *ccaR* pSPG by PCR **(a)** and restriction endonuclease digestion **(b)**. 1: Positive control of PCR (template DNA was genomic DNA of *S. clavuligerus*, 2: Negative control of PCR with no template DNA, 3: *ccaR* PCR product obtained by using pAK23 as template DNA, 4: 100 bp DNA ladder plus, 5: λ *Pst*I DNA ladder, 6: *Nde*I and *Spe*I digested pSPG.

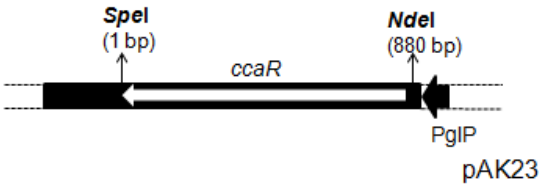


Figure 3. 6. The location of *ccaR* in cloning sites of pAK23 vector.

3.1.2. Chromosomal integration of *ccaR* gene with its own promoter (p*ccaR*) in *S. clavuligerus*

ccaR gene with its own promoter (p*ccaR*) was PCR amplified by using genomic DNA of *S. clavuligerus* as the template (Figure 3.7). The purified PCR product of p*ccaR* was ligated to pGEM-T easy vector system and transferred into *E. coli* DH5α cells via transformation. The recombination was verified by PCR, restriction enzyme digestion (Figure 3.8) and sequencing.

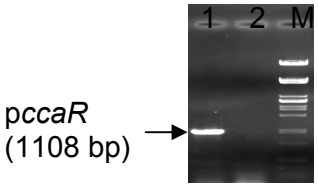


Figure 3. 7. PCR amplification of p*ccaR* by using genomic DNA of *S. clavuligerus* as template. 1: p*ccaR* PCR product using genomic DNA of *S. clavuligerus* as template, 2: Negative control with no template DNA, M: λ *Pst*I DNA ladder.

The resulting recombinant was named as pG15 and the schematic representation of orientation of the gene on MCS of the recombinant plasmid is given in Figure 3.9.

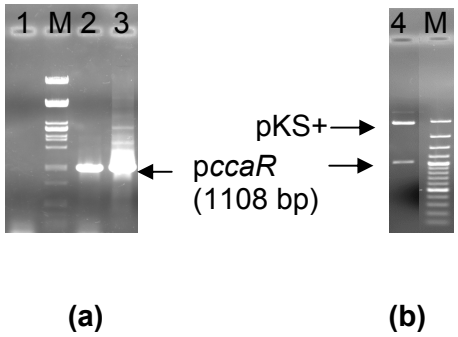


Figure 3. 8. Verification of cloning of p*ccaR* into pGEM-T easy vector via PCR (a) and double digestion (b). **(a)** 1: Negative control with no template DNA, M: λ *Pst*I DNA ladder, 2: p*ccaR* PCR product by using pG15 as template DNA, 3: p*ccaR* PCR product by using genomic DNA of the wild type as template, **(b)** 4: *Eco*RI-*Xba*I digested pG15, M: 100 bp DNA ladder.

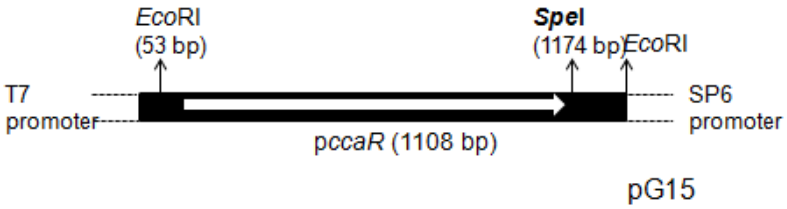


Figure 3. 9. The orientation of p*ccaR* gene in MCS of pG15 plasmid.

Several attempts to clone p*ccaR* rescued form the pG15 vector into the *Eco*RI site of pSET152 integration vector was unsuccessful. Thus, it was cloned to another *E. coli* cloning vector, pBlueskriptIIKS+ (pKS+), which have a wide repertoire of enzyme recognition sites in its MCS, to provide available restriction regions for p*ccaR* for following ligation process to pSET152. p*ccaR* was cloned within the *Eco*RI-*Spe*I sites of MCS of pKS+ vector generating pKS15. Afterwards, the gene was digested with *Eco*RI and *Xba*I restriction enzymes and ligated to pSET152 linearized with the same enzymes (Figure 3.10). After introducing of this to *E. coli* DH5α cells via transformation, the resulting colonies grown on apramycin containing LA plates were picked, cultured and the isolated plasmids from these cultures were screened. The recombination was verified by PCR and enzyme digestion reactions (Figure 3.11). One of the recombinant colonies was selected and named as pSET-PC (Figure 3.12).

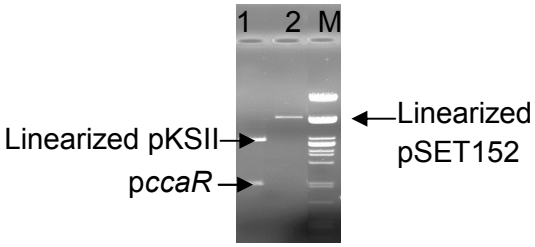


Figure 3. 10. Digestion of pG15 and pSET152 with *Eco*RI and *Xba*I enzymes. 1: *Eco*RI-*Xba*I digested pG15, 2: *Eco*RI-*Xba*I digested pSET152, M: λ *Pst*I DNA ladder.

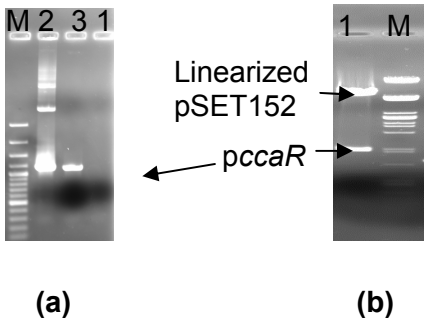


Figure 3. 11. Verification of cloning of p*ccaR* into pSET152 integration vector via PCR (a) and double digestion (b). **(a)** M: 100 bp DNA ladder plus, 1: Negative control with no template DNA, 2: p*ccaR* PCR product by using pSET-PC as template DNA, 3: p*ccaR* PCR product by using genomic DNA of the wild type as template, **(b)** 1: *Eco*RI-*Xba*I digested pPSET-PC, M: λ *Pst*I DNA ladder.

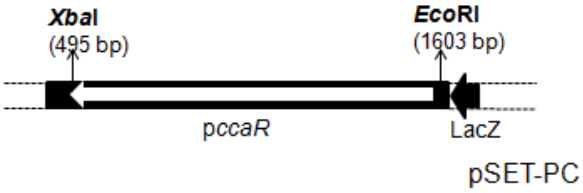


Figure 3. 12. The orientation of p*ccaR* gene in MCS of pSET-PC plasmid.

3.1.3. Intergeneric conjugation between recombinant *E.coli* cells and *S. clavuligerus*

Recombinant pAK23 and pSET-PC plasmids as well as pSPG and pSET152 were introduced into methylation deficient *E. coli* strain ET12567/pUZ8002 prior to conjugation reaction. This is a crucial step to avoid restriction barrier of *S. clavuligerus* cells. pUZ8002 is a RK2 derivative (with a mutaion in *oriT*) transfer plasmid that provides mobilization of the resident plasmid during the conjugation

reaction. It has an inefficient transfer property due to a mutation in its own *ori*T site. Still, a low level of self-transfer ability renders pUZ8002 to introduce into *E. coli* ET12567 (Bierman *et al*., 1992; Paget *et al*., 1999; Paranthaman and Dharmalingam, 2003). After transformation, all four recombinant *E. coli* ET12567/pUZ8002 strains were verified by PCR analyses (data not shown). Afterwards, conjugation was performed as described by Flett *et al*. (1997). Resulting exconjugants were selected on apramycin containing TSA plates. Experiments were performed to verify pSPG and pAK23 carrying recombinant *S. clavuligerus* cells by isolating their total DNA and transforming them into *E. coli* DH5α cells. Plasmids isolated from these recombinant *E. coli* DH5α cells were used as template for PCR and used for restriction digestion to confirm recombination (data not shown). In addition, verification of recombinant *S. clavuligerus* cells were also performed by PCR in which a reverse primer of the cloned gene and a primer designed from internal region of apramycin sequence was used to amplify the desired region (Figure 3.13).

pAK23 containing recombinant *S. clavuligerus* was designated as *S. clavuligerus* C11 while other recombinant *S. clavuligerus* that has pSET-PC vector integrated into its chromosome was named as *S. clavuligerus* PC. On the other hand, the control strains (pSPG containing *S. clavuligerus* and *S. clavuligerus* having pSET152 in its *att*B site(s)) would be used for fermentation studies as vector controls, which were designated as pGV and pTV, respectively.

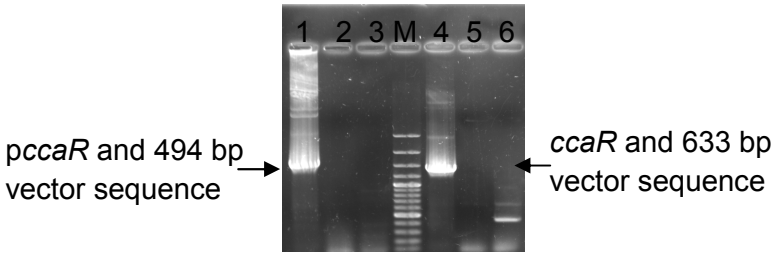


Figure 3. 13. Verification of recombinant *S. clavuligerus* C11 and PC cells by PCR. 1: PCR product including whole p*ccaR* and 494 bp vector sequence obtained by using PC total DNA, 2: Negative control with no template DNA, 3: Negative control by using genomic DNA of the wild type as template DNA, M: 100

bp DNA ladder plus, 4: PCR product including whole *ccaR* and 633 bp vector sequence obtained by using C11 total DNA, 5: Negative control with no template DNA, 6: Negative control by using genomic DNA of the wild type as template.

3.1.4. Comparison of cephamycin C and clavulanic acid production capacities of the recombinant *S. clavuligerus* strains grown in TSB and SA media by bioassay and HPLC analyses

The regulatory effect of CcaR protein on cephamycin C as well as CA productions was monitored by fermentation studies in which wild type *S. clavuligerus* NRRL3585 and recombinant *S. clavuligerus* C11, pGV, PC and pTV strains were grown in SA and TSB for 168 h. Three biological replicates for each strain were fermented and the samples taken for 24 h intervals during the course of fermentation were used to perfom bioassay, HPLC and growth determination via DNA concentration. As there is no cephamycin C standard available in our hand, HPLC analysis was only performed for determination of clavulanci acid yields of the recombinants in comparison to the wild type by using potassium clavulanate (DEVA pharmaceuticals) as the standard. On the other hand, bioassay studies were performed to detect any increase in the cephamycin C titers of the recombinants compared to the wild type's cephamycin C production by using cephalosporin C as the standard.

The growth of PC in both media was higher than that of the wild type strain and its vector control (pTV) was showed a higher growth pattern in SA when compared to the wild type strain especially between 24 and 72 h of the fermentation, but in the following hours its growth was similar with that of the wild type (Figure 3.14a). The growth of C11 was drastically decreased during the course of fermentation in TSB, however, in SA defined media, it grew in a lesser extent than the wild type till 72nd h of fermentation and after 96 h, it did not show a drastic difference as compared to the wild strain in terms of its growth. Strikingly, in TSB, the growth of all strains gradually decreased after 48 h of fermentation (Figure 3.14b). The higher growth of the strains in TSB media was most probably due to the nutritional richness of the medium used.

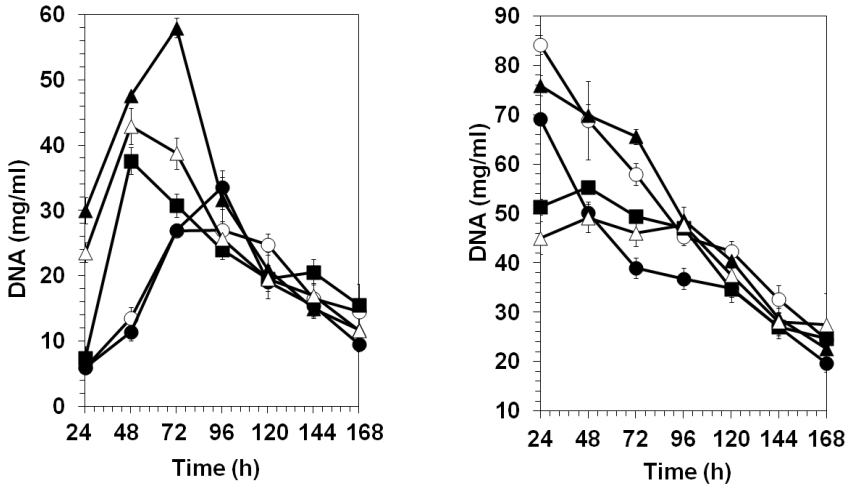


Figure 3. 14. Time-dependent growth of wild type *S. clavuligerus* NRRL3585 (■) and the recombinant strains, C11 (●), PC (▲), pGV (○) and pTV (△), in SA **(a)** and TSB **(b)**.

Volumetric cephamycin C production of pGV and C11 recombinant strains were approximately 2 and 3 times higher than that of the wild type strain in SA medium, respectively. However, the volumetric cephamycin titers of PC and pTV strains were similar to the wild type although PC had pSET-PC integrated to its chromosome (Figure 3.15a). Antibiotic production capacities of the cells are better distinguished by examining the specific production patterns of the strains as it gives the µg cephamycin C produced per mg DNA of bacteria. According to the specific cephamycin C titers obtained, C11 having *ccaR* on a multicopy plasmid under a strong glycerol promoter incerased its production more than 3-fold at the end of 120 h fermentation when compared to the wild strain. Besides, the specific cephamycin C production in pGV was 1.5 times higher than that of the wild type strain at the same incubation time. It is known that overexpression of the recombinant gene on a multicopy plasmid generally leads an extra cost to the host cell physiology, most commonly defined as “metabolic burden”. As a consequence of metabolic burden, the maintanence and synthesis of a multicopy plasmid in the host cell seriously effects secondary metabolite pathways (Baltz, 1998; Williams *et al*., 2009; Balderas-Hernandez *et al*., 2009; Özcengiz *et al*.,

2010). The integration of an extra copy(ies) of p*ccaR* to the chromosome of *S. clavuligerus* did not exert any remarkable increase in the antibiotic production, confirming that SA medium does not favor cephamycin C biosynthesis by the cells as previously reported by Perez-Llarena *et al*. (1997) (Figure 3.15b).

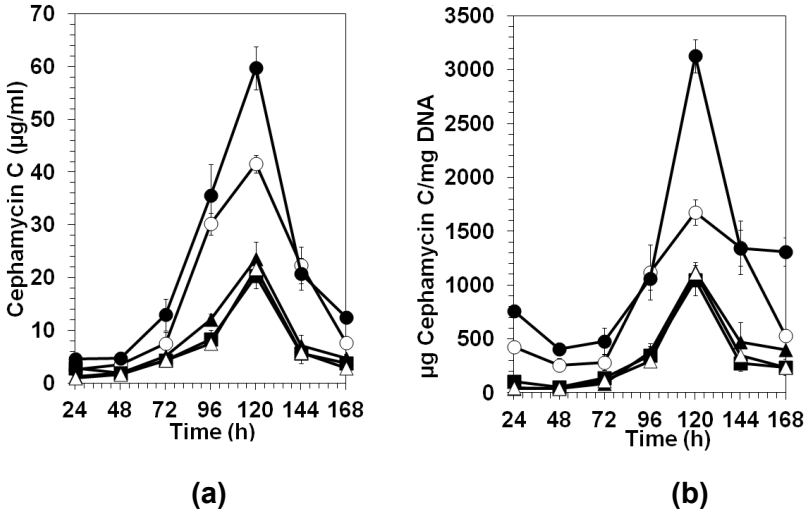
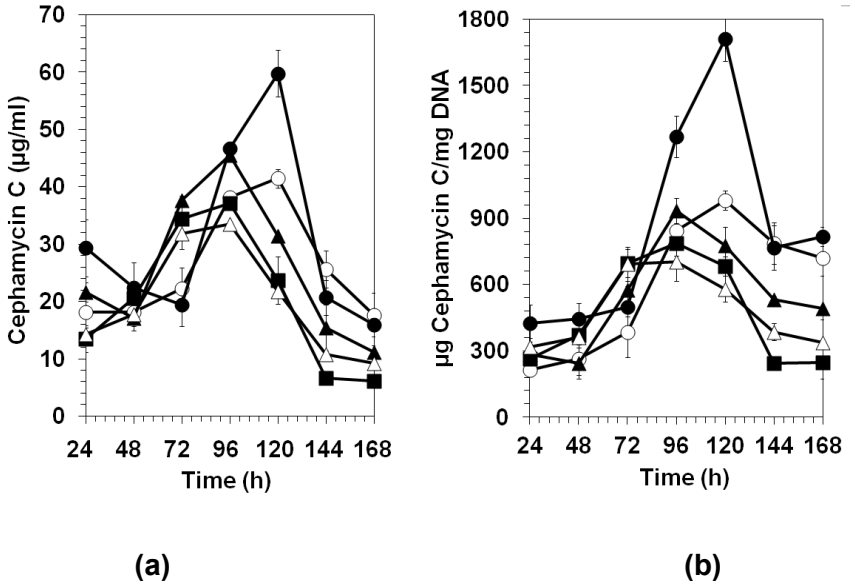


Figure 3. 15. Comparison of the volumetric **(a)** and specific **(b)** cephamycin C titers of the recombinant C11 (●), PC (▲), pGV (○) and pTV (△) straines with that of the wild type strain (■) grown in SA medium by bioassay during the course of fermentation.

In TSB medium, volumetric cephamycin C production capacities of the strains were different from those obtained when grown in SA medium. Of the strains compared, C11 was the best by giving the highest cephamycin C yield at 120th h which was 2.3 times higher than that was found in the wild type (Figure 3.16a and b). PC strain having extra copy of p*ccaR* in *attB* site of its chromosome also showed a 1.2 to 2.1 increase (Figure 3.16a and b).



(a) **(b)**

Figure 3. 16. Comparison of the volumetric **(a)** and specific **(b)** cephamycin C titers of the recombinant C11 (●), PC (▲), pGV (○) and pTV (△) strains with that of the wild type strain (■) grown in TSB by bioassay during the course of fermentation.

Afterwards, clavulanic acid titers produced by the recombinants grown in SA and TSB during 168 h fermentation were determined by both bioassay (Figure 3.17) and HPLC analyses (Figure 3.18). Clavulanic acid titer of the recombinant C11 strain was found to be approximately 6-fold higher than that of the wild strain at the end of 120 h of fermentation in both media. PC was capable of producing 2 to 3 times more clavulanic acid as compared to the wild type strain at 96th h and 120th h of incubation in TSB and SA, respectively.

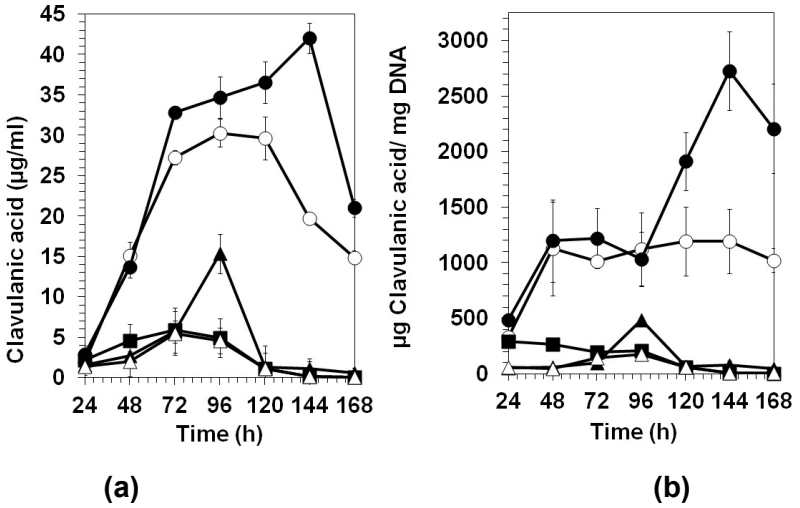


Figure 3. 17. Comparison of the volumetric **(a)** and specific **(b)** clavulanic acid titers of the recombinant C11 (●), PC (▲), pGV (○) and pTV (△) strains with that of the wild type strain grown (■) in SA by bioassay during the course of fermentation.

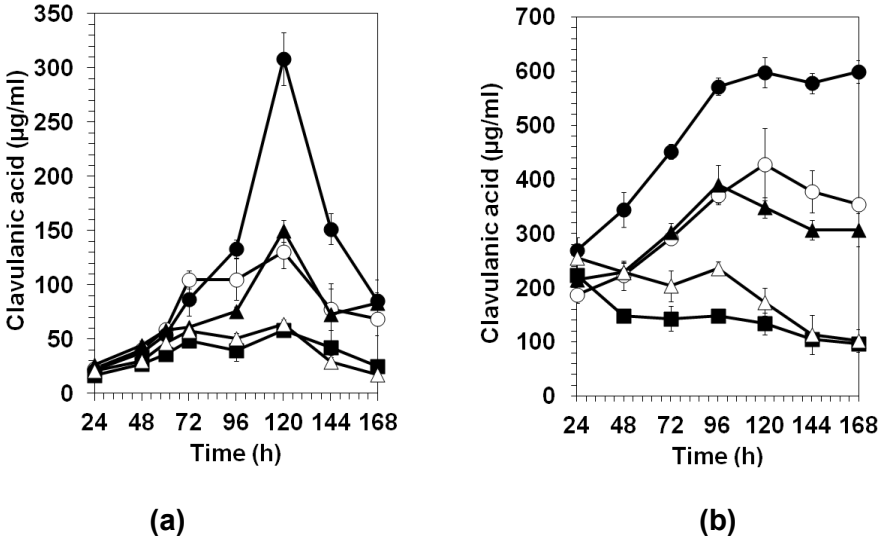


Figure 3. 18. Comparison of the clavulanic acid titers of the recombinant C11 (●), PC (▲), pGV (○) and pTV (△) pTV strains with that of the wild type strain (■) grown in SA **(a)** and TSB **(b)** by HPLC during the course of fermentation.

pSET152 is an integrative plasmid which is unable to replicate in *Streptomyces*. With the availibility of ϕC31 *attP-int* locus in its sequence, it can integrate into the *attB* attachment site in *Streptomyces* chromosome. Like *S. coelicolor* and *S. lividans*, many of the *Streptomyces* spp. genome harbors several secondary or pseudo-*attB* sites (Combes *et al*., 2002). The use of integration vector for insertion of single copy of a desired gene into the bacterial chromosome provides stable recombinants and avoids the problems encountered with the presence of multicopy plasmids in the host cell (Bierman *et al*., 1992). There are many studies reported so far in which the use pSET152 for insertion of cloned genes of a specific antibiotic cluster into the chromosome positively affected the yield of the related antibiotic without giving any deleterious effect to the cell (Luzhetskii *et al*., 2001; Paranthaman and Dharmalingam, 2003; Liao *et al*., 2010).

The insertion of p*ccaR* harbored by the recombinant PC to the *S. clavuligerus* chromosome did cause a 1.2 to 2.1 fold increase in the cephamycin C yield in TSB medium. However, HPLC quantification of clavulanic acid produced by PC strain grown in SA medium showed 3 fold higher level of clavulanic acid yield than that of the wild strain. C11 produced 2 to 3, and 6 times higher level of cephamycin and clavulanic acid, respectively, as compared to the wild type strain in both media. According to data obtained from bioassay and HPLC, the integration of extra copy(ies) of *ccaR* to the chromosome of *S. clavuligerus* or its overexpression in the cell enhanced clavulanic acid biosynthesis at higher extent than biosynthesis of cephamycin C antibiotic.

3.2. Total RNA isolation for Reverse transcription PCR (RT-PCR) and qRT-PCR

The sampling times for RNA isolation to be used for RT-PCR and qRT-PCR analysis were decided by examining the strains' growth and specific cephamycin C production at early hours of fermentation (Figure 3.19a,b). The µg RNA per mg of DNA was also determined and plotted in Figure 3.19c. At early hours of fermentation, the specific cephamycin C production by C11 was remarkably higher than that in the wild type. However, its growth decreased very sharply after

24 h. According to the data, 15th h, 24th h and 30th h of fermentation were selected for sampling time for further analyses.

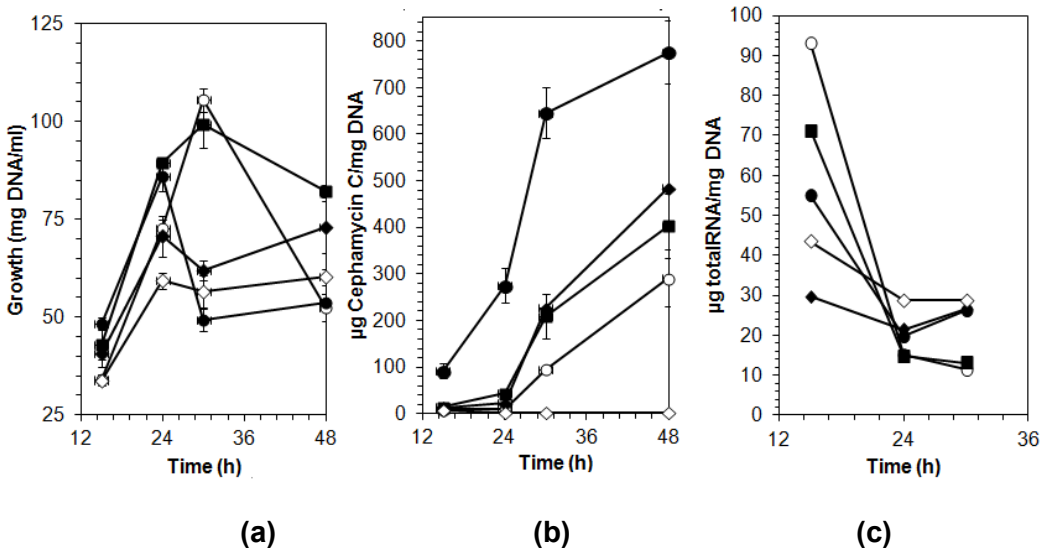


Figure 3. 19. Growth **(a)**, specific cephamycin C titers **(b)** and µg RNA per mg DNA **(c)** of the both wild type *S. clavuligerus* (ATCC27064 (◆) and NRRL3585 (■)) strains, C11 (●), pGV(○) recombinants and *ccaR*-disrupted mutant strain of *S. clavuligerus* (◇).

The samples taken at these time intervals from wild type and recombinant *S. clavuligerus* culture were used for RNA isolation by using RNeasy mini kit according the instruction of manufacturers with some modifications described in the Section 2.16. The isolated RNAs were run in 1% agarose gels (Figure 3.20 and 3.21), their quality and quantity were determined by spectrophotometric measurement in NanoDrop® ND-2000 (ThermoScientific).

3.3. Transcriptional analysis of the regulatory effect of *ccaR* on the cephamycin C gene cluster by RT-PCR

The effect of *ccaR* gene in the transcriptional regulation of the cephamycin C gene cluster was examined by performing RT-PCR studies in which total RNAs of *ccaR::aphII* mutant of *S. clavuligerus, S. clavuligerus* C11 having extra *ccaR* gene on a multicopy plasmid and the wild type strains *S. clavuligerus* NRRL3585 and ATCC27064 were used as the templates.

3.3.1. Transcriptional profiles of the genes in cephamycin C gene cluster in *ccaR:: aphII* mutant of *S. clavuligerus* ATCC27064

The transcriptional profile of the cephamycin C gene cluster was examined in three sections: (i) the region that begins with *ccaR* gene and ends up with *pcbR*, (ii) the middle part of the cluster in which *cmcI*, *cmcJ*, *cefF* and *cmcH* genes are located, (iii) the third part that *cefD, cefE, pcd*, *cmcT*, *pbpA* and *bla* genes are present.

There was almost no expression of *ccaR* and *blp* genes in the absence of an intact *ccaR* gene in the cell. The transcripts of *lat* and *pcbAB* genes were repressed in the mutant strain while *pcbC* gene was expressed as a lesser extent in the *ccaR*-disrupted mutant as compared to the wild type strain. However, a similar expression pattern of *orf10* was observed in both strains for all the hours tested. *pcbR* gene was expressed similarly at 15 h in the *ccaR*-disrupted strain and the wild type, but interestingly, in the following hours, its expression was found to be higher in the wild strain (Figure 3.20).

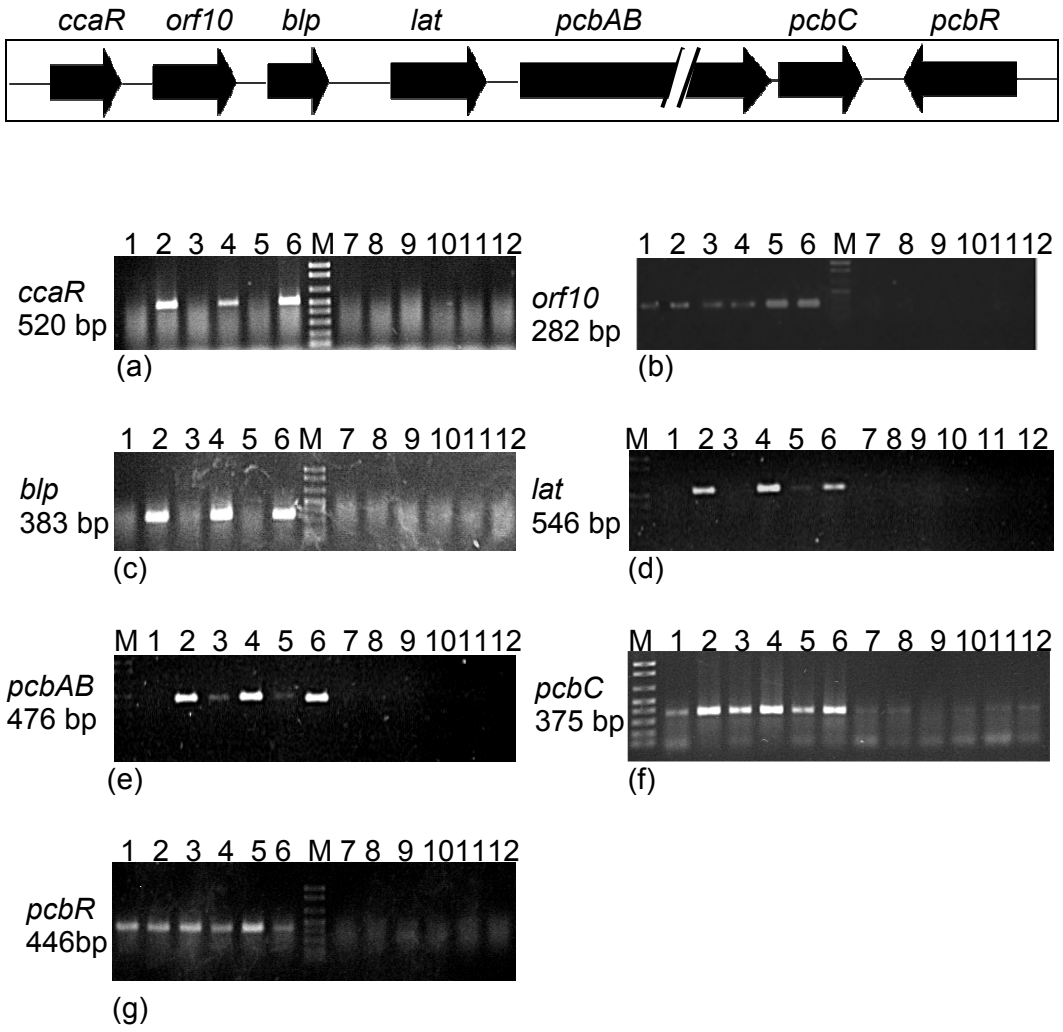


Figure 3. 20. Comparison of the gene expressions on the cephamycin C pathway in a *ccaR:: aphII* mutant of *S. clavuligerus* ATCC27064 and its wild type strain by RT-PCR during the time course. Lanes refer to the expression of related genes in the *ccaR::aphII* mutant (1, 3, 5) and in the wild type (2, 4, 6) at T_{15}, T_{24} and T_{30}, respectively. 7, 9, 11: RT minus controls (used for detection of possible DNA contamination in RNA samples) of lanes 1, 3 and 5, respectively. 8, 10, 12: RT minus controls of lanes 1, 3 and 5, respectively. (a) *ccaR*, (b) *orf10*, (c) *blp*, (d) *lat*, (e) *pcbAB*, (f) *pcbC*, (g) *pcbR*.

The transcription of *cmcI, cmcJ, cefF* and *cmcH* genes were repressed in the mutant strain, as shown in Figure 3.21. The decrease in transcription of *cmcI* and

cmcJ genes in the absence of an intact *ccaR* seemed to be more serious than that of *cefF* and *cmcH* genes.

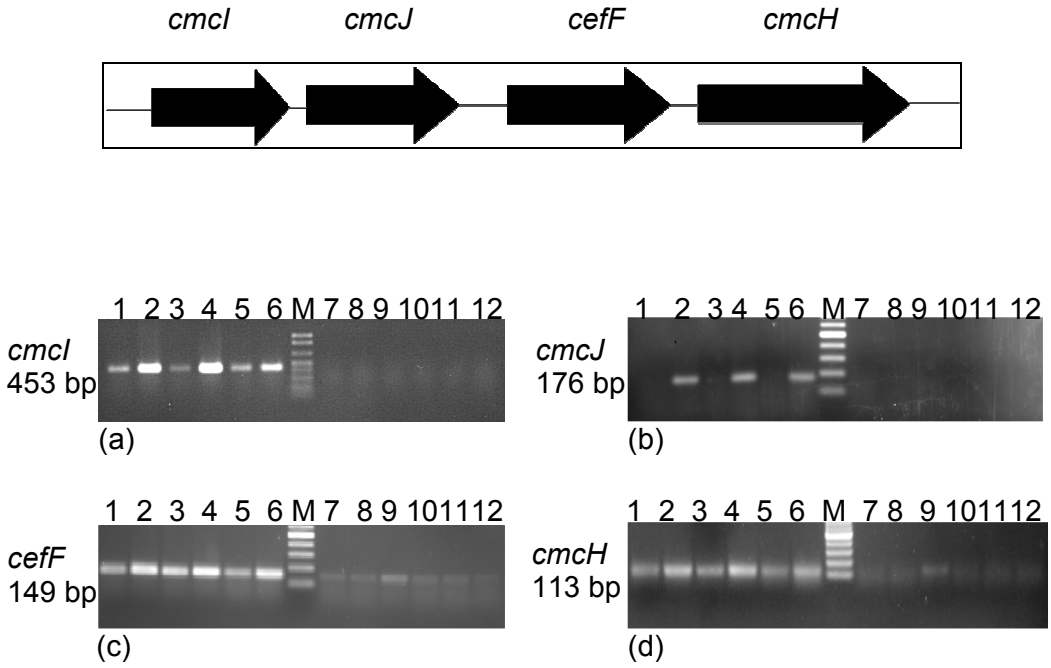


Figure 3. 21. Comparison of the gene expressions on the cephamycin C pathway in a *ccaR::aphII* mutant of *S. clavuligerus* ATCC27064 and its wild type strain by RT-PCR during the time course. Lanes refer to the expression of related genes in the *ccaR::aphII* mutant (1, 3, 5) and in the wild type (2, 4, 6) at T_{15}, T_{24} and T_{30}, respectively. 7, 9, 11: RT minus controls of lanes 1, 3 and 5, respectively. 8, 10, 12: RT minus controls of lanes 1, 3 and 5, respectively. (a) *cmcI*, (b) *cmcJ*, (c) *cefF*, (d) *cmcH*.

The transciption of *cefD* and *cefE* genes significantly decreased in the *ccaR*-disrupted strain in all hours of fermentation. Besides, the expression of *cmcT* in the mutant was slightly less than that of the wild type. In addition, it was observed that the absence of CcaR protein did not exert a detectable inhibitory effect in transcription of *pcd*, *pbpA* and *bla* (Figure 3.22).

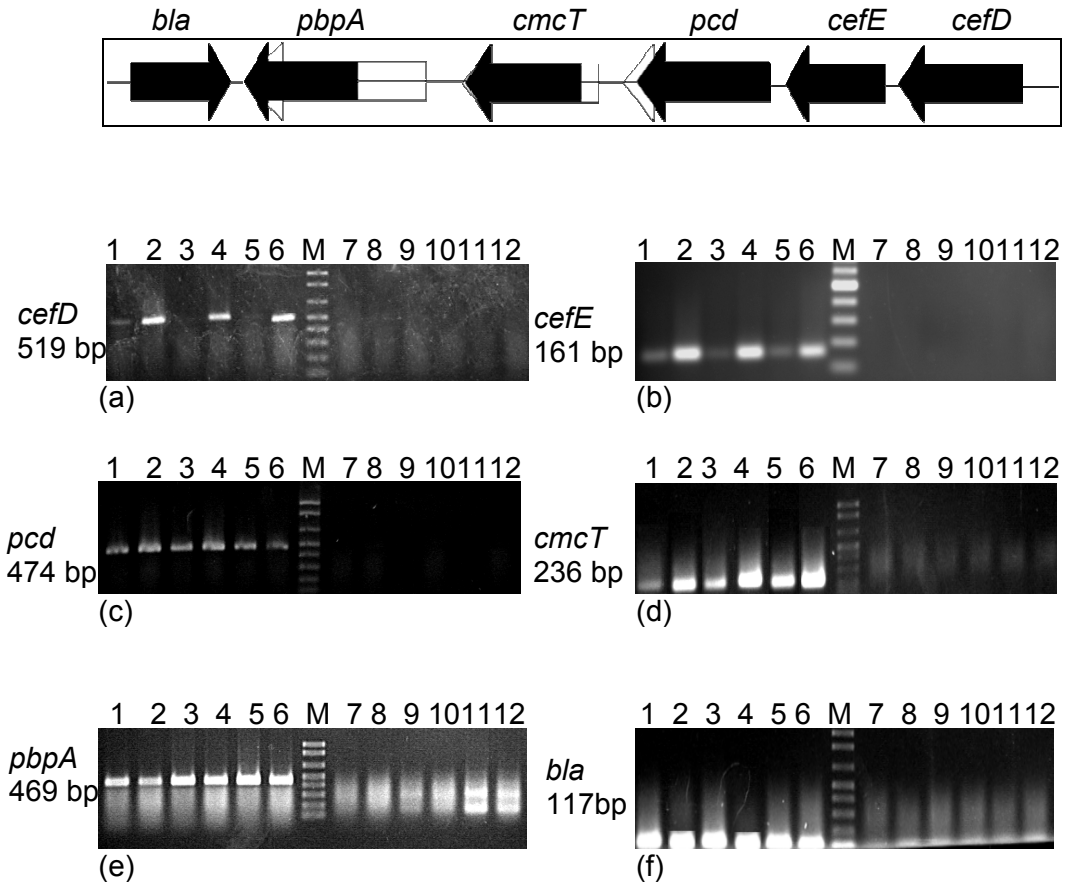


Figure 3. 22. Comparison of the gene expressions on the cephamycin C pathway in a *ccaR::aphII* mutant of *S. clavuligerus* ATCC27064 and its wild type strain by RT-PCR during the time course. Lanes refer to the expression of related genes in the *ccaR::aphII* mutant (1, 3, 5) and in the wild type (2, 4, 6) at T_{15}, T_{24} and T_{30}, respectively. 7, 9, 11: RT minus controls of lanes 1, 3 and 5, respectively. 8, 10, 12: RT minus controls of lanes 1, 3 and 5, respectively. (a) *cefD*, (b) *cefE*, (c) *pcd*, (d) *cmcT*, (e) *pbpA*, (f) *bla*.

As a summary, RT-PCR analysis showed that in the absence of CcaR protein, a significantly low level of expression pattern was observed in its own expression as well as *blp*, *lat*, *pcbAB*, *cefD*, *cefE*, *cmcI*, *cmcJ*, *cefF* and *cmcH* expressions in the cell. These genes encode enzymes that have roles in the first (*lat, pcbAB*), intermediary (*cefD, cefE, cefF*) and final (*cmcI, cmcJ, cmcH*) steps of the cephamycin C biosynthesis. Furthermore, in the mutant strain, an observable decrease was determined in the transcriptional profiles of *pcbC* and *cmcT* which

are the biosynthetic and transporter genes, respectively. However, the possible differential in the expression patterns of the remaining genes of the cluster, namely, *pcd*, *pbpA*, *bla*, *pcbR* in the *ccaR* mutant and the wild type was not clear enough to detect by RT-PCR.

3.3.2. Co-transcriptional profile of the genes in cephamycin C gene cluster in *S. clavuligerus* ATCC27064

Some of the genes are closely located to each other in the cephamycin C gene cluster without leaving enough space for location of a promoter in their 5' sequences. However, majority of the intergenic regions are large enough to harbour related promoters (Table 1.4). RT-PCR experiments were performed to determine intergenic region amplification of the genes in cephamycin C gene cluster (Figure 3.23). RNAs isolated from 24 h cultures of *S. clavuligerus* ATCC27064 were used as the template in the RT-PCR.

All genes in the cluster were shown to be bicistronically transcribed at 24 h in wild type *S. clavuligerus*. As the amplicons obtained by using the primers designed to amplify intergenic regions between *cmcT-pbpA and cmcH-ccaR* were larger than 1 kb in size, it was difficult to get a band by one step RT-PCR reaction. As an alternative, the cDNA was synthesized by using random primers and then PCR was performed by using Taq polymerase (Fermentas). As a result, a very faint band could be obtained representing the intergenic regions between *cmcH-ccaR* and *cmcT-pbpA*.

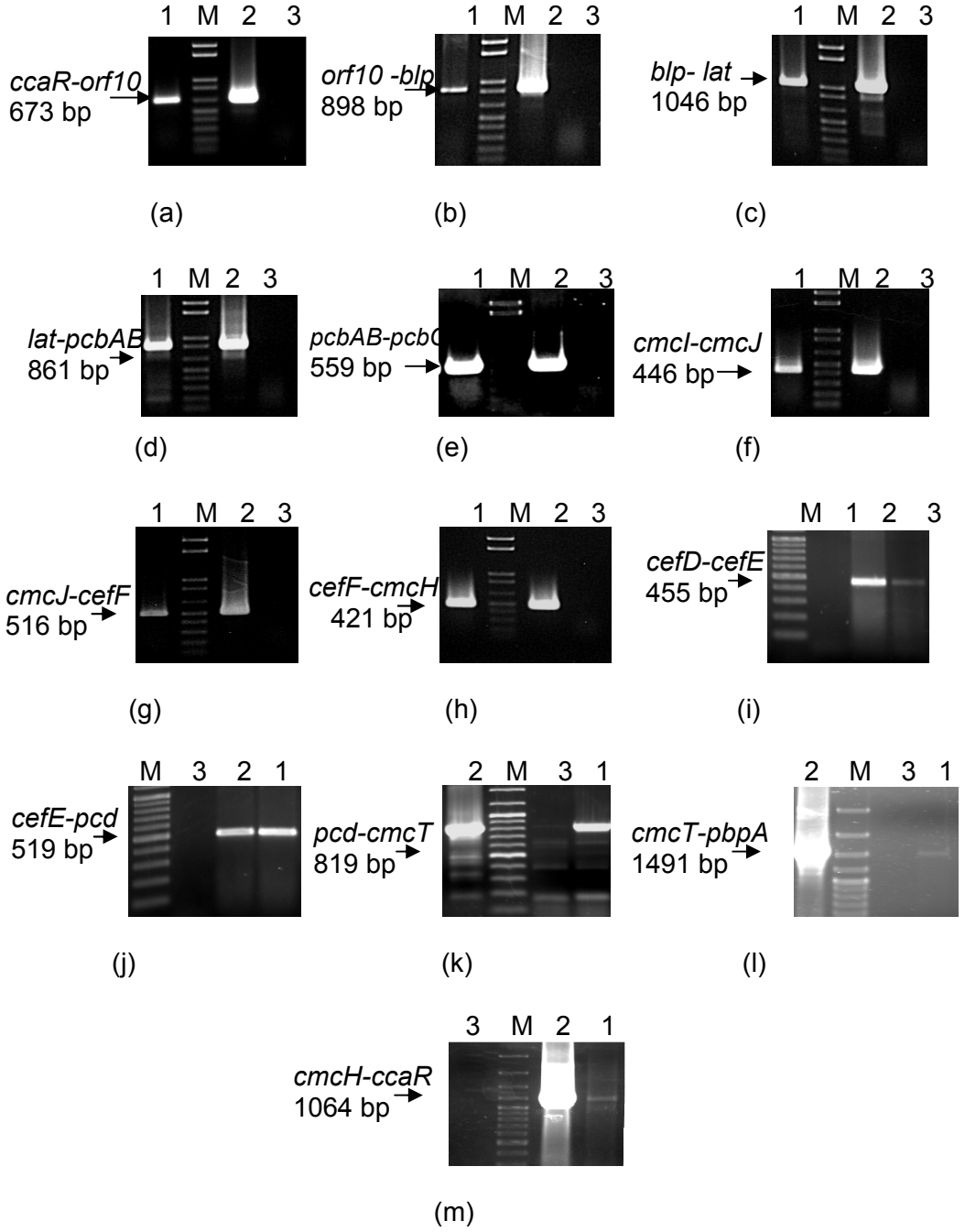


Figure 3.23. Co-transcriptional profile of cephamycin C gene cluster in *S. clavuligerus* ATCC27064 at T_{24} as shown by RT-PCR. 1: Expression of the related intergenic region by using RNA of the wild type as template, 2: Expression of the related intergenic region by using genomic DNA of the wild type as template in RT-PCR, 3: RT minus control of lane 1. (a) *ccaR-orf10*, (b) *orf10-blp*, (c) *blp-lat*, (d) *lat-pcbAB*, (e) *pcbAB-pcbC*, (f) *cmcI-cmcJ*, (g) *cmcJ-cefF,* (h) *cefF-cmcH,* (i) *cefD-cefE,* (j) *cefE-pcd,* (k) *pcd-cmcT,* (l) *cmcT-pbpA*, (m) *cmcH-ccaR*.

3.3.3. Transcriptional profiles of the genes in cephamycin C gene cluster in *S. clavuligerus* C11

Visible alterations in the expressions of the cephamycin C genes were observed in the presence of an extra *ccaR* gene on a multicopy plasmid carrying recombinant *S. clavuligerus* C11 strain by RT-PCR analyses (Figure 3.24, 3.25 and 3.26).The increment of the *blp* expression in the recombinant strain was apparent in all hours of the incubation examined (Figure 3.24).

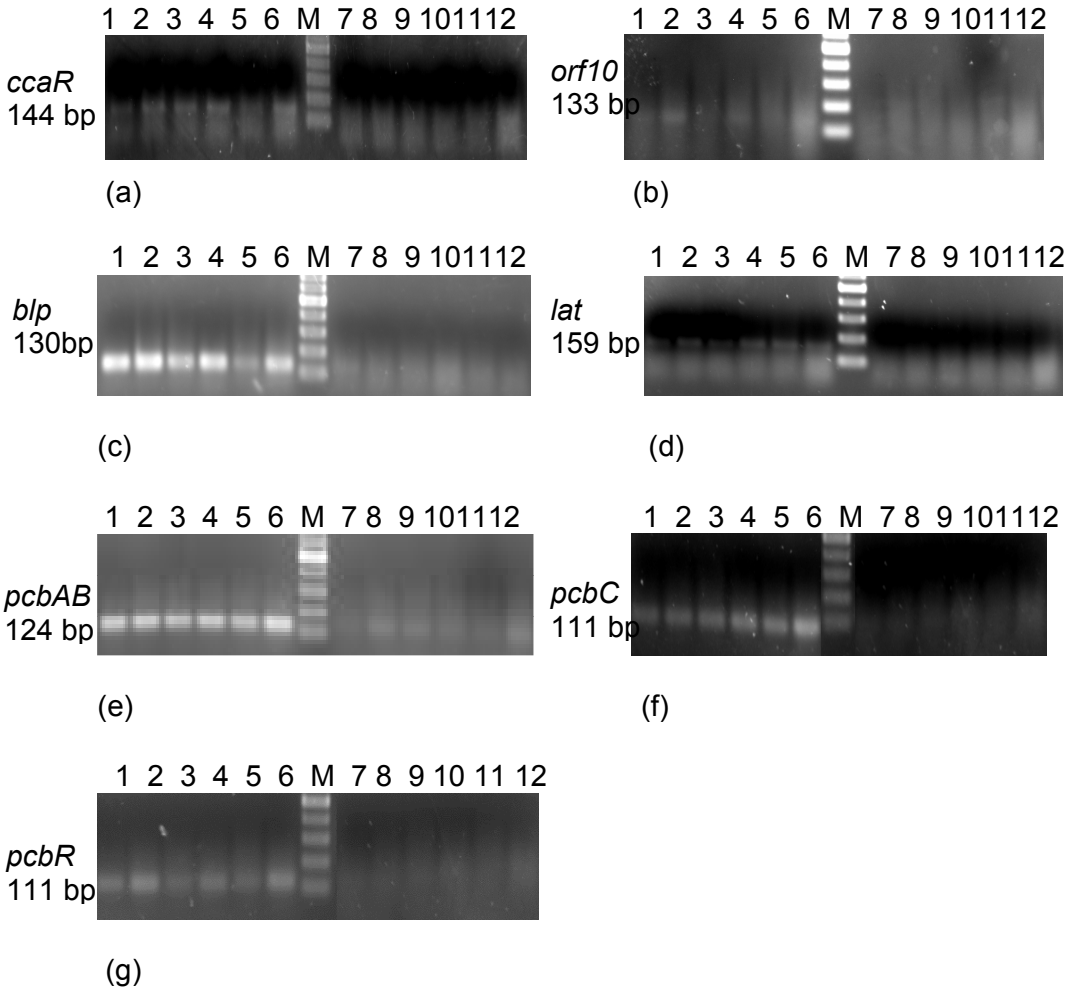


Figure 3. 24. Comparison of the gene expressions on the cephamycin C pathway in multicopy *ccaR* containing recombinant *S. clavuligerus* NRRL3585 and its wild type strain by RT-PCR during the time course. Lanes refer to the expression of related genes in the recombinant (1, 3, 5) and in the wild type (2, 4, 6) at T_{15}, T_{24} and T_{30}, respectively. 7, 9, 11: RT minus controls of lanes 1, 3 and 5,

respectively. 8, 10, 12: RT minus controls of lanes 1, 3 and 5, respectively. (a) *ccaR*, (b) *orf10*, (c) *blp*, (d) *lat*, (e) *pcbAB*, (f) *pcbC*, (g) *pcbR*.

No observable changes were obtained by RT-PCR in the expression profiles of *cmcJ*, *cefF* and *cmcH* genes in the recombinant strain as compared to the wild type while an increment in the *cmcI* transcription was apparent in the recombinant strain.

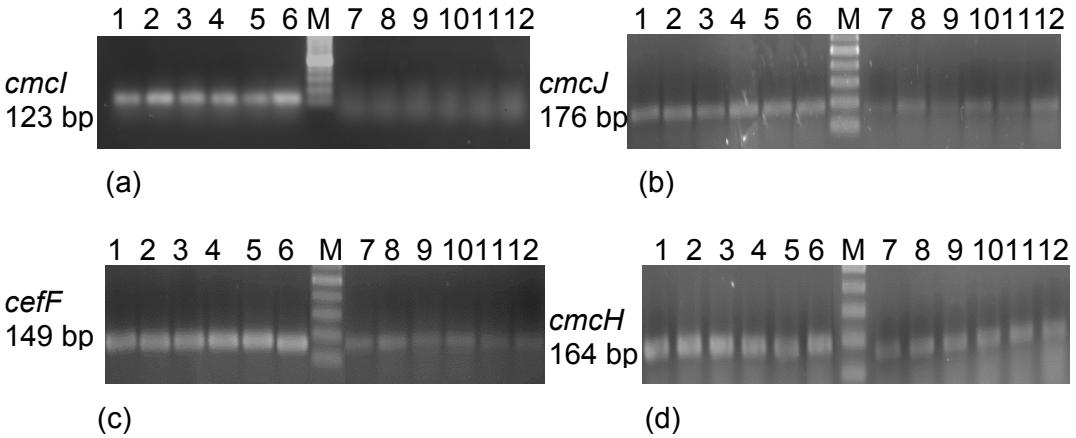


Figure 3. 25. Comparison of the gene expressions on the cephamycin C pathway in multicopy *ccaR* containing recombinant *S. clavuligerus* NRRL3585 and its wild type strain by RT-PCR during the time course. Lanes refer to the expression of related genes in the recombinant (1, 3, 5) and in the wild type (2, 4, 6) at T_{15}, T_{24} and T_{30}, respectively. 7, 9, 11: RT minus controls of lanes 1, 3 and 5, respectively. 8, 10, 12: RT minus controls of lanes 1, 3 and 5, respectively. (a) *cmcI*, (b) *cmcJ*, (c) *cefF*, (d) *cmcH*.

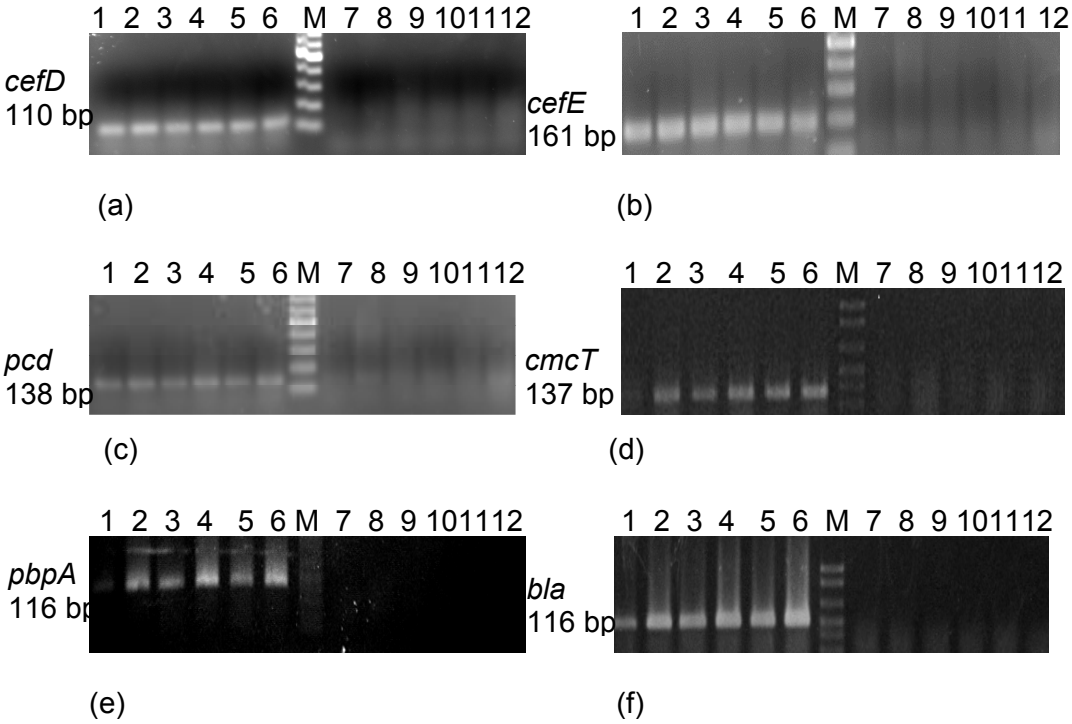


Figure 3. 26. Comparison of the gene expressions on the cephamycin C pathway in multicopy *ccaR* containing recombinant *S. clavuligerus* NRRL3585 and its wild type strain by RT-PCR during the time course. Lanes refer to the expression of related genes in the recombinant (1, 3, 5) and in the wild type (2, 4, 6) at T_{15}, T_{24} and T_{30}, respectively. 7, 9, 11: RT minus controls of lanes 1, 3 and 5, respectively. 8, 10, 12: RT minus controls of lanes 1, 3 and 5, respectively. (a) *cefD*, (b) *cefE*, (c) *pcd*, (d) *cmcT*, (e) *pbpA*, (f) *bla*.

As a summary, the intensity of the bands obtained from RT-PCR of *ccaR, lat, blp, cmcI, cefD*, *pcbR* genes in the recombinant *S. clavuligerus* C11 strain carrying multicopy *ccaR* was found to be higher than those in the wild type strain.

3.3.4. Co-transcriptional profile of the genes in cephamycin C gene cluster in *S. clavuligerus* NRRL3585

In order to analyze co-transcriptional pattern of cephamycin C cluster expression in the wild type *S. clavuligerus* NRRL3585, RT-PCR experiments were performed by using RNA samples isolated from 24 h cultures as template (Figure 3.27,3.28). Positive control reaction in which the genomic DNA of the wild type was used as template DNA and a negative control reaction including RNA as the template and only platinum Taq polymerase enzyme was included in the experiment for each intergenic region.

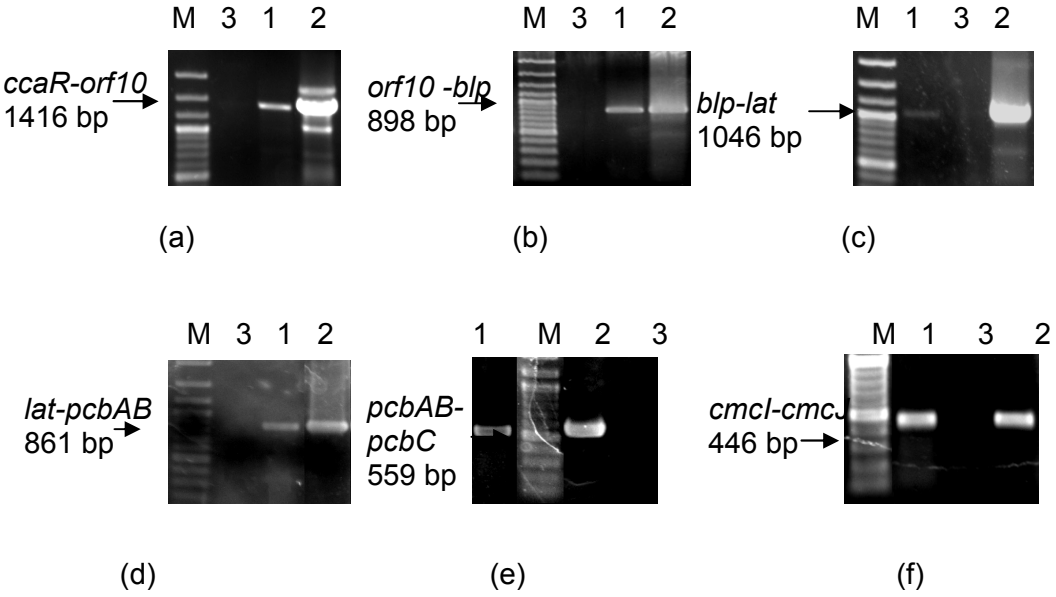


Figure 3. 27. Intergenic region amplification of cephamycin C genes in *S. clavuligerus* NRRL3585 at T_{24} as shown by RT-PCR. 1: Expression of the related intergenic region by using RNA of the wild type as a template in RT-PCR, 2: Expression of the related intergenic region by using genomic DNA of the wild type as a template in RT-PCR, 3: RT minus control of lane 1. (a) *ccaR-orf10*, (b) *orf10-blp*, (c) *blp-lat*, (d) *lat-pcbAB*, (e) *pcbAB-pcbC*, (f) *cmcI-cmcJ*.

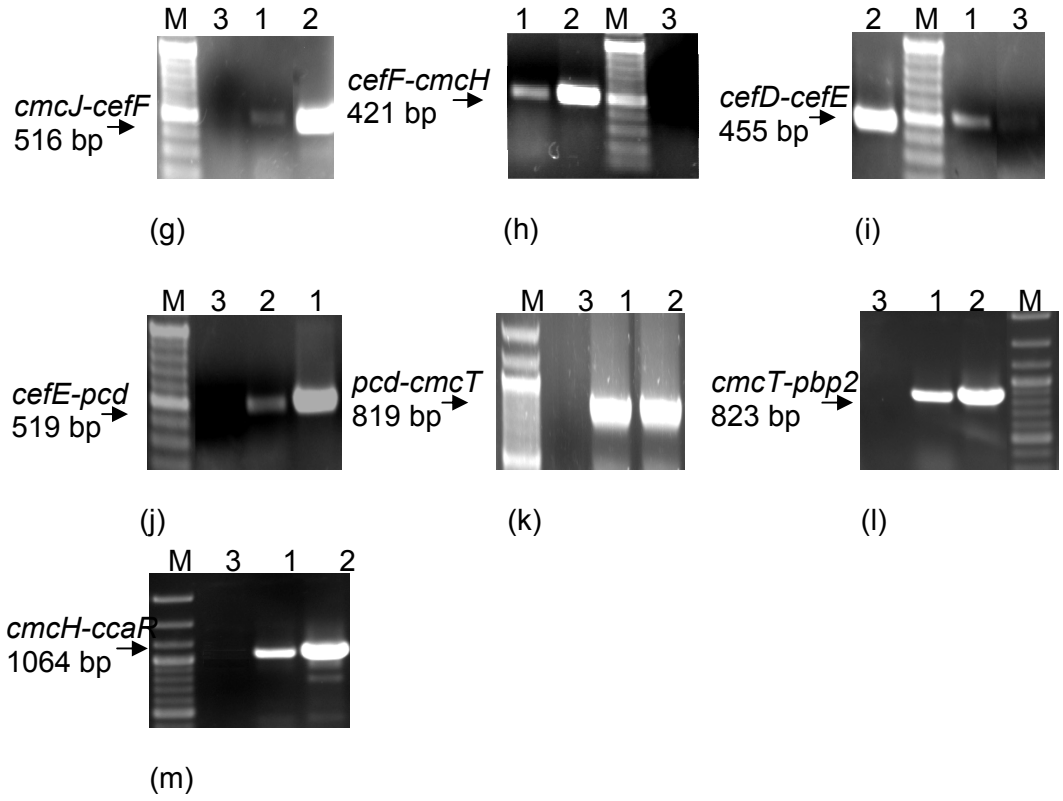


Figure 3. 28. Intergenic region amplification of cephamycin C genes in *S. clavuligerus* NRRL3585 at T_{24} as shown by RT-PCR. 1: Expression of the related intergenic region by using RNA of the wild type as a template in RT-PCR, 2: Expression of the related intergenic region by using genomic DNA of the wild type as a template in RT-PCR, 3: RT minus control of lane 1. (g) *cmcJ-cefF,* (h) *cefF-cmcH,* (i) *cefD-cefE,* (j) *cefE-pcd,* (k) *pcd-cmcT,* (l) *cmcT-pbp2*, (m) *cmcH-ccaR* intergenic regions.

Co-transcriptional analyses of the cephamycin C gene cluster by RT-PCR showed that all successive genes were transcribed together in the wild type strain. The intensity of the bands are related with the efficiency of the PCR reaction performed.

A general view of CcaR regulation on the cephamycin C gene cluster was demonstrated by RT-PCR analysis (Figure 3.29). However, the data represented here by RT-PCR were based on end point detection and were unable to give a

quantitative measurement of the expression of the genes of cephamycin C biosynthesis. Hence, the effect of *ccaR* on the expression of the cephamycin C genes were further analysed by subsequent qRT-PCR experiments as it allows simultaneous amplification and detection of DNA templates on the basis of flourescence emmission (Wong and Medrano, 2005).

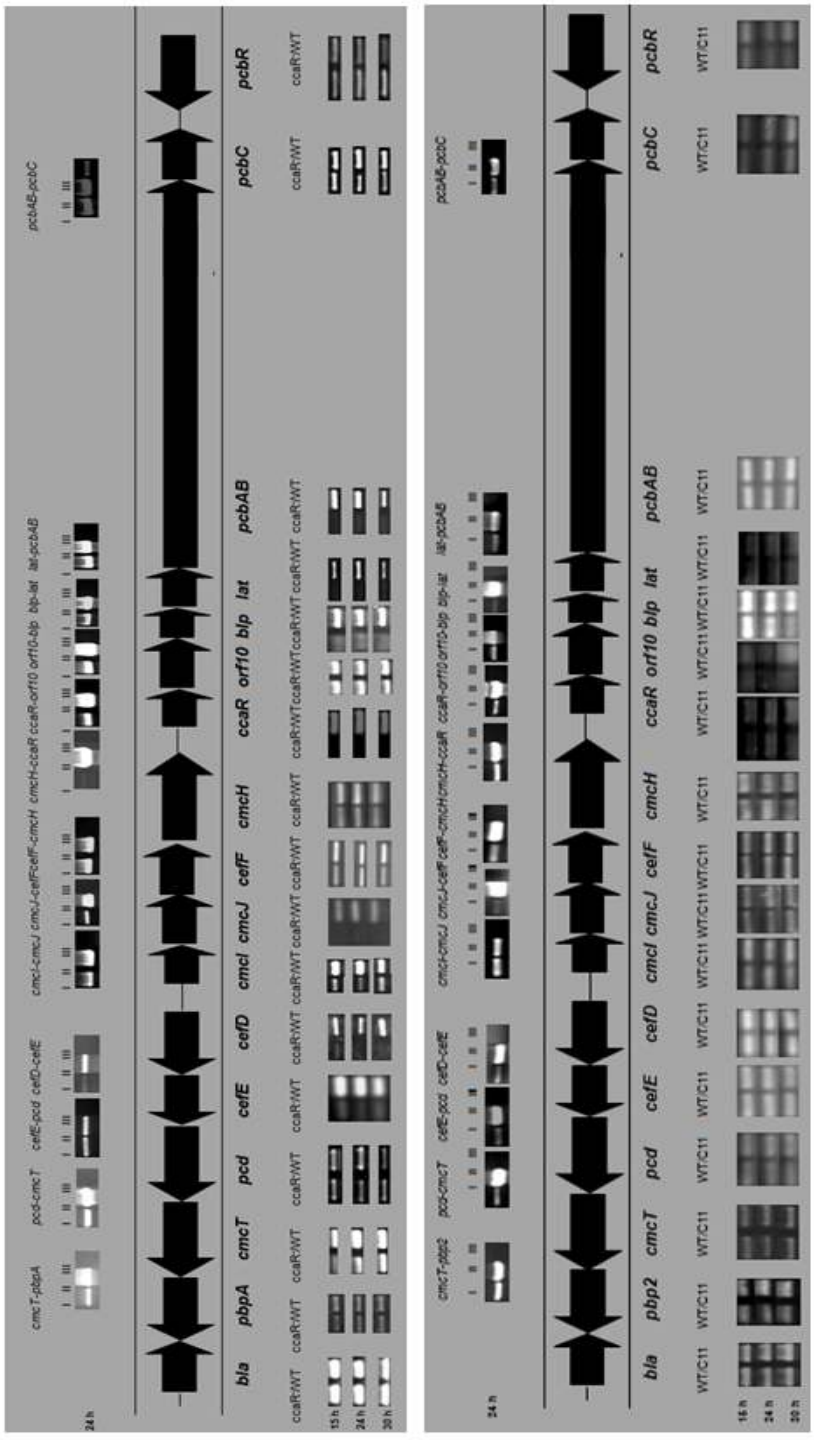
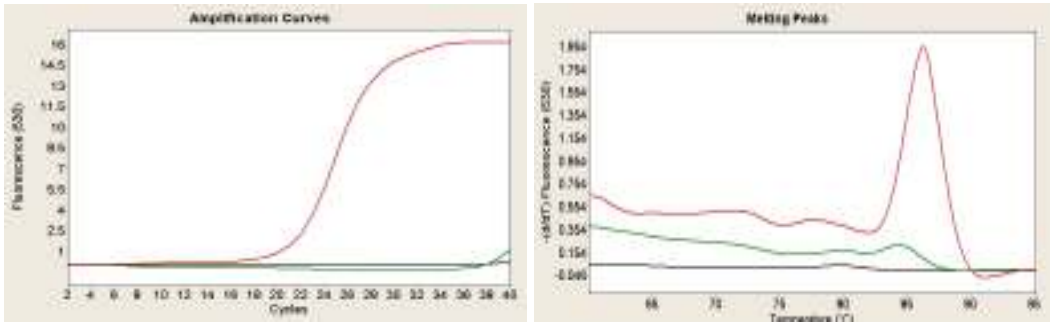


Figure 3. 29. RT-PCR analysis of the transcriptional organization of the intergenic regions on the cephamycin C gene cluster as well as the effect of CcaR activator on the cephamycin C gene transcriptions in *ccaR::aphII* and C11 strains as compared to the wild type.

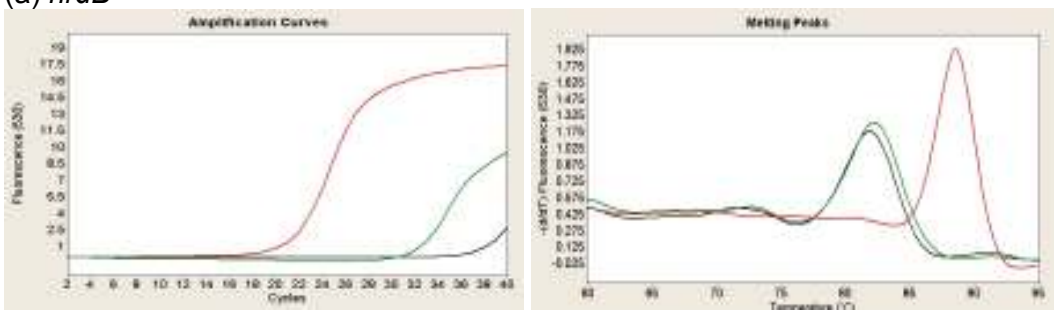
3.4. Expression analysis of the regulatory effect of *ccaR* on the cephamycin C gene cluster by qRT-PCR

3.4.1. Optimized qRT-PCR conditions

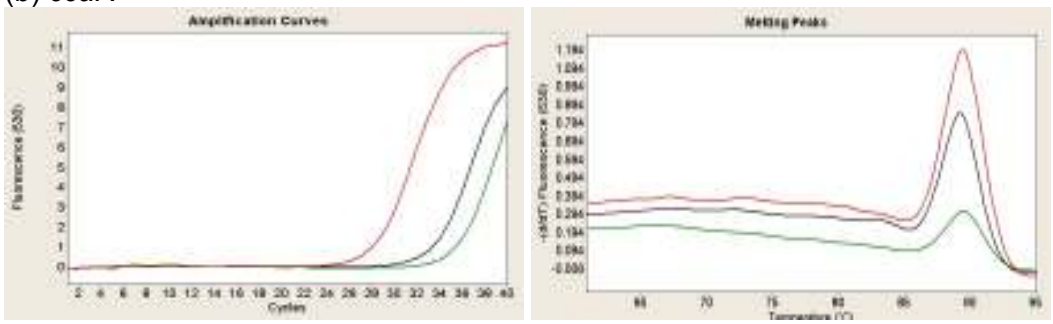
Optimization of qPCR conditions is a very crucial step to get reproducible, sensitive and efficient results following the assay. The primer-probe concentrations, cycling conditions and buffer composition of the reagents plus $MgCl_2$ concentration are the major parameters to be considered in the optimization process. Furthermore, the RNA quality is also essential for obtaining reliable data by qRT-PCR. These kind of preliminary optimization steps are important to eliminate test to test variations (Edwards and Logan, 2009). The use of phenol-chloroform extration plus DNase treatment during the RNA isolation procedure by RNeasy mini kit further increased the quality and quantity of RNA obtained in this study. Besides, a two-step qPCR method was preferred for cDNA synthesis; 250 ng random primer and 2 µg total RNA were used in the reaction. As the SYBR premix buffer already contains 2 mM $MgCl_2$ there was no need to optimize the concentration of this divalent. However, different concentrations of primers (25 nM, 150 nM, 250 nM and 350 nM) were tested for all the genes of the cluster as well as the reference gene. Following the preliminary experiments, 150 nM was found to be the most appropriate primer concentration for the amplification of all the genes except for *cmcT* and *orf10*, of which, 350 nM and 250 nM of primers, respectively, provided the best amplifications. On the other hand, 60 °C as an annealing temperature and a cycle number of 40 were chosen as the parameters applied in qPCR assays. The selection of right reference gene was a very important factor to be considered. In Figure 3.30-3.34, amplification plots and dissociation curves for each gene under the optimized conditions are given. Ct and Tm values of the optimized amplification plots and melting peaks derived from the preliminary studies were also tabulated in Table 3.1. In the literature, 23S-16S rRNA or *hrdB* encoding a major sigma factor were most commonly used internal control genes to determine the relative expression of the relevant genes by qRT-PCR (Huang *et al*., 2005; Rintala and Nevalainen, 2006; Nazari *et al*., 2011). In this study, *hrdB* was found to be the most appropriate reference gene to be used in the qRT-PCR experiments as compared to the 16S rRNA encoding gene (data not shown).



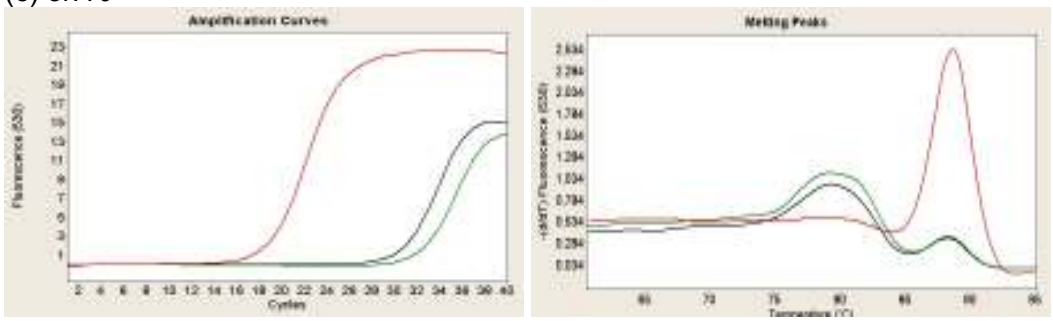
(a) *hrdB*



(b) *ccaR*

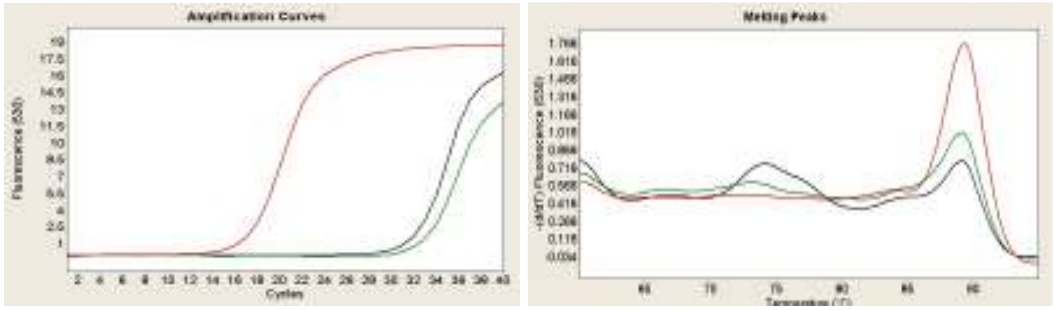


(c) *orf10*

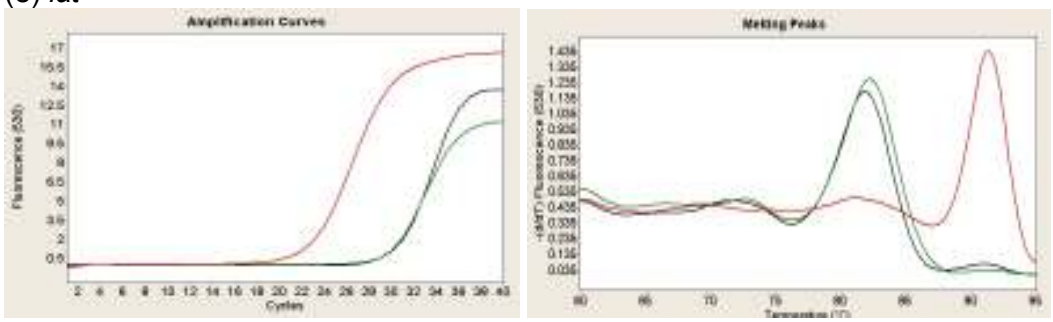


(d) *blp*

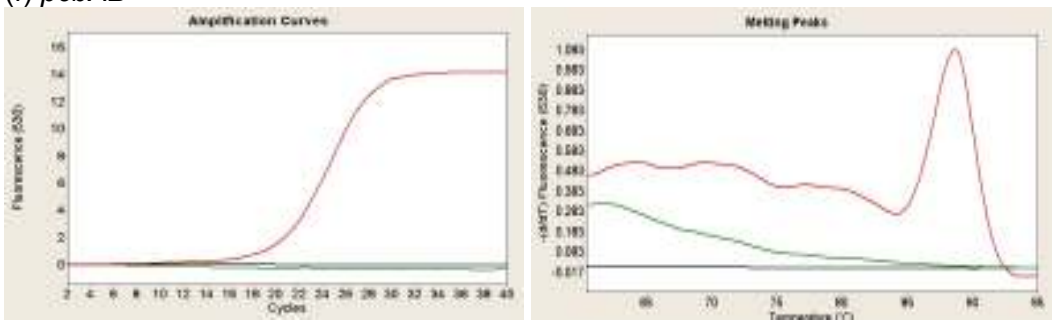
Figure 3. 30. Amplification plots and dissociation curves of reference gene and cephamycin C genes obtained under the optimized conditions of qRT-PCR. Black lines represent NTC (no template control), green lines represent RTC (RNA template control) and red lines represent target genes. (a) *hrdB*, (b) *ccaR*, (c) *orf10*, (d) *blp.*



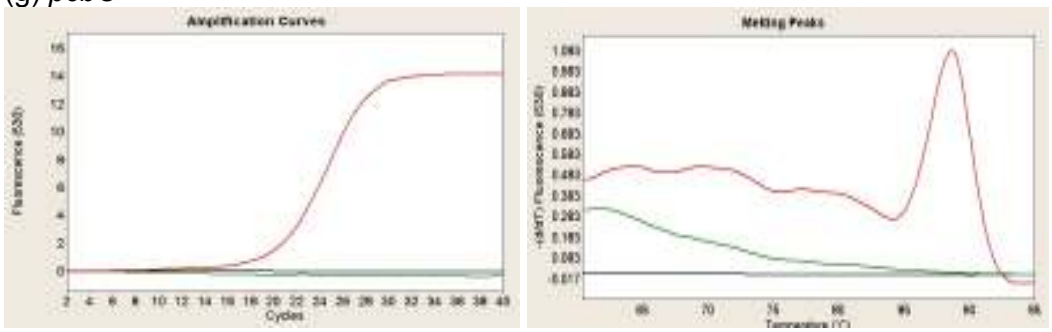
(e) *lat*



(f) *pcbAB*

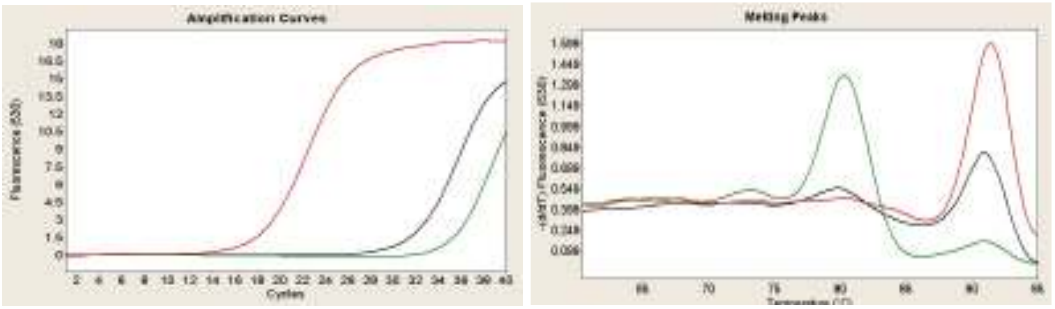


(g) *pcbC*

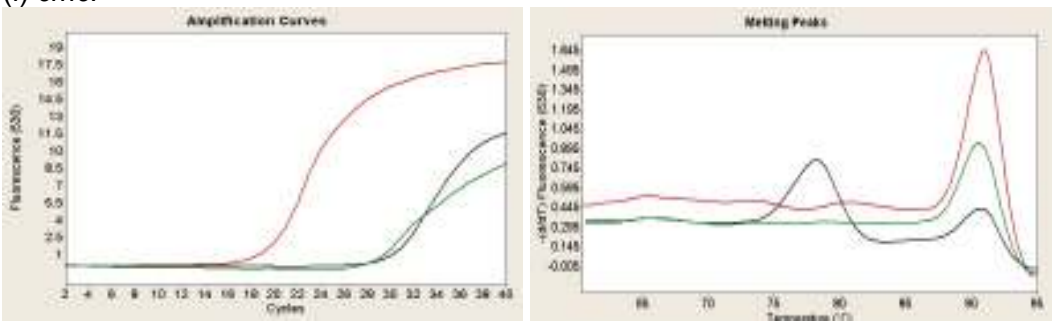


(h) *pcbR*

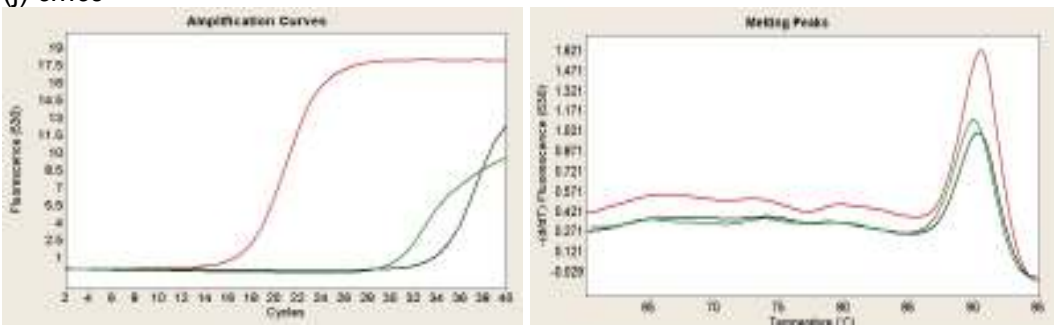
Figure 3. 31. Amplification plots and dissociation curves of reference gene and cephamycin C genes obtained under the optimized conditions of qRT-PCR. Black lines represent NTC (no template control), green lines represent RTC (RNA template control) and red lines represent target genes. (e) *lat*, (f) *pcbAB*, (g) *pcbC*, (h) *pcbR*.



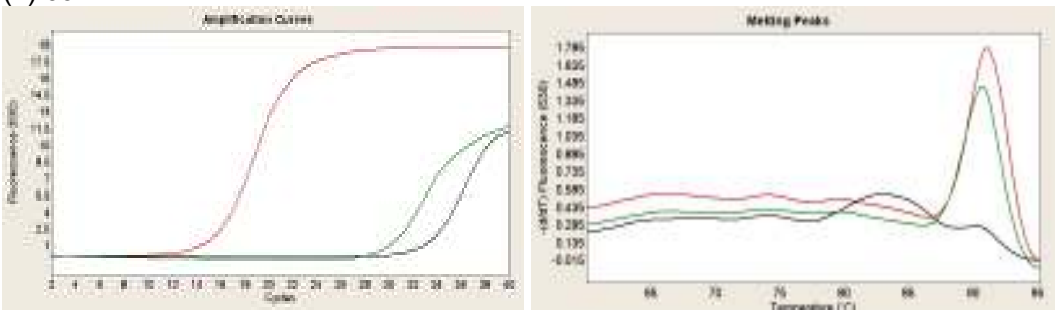
(i) *cmcI*



(j) *cmcJ*

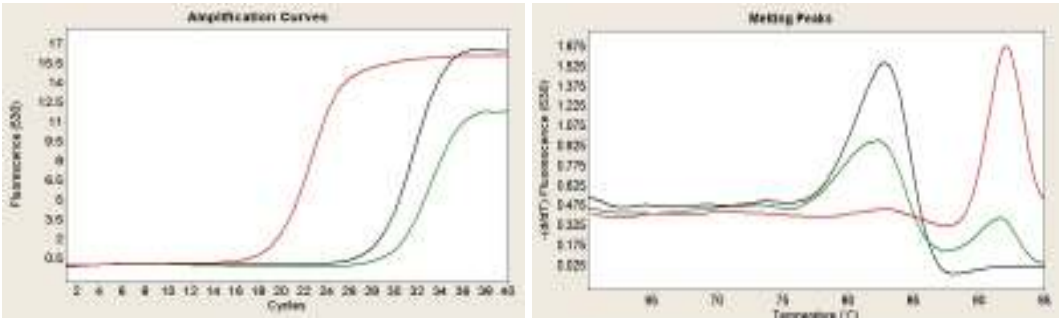


(k) *cefF*

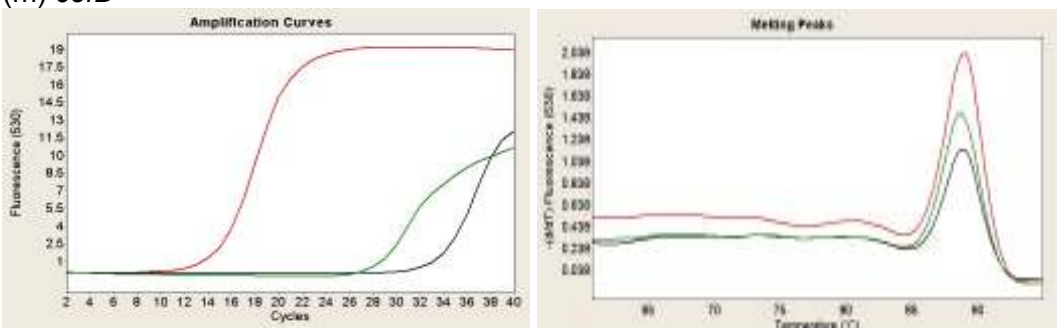


(l) *cmcH*

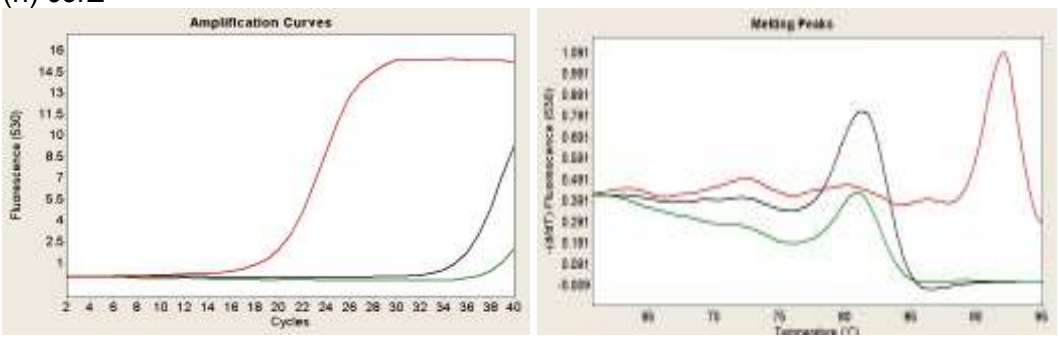
Figure 3. 32. Amplification plots and dissociation curves of reference gene and cephamycin C genes obtained under the optimized conditions of qRT-PCR. Black lines represent NTC (no template control), green lines represent RTC (RNA template control) and red lines represent target genes. (i) *cmcI*, (j) *cmcJ*, (k) *cefF*, (l) *cmcH*.



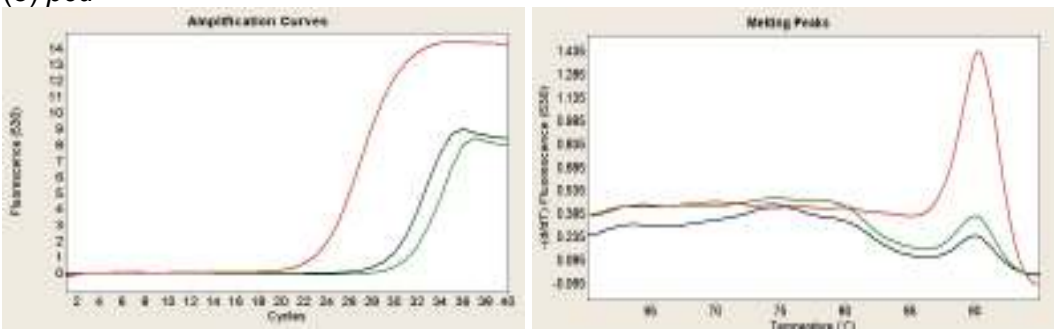
(m) *cefD*



(n) *cefE*

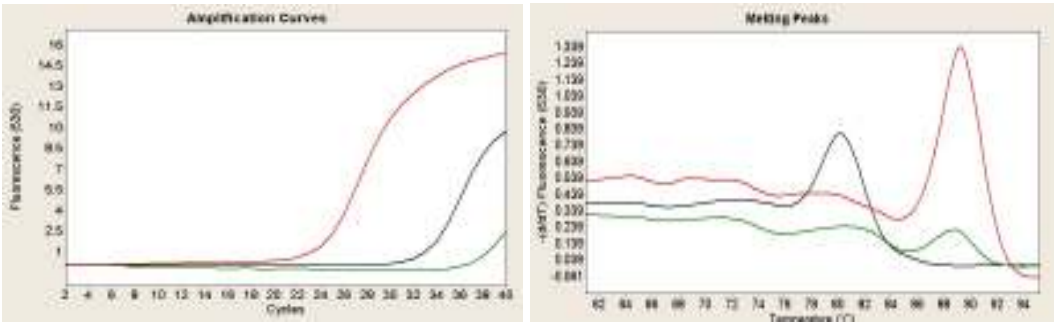


(o) *pcd*

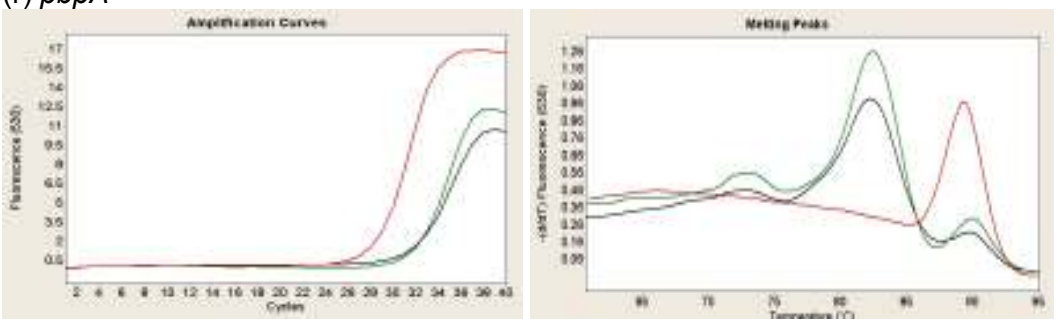


(p) *cmcT*

Figure 3. 33. Amplification plots and dissociation curves of reference gene and cephamycin C genes obtained under the optimized conditions of qRT-PCR. Black lines represent NTC (no template control), green lines represent RTC (RNA template control) and red lines represent target genes. (m) *cefD*, (n) *cefE*, (o) *pcd*, (p) *cmcT*.



(r) *pbpA*



(s) *bla*

Figure 3. 34. Amplification plots and dissociation curves for each gene obtained by qRT-PCR in optimized conditions. Black lines represent NTC (no template control), green lines represent RTC (RNA template control) and red lines represent target genes. (r) *pbpA*, (s) *bla*.

Table 3. 1. Ct and Tm values of the optimized amplification plots-melting peaks

	Ct			Tm		
	Target	NTC	RTC	Target	NTC	RTC
hrdB	20.61	35.00	35.00	86.35	79.82	84.34
ccaR	23.61	35.00	35.00	89.67	89.11/79.79	89.04/79.65
orf10	27.32	32.39	35.00	89.40	89.22	89.35
blp	17.52	29.61	30.96	88.42	88.25/79.24	88.33/79.40
lat	16.03	30.73	31.44	89.31	89.01/74.16	89.23
pcbAB	20.70	29.45	28.80	91.52	81.90	82.13
pcbC	19.97	35.00	35.00	88.74	70.08	70.52
pcbR	20.74	31.38	32.71	88.97	79.86	79.71
cmcI	17.76	31.03	35.00	91.40	91.02/79.66	80.27
cmcJ	18.35	29.20	27.17	90.93	90.62/78.13	90.52
cefF	16.71	34.34	28.97	90.60	90.45	90.06
cmcH	14.56	31.92	28.68	91.14	82.72	90.69
cefD	18.27	27.51	28.93	92.11	83.06	91.60/82.17
cefE	13.77	32.41	26.89	89.02	88.93	88.59
pcd	19.16	35.00	35.00	91.94	81.28	81.03
cmcT	22.62	28.45	29.75	90.28	90.07/75.09	90.06/77.58
pbpA	23.10	31.86	35.00	89.26	80.13	88.69
bla	27.21	29.56	30.56	89.66	89.97/82.11	90.01/82.45

3.4.2. Amplification efficiency calculation on the basis of standard curve formation

Efficiency correction based quantification model (Pfaffl, 2001) was preferred to evaluate qRT-PCR data in the present study. The efficiency values of target and control samples may not be equal most of the time and minor differences in the amplification efficiencies of both target and reference genes might lead to significant fluctuations in the Ct values (Platts *et al*., 2008). Amplification efficiency was determined according to the formula $E = 10^{[-1/slope]}$ (Pffafl, 2001) by using the slope of the standard curve obtained from serially diluted DNA samples. Hence genomic DNA of *S. clavuligerus* was used as a template to plot standard curve for each primer set of the related genes (Lopez-Garcia *et al*., 2010) (Figure 3.35-3.37).

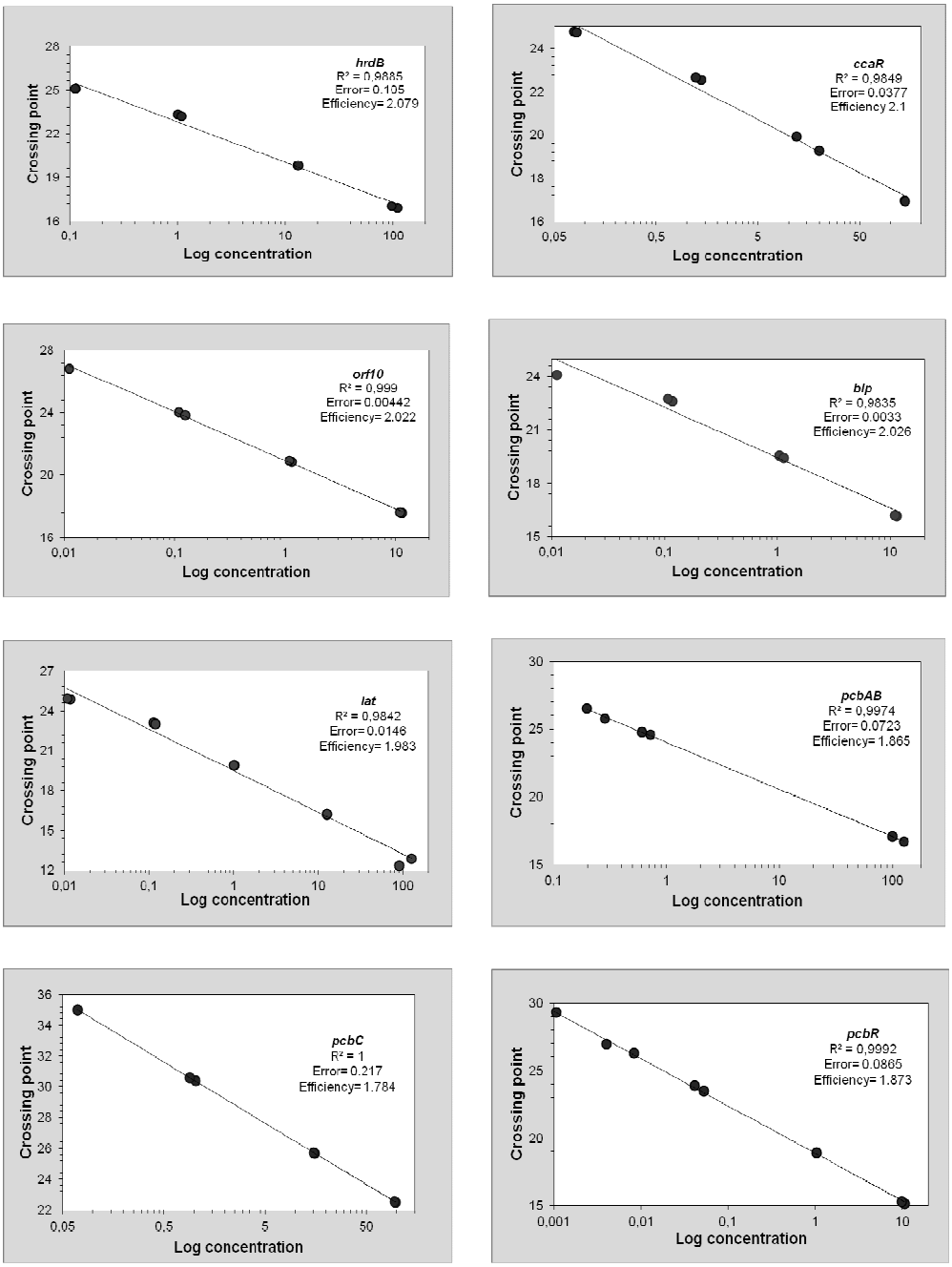


Figure 3. 35. Standard curves for the genes (*hrdB, ccaR, orf10, blp, lat, pcbAB, pcbC,* and *pcbR*) in the cephamycin C cluster and the corresonding amplification efficiencies of each primer set.

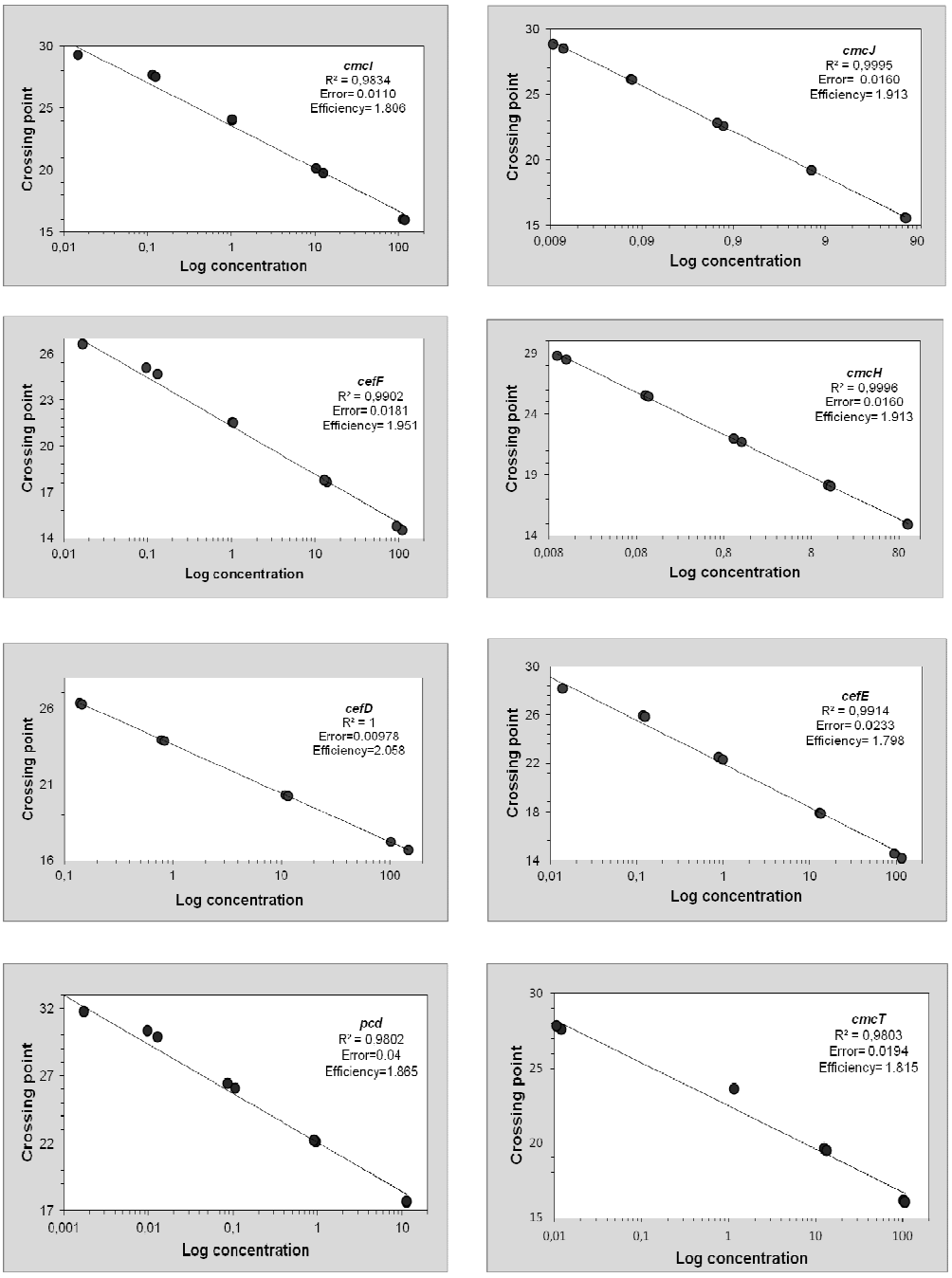


Figure 3. 36. Standard curves for the genes (*cmcI, cmcJ, cefF, cmcH, cefD, cefE, pcd* and *cmcT*) in the cephamycin C cluster and the corresonding amplification efficiencies of each primer set.

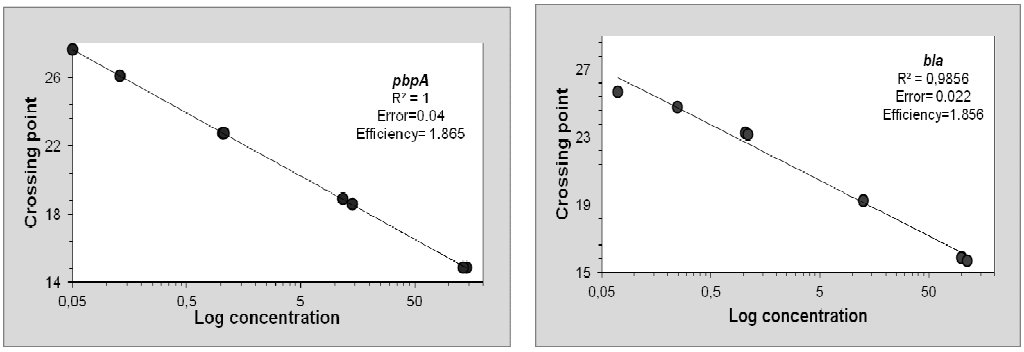


Figure 3. 37. Standard curves for the genes (*pbpA* and *bla*) in the cephamycin C cluster and the corresonding amplification efficiencies of each primer set.

R^2 is coefficient of determination and indicates linearity of a standard curve. It is one of the parameters considered in evaluation of PCR efficiency. A value of R^2 >0.98 is desired for good amplification efficiency (Meijerink *et al*., 2001). As shown in Table 3.2, all amplification efficiency values acquired by using the slope of the plots were in a range of 1.6 to 2.1, i.e. within the acceptable borders reported previously (Pfaffl, 2004).

Table 3. 2. Amplification efficiencies of primer sets for each gene on the cephamycin C cluster

Gene Name	Amplification efficiency	Gene Name	Amplification efficiency
bla	1.856	*cmcH*	1.913
pbpA	1.865	*ccaR*	2.1
cmcT	1.815	*orf10*	2.022
pcd	1.865	*blp*	2.026
cefE	1.798	*lat*	1.983
cefD	2.058	*pcbAB*	1.865
cmcI	1.806	*pcbC*	1.784
cmcJ	1.913	*pcbR*	1.873
cefF	1.951	*hrdB*	2.079

3.4.3. Quantification of time-dependent expression pattern of the cephamycin C biosynthetic gene cluster in the wild type strain and its *ccaR*-negative mutant by qRT-PCR

Time-dependent changes in the expression profile of cephamycin C cluster in the *ccaR*-disrupted mutant as compared to the wild type strain are represented in Figure 3.38 to 3.41. The relative expression values were obtained very low to display in a linear scale. Hence, logaritmic transformation was preferred to signify the relative expression ratio of the genes in the graphs (Derveaux *et al*., 2010). The expression value for the control was assumed as 1. There are significant differences in gene expression levels between wild type and its *ccaR*-minus mutant. Besides, a significant level of difference between three sampling time in gene expression in the mutant strain. Except for *pbpA* ve *bla* genes at 15 h and 30 h, the significance level of the relative expression ratio for all genes at all hours were $p<0.001$ and was indicated as "***" in the graphs.

When looked at the qRT-PCR results, the disruption of *ccaR* gene, thereby the absence of CcaR protein in the cell was led to 2212-, 1718- and 1291- fold decrease at T_{15}, T_{24} and T_{30} in the expression level of *lat* gene encoding the first enzyme in the cephamycin C biosynthesis that mediates the formation of α-aminoadipic acid from lysine in a two step reaction (Figure 3.38). The *lat* expression exerted the most drastic decline profile among the expression data derived from the *ccaR* minus strain by qRT-PCR. Consequently, cephamycin C synthesis was not possible in the lack of the rate limiting enzyme, LAT, in the cell (Figure 3.19b). It was previously shown that the disruption of *lat* gene resulted in the blockage of cephamycin C production as well as a 2 to 2.5 fold high clavulanic acid titer in the mutant strain of *S. clavuligerus* (Paradkar *et al*., 2001). In this respect, our data on *lat* expression in the mutant strain that obtained by qRT-PCR were consistent with the previous reports.

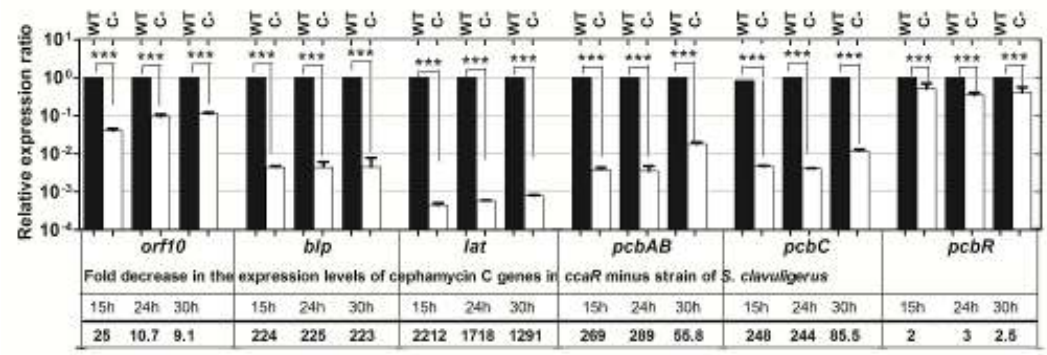


Figure 3. 38. Quantification of *orf10*, *blp*, *lat*, *pcbAB*, *pcbC* and *pcbR* gene expressions in the wild type and its *ccaR*- mutant by qRT-PCR during the course of fermentation. Two independent qRT-PCR runs were performed and each run included two biological replicates and three technical replicates for each sample. One way repeated ANOVA with Tukey post hoc was performed on the normalized gene expression to check whether the expressions were statistically different between the wild type and *ccaR* minus strain of *S. clavuligerus* (***= p <0.001).

orf10 encodes for a protein with an unknown function. The *orf10* gene expression was 25-fold lower in the mutant strain than that of the wild type at T_{15}. The extent of decrease was 10-fold at T_{24} and 9-fold at T_{30} in the mutant strain. Another point is that the *orf10* mRNA expression was possibly quite unstable in that a distinct band was hardly seen both in the wild type and manipulated strains in RT-PCR expreriments (Figure 3.20 and 3.24). This may also point to the possibility of the presence of a terminator sequence within the intergenic region between *ccaR* and *orf10* genes unavailing the negative effect of *ccaR* disruption on the *orf10* expression.

blp encodes a protein that resembles BLIP (β-lactamase inhibitory protein) in its sequence but does not have any β-lactamase inhibitory activity. Deletion of neither *blp* nor *orf10* did not cause any deleterious effect in the cephamycin C production (Alexander and Jensen, 1998). In contrast to *orf10* expression, a moderate and fairly constant decrease in the *blp* gene expression, corresponding to a 224-fold reduced expression level, was detected by qRT-PCR assays

throughout the course of fermentation in the mutant strain. The presence of a wide intergenic region, 346 bp in length (Perez-Llarena *et al*., 1997; Alexander and Jensen, 1998), between *orf10* and *blp* genes on the cephamycin C cluster probably indicates the presence of a CcaR dependent promoter ahead of the *blp* gene.

The early step enzyme encoding genes, *pcbAB* and *pcbC* displayed a moderately decreased level of expression during the course of fermentation. Fold decrease in the expression levels of *pcbAB* gene was found as 269 at T_{15}, 289 at T_{24}, and 55.8 at T_{30}. A similar low expression pattern of *pcbC* gene was also recorded (248-fold less at T_{15}, 244-fold less at T_{24}, and 85.5 fold less at T_{30}) confirming the RT-PCR results. According to the data obtained by qRT-PCR, the expression of the both genes was controlled by CcaR activator and this control was most probably carried out by *lat* promoter region. Given that *pcbAB* gene is approximately 11 kb in size, the absence of a wide intergenic region between the two genes might imply the existence of a possible promoter region of *pcbC* inside *pcbAB* gene for an efficient control of *pcbC* (Petrich, 1992).

pcbR gene, encoding an enzyme anchored in the membrane and acting on resistance of the cell against its own cephamycin C, was shown to be expressed in *ccaR* mutant with only 2 to 3 fold lower level of expression throughout the course of fermentation.

In the cephamycin C gene cluster, *cmcJ* gene which is located adjacent to *cmcI* was expressed relatively high levels in the mutant strain compared to the *cmcI* expression. The fold decrease in its expression profile in *ccaR* mutant was in a range of 120 and 260 (Figure 3.39). *cefF* and *cmcH* genes encode enzymes that function successively in the cephamycin C biosynthesis. A remarkable decrease in the *cefF* gene expression was detected at T_{24} (354-fold reduction in expression). Interestingly, this drastic decrease in its expression continued with a 314-fold reduction, at T_{30}, as well. In addition, the *cmcH* expression decreased up to 239-fold at T_{24}. A 132-fold decrease at T_{15} and 148-fold decrease at T_{30} were detected in the *ccaR* minus strain.

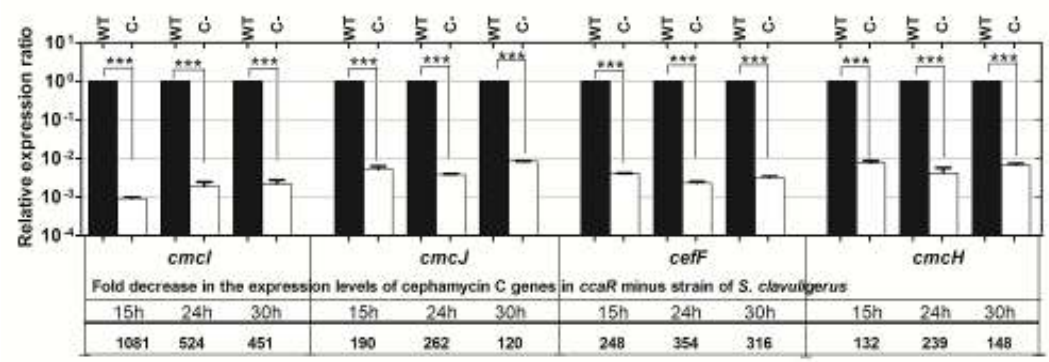


Figure 3. 39. Quantification of *cmcI*, *cmcJ, cefF* and *cmcH* gene expression in the wild type and its *ccaR*- mutant by qRT-PCR during the course of fermentation. Two independent qRT-PCR runs were performed and each run included two biological replicates and three technical replicates for each sample. One way repeated ANOVA with Tukey post hoc was performed on the normalized gene expression to check whether the expressions were statistically different between the wild type and *ccaR* minus strain of *S. clavuligerus* (***= p <0.001).

The binding of CcaR to *cefD-cmcI* bidirectional promoter was previously reported (Santamarta *et al*., 2002). Correspondingly, the low level expression of *cefD* and *cmcI* genes were detected in *ccaR*- strain. 205-, 299- and 105-fold decreases at T_{15}, T_{24} and T_{30} were observed in the expression level of *cefD* gene as compared to the wild type (Figure 3.40). A drastically low level of expression was obvious for *cmcI* gene at T_{15}, as being a 1081-fold lower expression level in the mutant strain, however, this reduction was not sustained during the time course. The relative expression levels of *cmcI* were found to decrease 524- and 451-fold in the mutant strain at T_{24} and T_{30}, respectively (Figure 3.39). *cefD* encodes IPN epimerase involved in conversion of isopenicillin N to penicillin N in the cephamycin C biosynthesis pathway (Usui and Yu, 1989). As being a crucial enzyme for the cephamycin C biosynthesis, *cefD* expression is under the control of CcaR (Alexander and Jensen, 1998). *cmcI* encodes a cephalosporin-7-α-hydroxylase enzyme that functions in hydroxylation of C-7´ position of β-lactam ring (Enguita *et al*., 1996). *cmcJ* encoded methyltransferase adds methoxyl group to the C-7 position to yield cephamycin C. The methoxylation step is very

important as it provides β-lactamase resistance to the cephamycin C producer. The role of *cmcI* at this point is the presence of metoxylation complex on CmcI (Öster *et al*., 2006). For this reason, our qRT-PCR data for *cmcI* expression in the *ccaR* minus strain might be considered quite logical and confirms the previous findings (Alexander and Jensen, 1998) in which no signal belonging to IPN epimerase was detected in different *ccaR* disrupted strains by Western blot analysis.

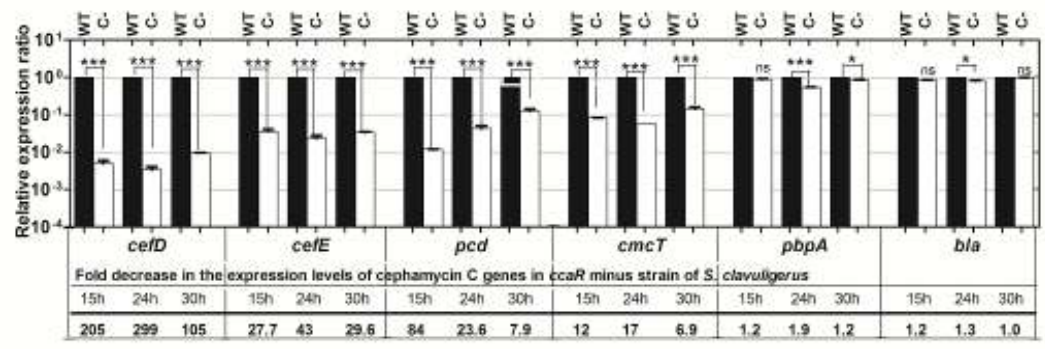


Figure 3. 40. Quantification of *cefD*, *cefE, pcd, cmcT, pbpA* and *bla* gene expression in the wild type and its *ccaR*- mutant by qRT-PCR during the course of fermentation. Two independent qRT-PCR runs were performed and each run included two biological replicates and three technical replicates for each sample. One way repeated ANOVA with Tukey post hoc was performed on the normalized gene expression to check whether the expressions were statistically different between the wild type and *ccaR* minus strain of *S. clavuligerus* (***= p <0.001, *= p <0.05, ns= p > 0.05).

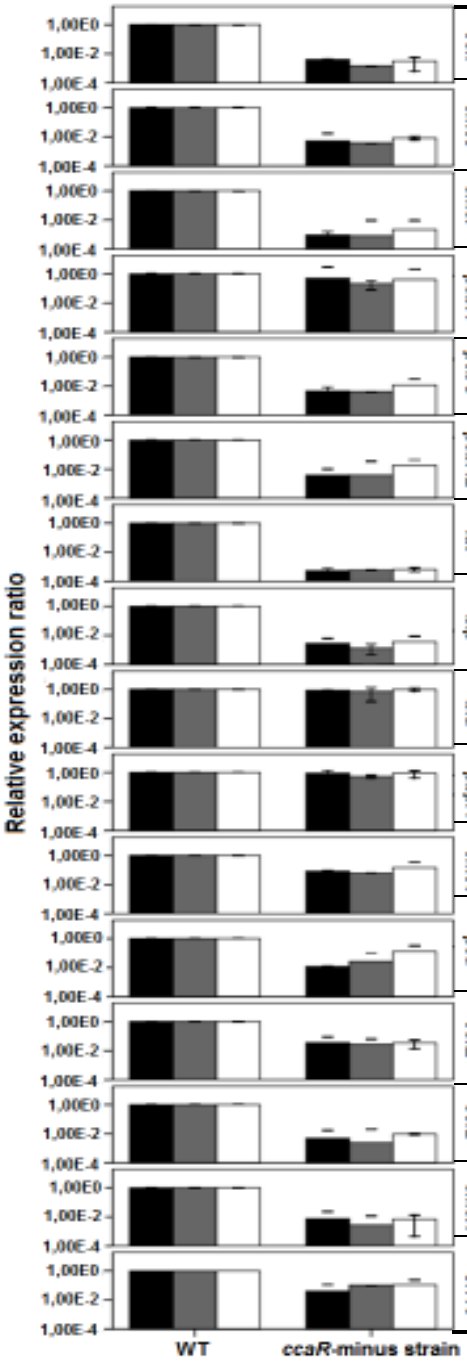
On the other hand, the data obtained by qRT-PCR assay of *cefE, pcd, cmcT* and *bla* genes were consistent with the results of RT-PCR experiments in which no significant decrease in the expression profiles during the time course of fermentattion was observed. *cefE* expression was lowered at relatively minor levels, corresponding to 27.7- to 43-fold decrease at all hours examined. It encodes for penicillin N expandase enzyme involved in formation of deacetoxycephalosporin C (DAOCS) from penicillin N, in the middle steps of cephamycin C biosynthesis. In contrast to Western blot analysis in which no

signal of DAOCS was detected in the *ccaR* mutants, no drastic effect of *ccaR* disruption in the expression level of *cefE* was found in our qRT-PCR analysis. The findings on *pcd* expression in the *ccaR* mutant by qRT-PCR were as expected since its absence led to a 30-70 % reduction in cephamycin C production in the cell (Alexander *et al*., 2007). It encodes an enzyme involved in formation of αAAA from L-lysine thereby acting in the first step of cephamycin C biosynthesis. According to the qRT-PCR analysis, *pcd* gene exhibited 84-fold at T_{15}, 23.6-fold at T_{24} and 7-fold at T_{30} lower expression levels in the mutant strain.

The expression of *cmcT* gene encoding an antibiotic transport protein decreased 12- and 17- fold in the mutant strain as compared to the wild type at T_{15} and T_{24}, respectively. Its low expression level was continued at T_{30}, but in a lesser extent as being 7.9-fold. *pbpA* and *bla* genes that coding for enzymes involved in β-lactam reisitance and cell morphology, respectively were not much affected by *ccaR* mutation in that only 1.3 to 1.9-fold decrease was detected in their expression level in *ccaR* disrupted strain.

A whole picture of the effect of *ccaR* disruption on the expression of cephamycin C genes is shown in Figure 3.41. As depicted from the figure, the expression of all genes on the cephamycin C gene cluster were repressed at all hours of incubation investigated. Only *pbpA* (at T_{15}) and *bla* genes (at T_{15} and T_{30}) were found with no significant change in their expression profiles in the specified hours. The minor decrease in the expression of *cmcT*, *pbpA*, *bla* and *pcbR* in the absence of *ccaR* might be attributed to their accessory roles on the pathway. In contrast, the expression of biosynthetic genes in the cluster strictly required the presence of CcaR in the cell. There are several studies in the literature in which qRT-PCR technique was preferred for quantification of the actual amount of the transcripts in the antibiotic biosynthesis clusters. For instance, Dangel *et al*. (2011) studied on the transcriptional regulation of the novobiocin biosynthetic gene cluster by using regulator gene deficient strains by qRT-PCR experiments. In addition, Lopez-Garcia *et al*. (2010) investigated the effect of *adpA*-deletion on the morphological differentiation and clavulanic acid formation by *S. clavuligerus*. Ostash *et al*. (2011) conducted a study by disrupting *lndYR*, encoding a GntR-like regulator, to indicate its deleterious effect on sporulation and antibiotic

production. Du *et al*. (2011) used qRT-PCR technology in order to identify the complete natamycin biosynthetic gene cluster of *S. chattanoogensis* through the disruption of pathway-specific activator genes. However, the data obtained from qRT-PCR studies are better supported by characterization of DNA-binding sequences of the activator proteins of the specified clusters. Hence qRT-PCR data from *ccaR* minus strain necessiated the determination of CcaR binding regions on the cephamycin C gene cluster to better understand the regulatory effect of *ccaR* on the expression of cephamycin C gene cluster.



	Fold decrease in the expression level of *ccaR*- minus strain		
	15 h	**24 h**	**30 h**
cefF	248	354	316
cmcJ	190	262	120
cmcI	1081	524	451
pcbR	2	3	2.5
pcbC	248	244	85.5
pcbAB	269	289	55.8
lat	2212	1718	1291
blp	224	225	223
bla	1.2	1.3	1
pbpA	1.1	1.9	1.2
cmcT	12	17	7
pcd	84	23.6	7.9
cefE	27.7	43	29.6
cefD	205	299	105
cmcH	132	239	148
orf10	25	10.7	9.1

Figure 3. 41. Quantification of time-dependent expression pattern of the cephamycin C cluster in the *ccaR*- disrupted strain as compared to the wild type by qRT-PCR. Error bars represent 95 % confidence intervals. Black bars represent T_{15}, grey bars represent T_{24} and white bars represent T_{30}.

3.4.4. Quantification of time-dependent expression pattern of cephamycin C biosynthetic gene cluster in the wild type strain and the cephamycin C overproducer C11 strain by qRT-PCR

According to the bioassay results, multicopy expression of *ccaR* in the recombinant C11 cells favored cephamycin C production significantly especially at the early hours of fermentation (Figure 3.19). For instance, a 6.5-fold high level of specific cephamycin C production was detected in C11 fermentation broths at the first sampling hour of the fermentation, T_{15}, although a similar amount of DNA was measured for both strains at this time. C11 entered stationary phase after 24 h of incubation while the wild type strain switched to this phase at the end of 30 h with a delay of six hours. Another striking difference between the growth patterns of these strains was a sharp decline in the growth of recombinant strain between T_{24} and T_{30}. This drastic fall within a very short time interval during the fermentation process was accompanied by a 3-fold high level of specific cephamycin C titer. Thereby, the positive effect of multicopy expression of *ccaR* on the antibiotic production was expected to be exerted at a transcriptional level on the basis of CcaR regulation of the cephamycin C gene cluster.

The time-dependent changes in the expression levels of the cephamycin C genes in the recombinant C11 strain in comparison to the wild type *S. clavuligerus* were determined by qRT-PCR experiments (Figure 3.36a,b,c). The introduction of an empty pSPG vector into the *S. clavuligerus* cells (pGV) did not lead to any remarkable change in the expression of cephamycin C biosynthesis genes (Figure 3.42 to 3.45).

While the cephamycin C titer was found to be much higher in C11 strain as compared to the wild type at T_{15}, there was also a 3.2-fold increase in the expression level of *pcbR* gene in the recombinant strain (Figure 3.42). The expresssion of *pcbR* gene showed a relative increase of 1.8-fold at T_{24}, while at T_{30}, its expression was still high (2-fold increase), giving a statistically significant ($p<0.001$) value after analysis of reperated measures by ANOVA. Overexpression of *ccaR* in the cell brings about higher level of *pcbR* expression especially at T_{15} as compared to the wild strain.

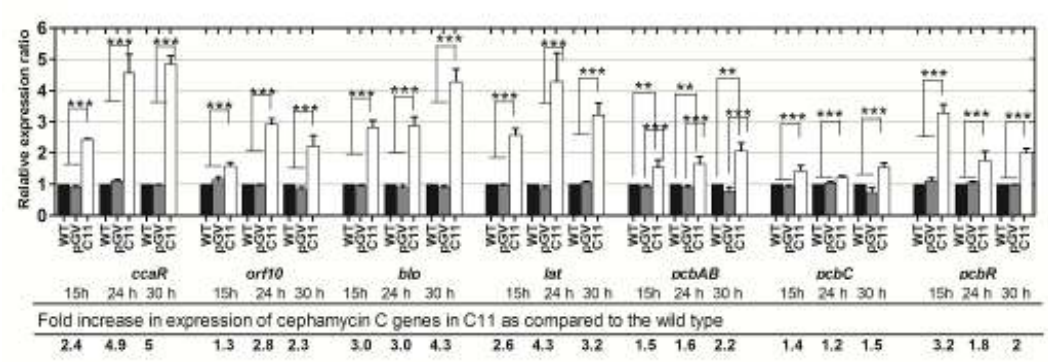


Figure 3. 42. Quantification of *ccaR*, *orf10, blp, lat, pcbAB, pcbC* and *pcbR* gene expression in the wild type, recombinant pGV and C11 strains by qRT-PCR during the course of fermentation. Two independent qRT-PCR runs were performed and each run included two biological replicates and three technical replicates for each sample. Two way repeated measures ANOVA with Bonferroni post hoc was performed on the normalized gene expression to check whether the expressions were statistically different between the wild type and C11, and pGV and C11 (** = $p < 0.01$, ***= $p < 0.001$).

Time-dependent expression analyses of *ccaR, lat, cmcI, cefD* and *blp* genes in recombinant C11 strain were found to be consistent with the data obtained from qRT-PCR based expression analyses of those genes in the *ccaR*-disrupted strain. In C11 strain, *ccaR* expression showed a significant relative increase of about 4.9- and 5-fold at T_{24} and T_{30}, while its expression was also high (a 2.4-fold increment) at T_{15}, in a statistically significant manner. The high levels of gene expression at T_{24} and T_{30} was maintained by *lat, cmcI, cefD* and *blp* genes in an order of 4.3-, 4.5-, 2.3- and 3-fold at T_{24}; and 3.2-, 5.1-, 3-, 4.3- fold at T_{30} (Figure 3.42-3.45) while *orf10* gene expression increased by 1.3 to 2.8-fold in C11 strain. *lat*, *cmcI* and *cefD* biosynthetic genes encode for enzymes involved in the first, middle and final steps of cephamycin C pathway. Thus, their increased level of expression in *ccaR* overexpression is expected. Although LAT and PCD encoding genes, *lat* and *pcd*, are involved in the initial two steps of cephamycin C biosynthesis, (conversion of lysine to α-AAA in a two step reaction), the multicopy expression of *ccaR* did not cause the same effect on the expression of *pcd* gene, thus confirming the data obtained from the *ccaR* disrupted strain by qRT-PCR. *pcbAB* gene showed approximately 1.5- to 2.2-fold increased expression level in C11 strain. Regarding *pcbC* gene, minor changes were detected in its expression

ranging from 1.2- to 1.5-fold increments, suggesting the presence of a promoter region independent from CcaR regulation which might be located at 3' end of *pcbAB*.

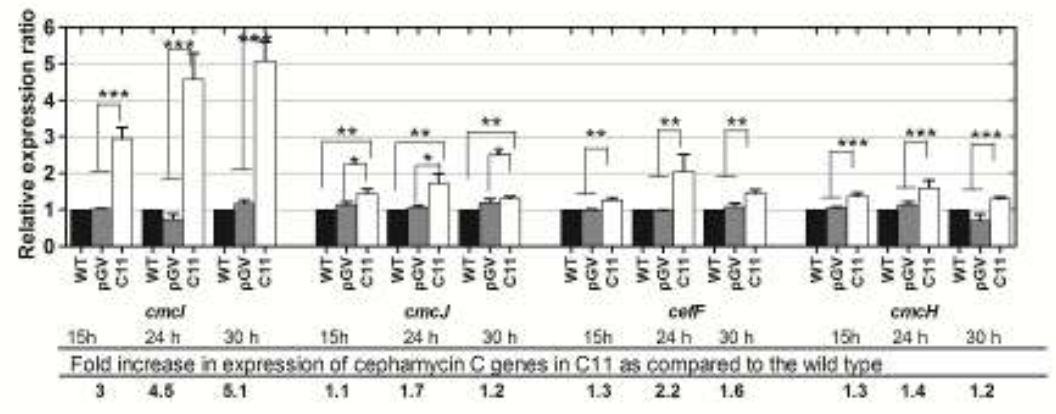


Figure 3. 43. Quantification of *cmcI*, *cmcJ, cefF* and *cmcH* gene expression in the wild type, recombinant pGV and C11 strains by qRT-PCR during the course of fermentation. Two independent qRT-PCR runs were performed and each run included two biological replicates and three technical replicates for each sample. Two way repeated measures ANOVA with Bonferroni post hoc test was performed on the normalized gene expression to check whether the expressions were statistically different between the wild type and C11, and pGV and C11 (* = $p < 0.05$, ** = $p < 0.01$, ***= $p < 0.001$).

Furthermore, *cmcJ* and *cefF* genes located after *cmcI* in the cluster were expressed at higher levels (1.7- and 2.2-fold, respectively) in the recombinant strain. A low level of increase (1.2 to 1.4 fold) in the expression of *cmcH* gene, encoding an enzyme that transfers a carbomoyl group to DAC during the final steps of the cephamycin C biosynthesis, was detected by qRT-PCR during the time course of fermentation.

cefE located next to the *cefD* gene in the cluster slightly increased its expression as a consequence of multicopy expression of *ccaR* in the cell and this increase had a significant value in statistical meaning ($p<0.05$). Though, *pcd*, *cmcT*, *pbpA* and *bla* genes were shown to be expressed at 2.5, 2.3, 1.8 and 1.4-times higher

levels in the recombinant than that of the wild type throughout the fermentation, corresponding to statictically different significance levels based on repeated measures of ANOVA. Since CmcT is a crucial protein that functions in the transport process of cephamycin C outside of the cell, overexpression of *ccaR* resulted in a 2.3-times higher level of *cmcT* expression in C11.

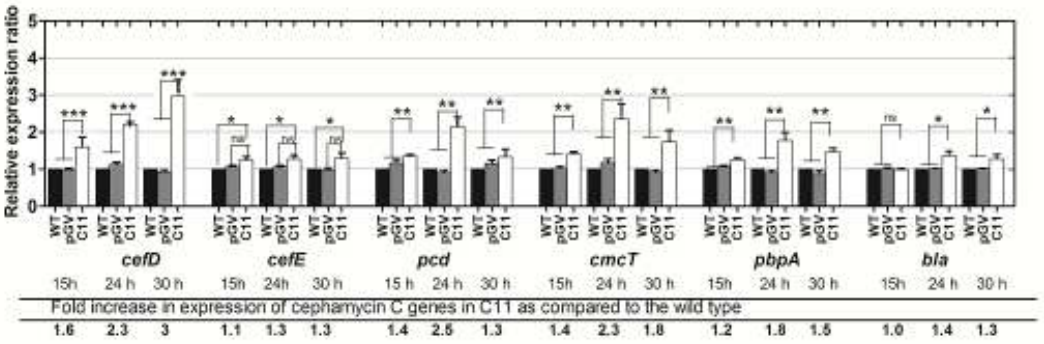
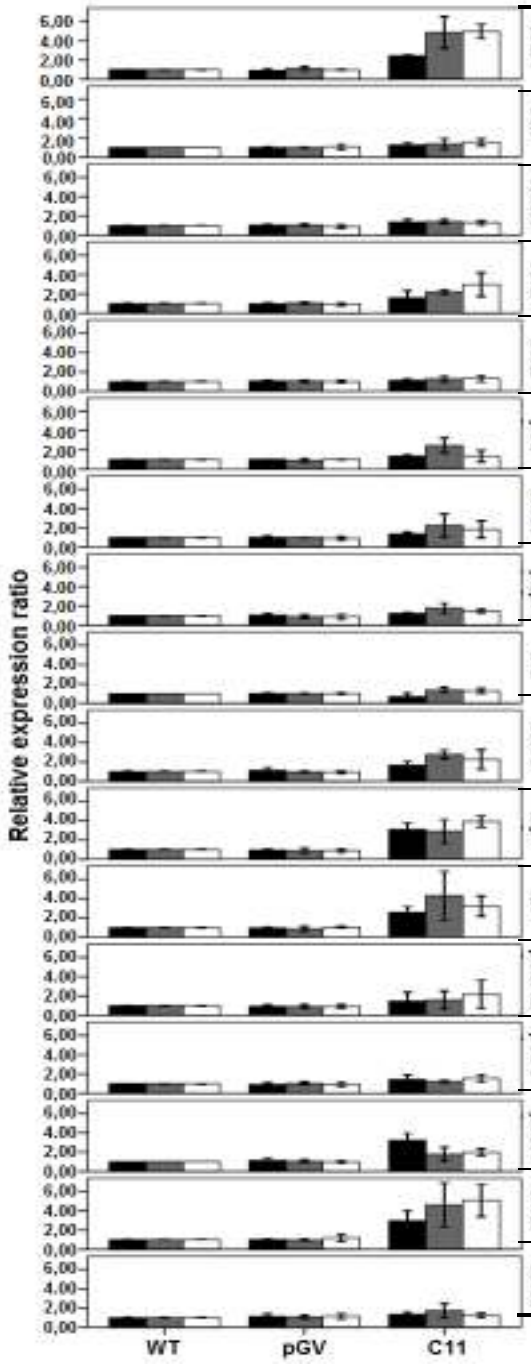


Figure 3. 44. Quantification of *cefD*, *cefE, pcd, cmcT, pbpA* and *bla* gene expression in the wild type, recombinant pGV and C11 strains by qRT-PCR during the course of fermentation. Two independent qRT-PCR runs were performed and each run included two biological replicates and three technical replicates for each sample. Two way repeated measures ANOVA with Bonferroni post hoc test was performed on the normalized gene expression to check whether the expressions were statistically different between the wild type and C11, and pGV and C11 (* = $p < 0.05$, ** = $p < 0.01$, ***= $p < 0.001$, ns= $p > 0.05$).

In Figure 3.45, a whole picture showing the time-dependent expression profile of cephamycin C gene cluster in the recombinant C11 strain by qRT-PCR is presented. To sum up, the expression of *ccaR*, *orf10*, *lat*, *cmcI*, *cefD, blp* and *pcbR* genes increased at a maximum of 5, 2.8, 4.3, 5.1, 3, 4.3 and 3.2 fold, respectively, in C11 as compared to the wild type strain and found to be statistically meaningful in ANOVA.

There are many reports indicating functional role of pathway-specific positive regulators on expression of secondary metabolite gene clusters. Jung *et al.* (2008) reported enhanced heterologous production of desosaminyl macrolides

and their hydroxylated derivatives by overexpression of *pikD* regulatory gene (integrated into the chromosome via pSET152 integration vector) in *S. venezuelae*. In the study of Park *et al*. (2009), stimulation of tautomycetin production by a global positive regulator, *afsR2*, via pathway-specific regulatory gene over-expression in *Streptomyces* sp. CK4412 was reported (by using a strong constitutive *ermE** promoter in an integrative expression vector). In a different study (Kitani *et al*., 2009), the positive effect of *aveR*, a LAL-family regulator, on the avermection production was demonstrated (*aveR*-constitutive expression plasmid was constructed for this purpose). In another study, Guo *et al* (2010) examined the effect of *aveR* on avermectin and oligomycin gene clusters. Besides, Malla *et al*. (2010) reported an improvement in doxorubicin productivity by overexpressing the regulatory genes (cloned to expression plasmid) in *S. peucetius*. In all of the above-mentioned studies, RT-PCR and qRT-PCR were the methods of choice to monitor the activities of secondary metabolite gene clusters.



Fold decrease in the expression level of C11

	15 h	24 h	30 h
ccaR	2.4	4.9	5
cefF	1.3	2.2	1.6
cmcH	1.4	1.3	1.2
cefD	1.6	2.3	3
cefE	1.1	1.3	1.3
pcd	1.4	2.5	1.3
cmcT	1.4	2.3	1.8
pbpA	1.2	1.8	1.5
bla	1	1.4	1.3
orf10	1.7	2.8	2.3
blp	3	3	4.3
lat	2.6	4.3	3.2
pcbAB	1.5	1.6	2.2
pcbC	1.4	1.2	1.5
pcbR	3.2	1.8	2
cmcI	3	4.5	5.1
cmcJ	1.1	1.7	1.2

Figure 3. 45. Quantification of time-dependent expression pattern of the cephamycin C cluster in the pGV and C11 strains as compared to the wild type by qRT-PCR. Error bars represent 95 % confidence intervals. Black bars represent T_{15}, grey bars represent T_{24} and white bars represent T_{30}.

3.5. Characterization of DNA-binding sequences of CcaR regulator on cephamycin C gene cluster

3.5.1. Cloning of *ccaR* gene to pET28a+ expression vector

ccaR gene was rescued from pG23 by *Not*I digestion (Figure 3.46). *Not*I digested *ccaR* was ligated to pET28a vector linearized with the same restriction enzyme and the ligation product was transferred to *E. coli* DH5α cells via transformation. Putative recombinant plasmids isolated from colonies grown on selective LA plates (containing kanamycin with a final concentration of 30 µg/ml) were screened. Recombination was confirmed by PCR (Figure 3.47). As the orientation of the *ccaR* gene within the MCS of the pET28a+ expression vector is very important for His Tag purification, digestion reactions were also performed to find the right clones (Figure 3.47).

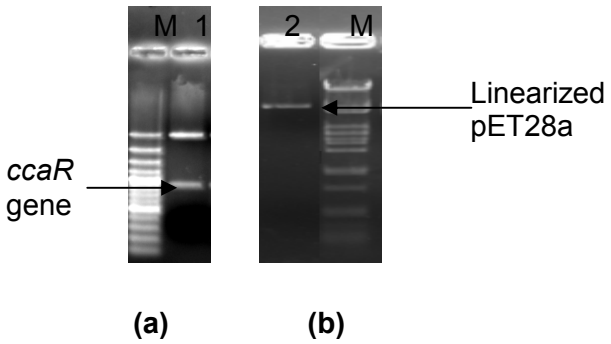


Figure 3. 46. Digestion of *ccaR* carrying pG23 **(a)** and pET28a+ **(b)** vectors with *Not*I enzyme. (a) M: 100 bp DNA ladder plus, 1: *Not*I digested pG23. (b) M: λ *Pst*I DNA ladder, 2: *Not*I digested pET28a+.

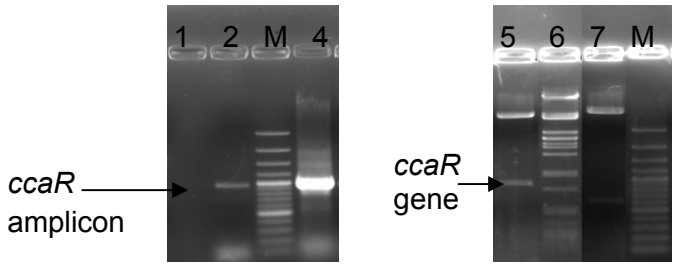


Figure 3. 47. Verification of cloning of *ccaR* to pET28a+ by PCR (a) digestion of the recombinant pET28a+ carrying ccaR to show the correct orientation of the *ccaR* in the MCS of the recombinant vector (b) 1: Negative control with no template DNA, 2: PCR product by using pET-C23 as template, M: 100 bp DNA ladder plus, 4: Positive control of PCR reaction in which genomic DNA of *S. clavuligerus* was used as template DNA. (b) 5: *Eco*RI digested pET-C23, 6: *λ/PstI* DNA ladder, 7: *Sac*I digested pET-C23, M: 100 bp DNA ladder plus.

The recombinant strain was designated as pET-C23 (Figure 3.48) and it was introduced to protease free *E. coli* BL21 cells for further expression analysis and purification of the CcaR protein.

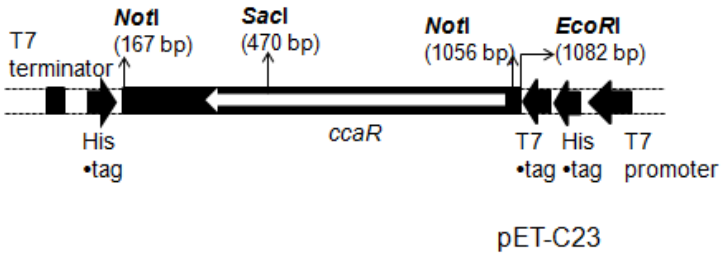


Figure 3. 48. The orientation of *ccaR* gene on the MCS of pET-C23.

3.5.2. Expression of CcaR in *E. coli* BL21 (DE3) cells and its purification by His Tag Affinity Chromatography

The expression of CcaR protein in *E. coli* BL21 cells was monitored by SDS-PAGE after 1 mM IPTG induction of the culture when reached to an OD_{600} value of 0.6, for 5 h at 37 °C and 200 rpm. Figure 3.49 shows the expression of CcaR protein in IPTG induced culture.

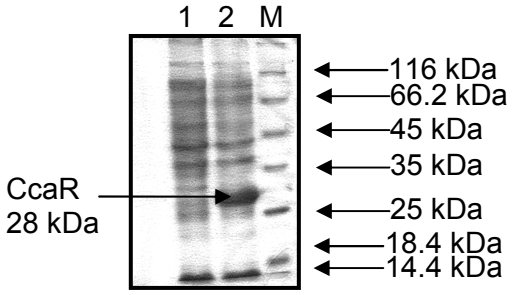


Figure 3. 49. CcaR expression in IPTG induced *E. coli* BL21 culture. M: Unstained protein molecular weight marker, 1: Control (uninduced sample) 2: Expressed CcaR protein.

CcaR protein was purified by using a Protino® Ni-TED 2000 purification column. The purification was performed under denaturing conditions. The LEW buffer used for resuspension of the pellet obtained after harvesting IPTG induced culture consisted of 8 M urea, 300 mM NaCl and 50 mM Na_2HPO_4, pH: 8. Elution buffer was prepared by adding 250 mM imidazole to the LEW buffer. However, the CcaR protein could not be eluted from the column with the standard protocol. Optimization studies were next conducted to obtain a partialy purified CcaR (Figure 3.50).

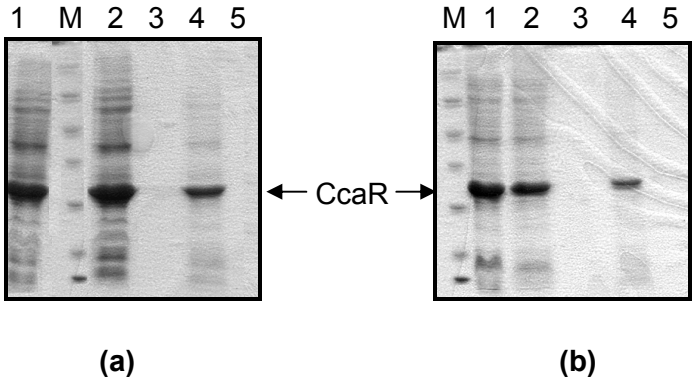


Figure 3. 50. Partial purification of CcaR by using His Tag affinity chromatography from the cell lysate containing 30 mM β-ME **(a)** or 1% Triton X-100 **(b)** with imidazole adjustment of the buffers used. M: Unstained protein molecular weight marker, 1: Induced sample, 2: Flow through sample, 3: Eluate, 4: Sample from 1st wash, 6: Sample from 2nd wash.

3.5.3. Electrophoretic Mobility Shift Assay (EMSA) to characterize DNA-binding sequences for CcaR on the cephamycin C cluster

EMSA experiments were conducted in order to support the data obtained from qRT-PCR analyses and explain regulation by CcaR of the cephamycin C cluster. The binding property of CcaR protein to the promoter regions of genes on the cephamycin C gene cluster were analyzed to observe possible interactions between them. Sequence specific oligonucleotides including upstream regions of related genes were designed and used in proof-reading PCR. The chromosomal DNA of *S. clavuligerus* NRRL3585 was used as template. A reaction buffer (Appendix D) supplemented with poly [d(I-C)] (1 µg/ µl) as competitive DNA, BSA (1 mg/ml) and 75 ng of promoter DNA and different concentrations of partially purified CcaR protein ranging from 1-2.5 µg were used in EMSA experiments. A separate gel retardation assay was performed by using *argR* probe as a negative control reaction due to the lack of any interaction of CcaR with *argR* promoter (Santamarta *et al*., 2011). As a result of EMSA represented here *ccaR*, *lat* promoters and *cefD-cmcI* bidirectional promoter on cephamycin C gene cluster

were found to be the targets for CcaR binding (Figure 3.51) confirming the findings reported previously (Kyung *et al*.,2001; Santamarta *et al*., 2002 and Santamarta *et al*., 2011).

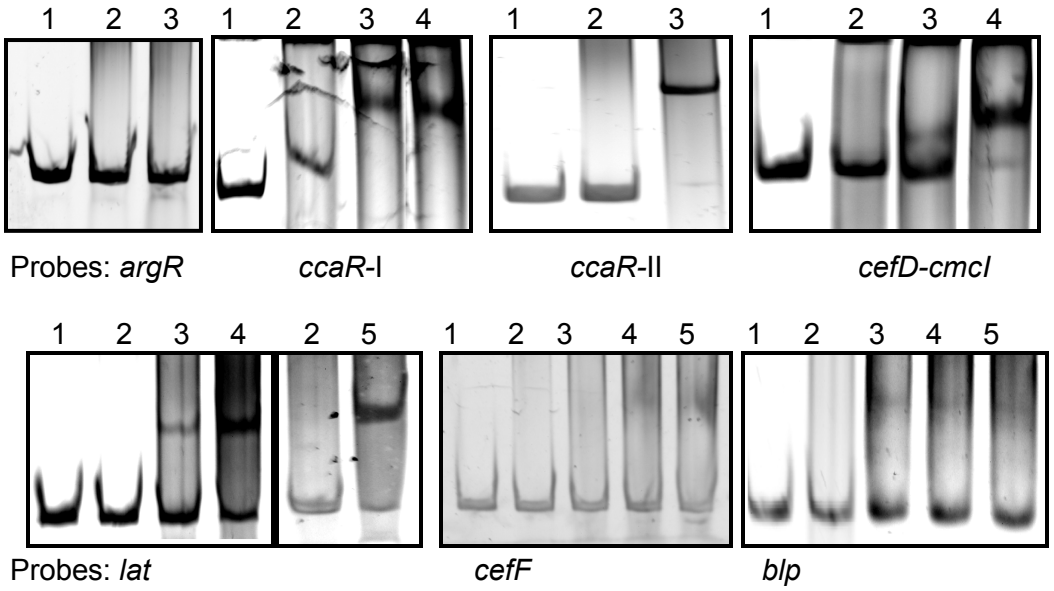


Figure 3. 51. EMSA analysis of CcaR protein with promoter regions (75 ng) on the cephamycin C gene cluster. 1: Free probe, 2: Reaction of probe with BSA (1 µg), 3: Reaction of probe with CcaR (1 µg), 4: Reaction of probe with CcaR (1.5 µg), 5: Reaction of probe with CcaR (2.5 µg).

In 2001, the interaction of CcaR with the *lat* promoter was reported by Kyung *et al*. in which crude cell extract containing His-tagged CcaR protein was used in DNA binding reaction. However, Santamarta *et al.* (2002) was unable to observe this binding property of the protein to the *lat* promoter in their experimental conditions when natural CcaR was used in the binding reaction. However, they showed the binding of CcaR to *cefD-cmcI* bidirectional promoter and its own promoter in the same study. Very recently, Santamarta *et al*. (2011) was able to observe the CcaR binding to the *lat* promoter by using recCcaR instead of the natural one in their EMSA experiments. In addition, they determined the interaction of CcaR protein with *cefF* promoter. However, the regulation of *cefF*

by CcaR was not clearly verified by gel retardation assay in our experimental conditions. Probably, refolding of recCcaR following dialysis process may not be proper for binding to *cefF* promoter. Unlike *cefF* promoter, a faint mobility shift of *blp* promoter with recCcaR was observed (Figure 3.51).

Alternative upstream regions of the genes on cephamycin C gene cluster were tested for binding of CcaR protein by following EMSA experiments. However, no additional binding of CcaR to other promoter regions on the cluster was detected (Figure 3.52).

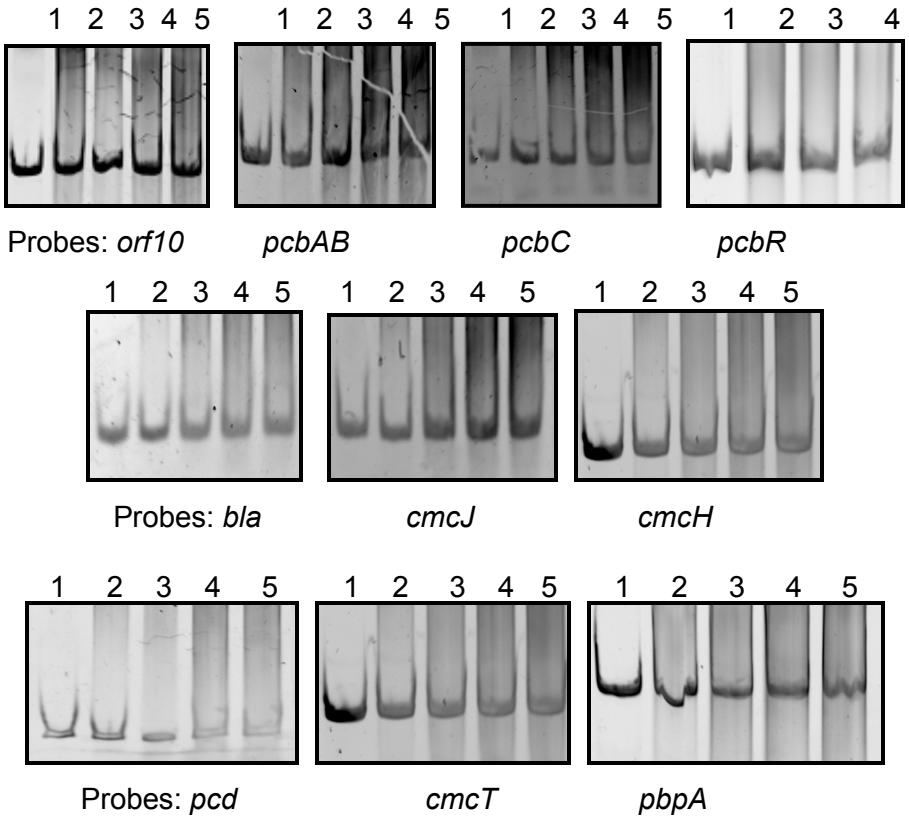


Figure 3. 52. EMSA analysis of CcaR protein with different promoters of cephamycin C gene cluster. 1: Free probe, 2: Reaction of probe with BSA (1 µg), 3: Reaction of probe with CcaR (1 µg), 4: Reaction of probe with CcaR (1.5 µg), 5: Reaction of probe with CcaR (2.5 µg).

CHAPTER 4

CONCLUSION

- *S. clavuligerus* NRRL3585 was engineered by expressing the *ccaR* gene either under a strong glycerol promoter in pSPG expression vector or with its own promoter in the integrative expression vector pSET152. The recombinant strains were designated as *S. clavuligerus* C11 and PC, respectively. In addition, the vector only recombinant strains were named as pGV and pTV. Next, comparison of cephamycin C and clavulanic acid production capacities of the recombinant *S. clavuligerus* strains grown in TSB and SA media by bioassay and/or HPLC analyses was performed.

- According to the specific cephamycin C titers obtained as a result of bioassay, the recombinant strain C11 produced 2- and 3-fold more antibiotic in TSB and SA, respectively, at T_{120}. However, the integration of an extra copy(ies) of p*ccaR* to the chromosome of *S. clavuligerus* resulted in less increase (1.2 to 2.1-fold) in specific cephamycin C yield in TSB.

- The clavulanic acid titer of C11 was found to be approximately 6-fold higher than that of the wild strain at T_{120} in both media. PC was capable of providing 2-3 times more clavulanic acid as compared to the wild type strain at 96^{th} h and 120^{th} h of incubation in TSB and SA, respectively.

- The effect of *ccaR* gene in the transcriptional regulation of cephamycin C gene cluster was examined by performing RT-PCR studies in which total

RNAs of *S. clavuligerus ccaR::aphII*, *S. clavuligerus* C11, the wild type strains *S. clavuligerus* NRRL3585 and ATCC27064, extracted from the samples harvested at 15, 24 and 30 h cultures, were used as template.

- In the absence of CcaR protein, a significantly low level of expression pattern was observed in the *ccaR*, *blp*, *lat*, *pcbAB*, *cefD*, *cefE*, *cmcI*, *cmcJ*, *cefF*, *cmcH* transcripts in the cell. An observable decrease was also determined in the transcriptional profiles of *pcbC* and *cmcT* which are biosynthetic and transporter genes, respectively. However, the possible difference in the expression patterns of the remaining genes, namely, *pcd*, *pbpA*, *bla*, *pcbR* in the *ccaR* mutant and the wild type was not clear enough to detect by RT-PCR.

- The intensity of the bands obtained from RT-PCR of *ccaR, lat, blp, cmcI, cefD*, *pcbR* genes in the recombinant *S. clavuligerus* C11 strain carrying multicopy *ccaR* seemed to be higher than that of the wild type strain.

- In order to detect co-transcriptional pattern of cephamycin C genes in the wild type *S. clavuligerus* strains, RT-PCR experiments were performed by using RNAs isolated from 24 h cultures as template. Bicistronic transcripts were obtained from the genes located adjacent to each other in the cluster. In contrast to the previous data with Northern blot analysis (Perez-Llarena *et al*., 1997), *ccaR* was found to be transcribed with *orf10*, in addition, *blp* was co-transcribed with *orf10* and *lat* genes in the cluster. Only *bla* and *pcbR* gave monocistronic transcripts.

- The role of CcaR activator in time-dependent transcriptional regulation of the cephamycin C biosynthetic gene cluster was further analysed by subsequent qRT-PCR experiments which allow simultaneous amplification and detection of DNA templates on the basis of flourescence emmission. The expression levels of the cephamycin C genes in the *ccaR* mutant strain and the recombinant C11 strain were quantified relative to that of the wild type *S. clavuligerus* strains. The expression of all genes on the cephamycin C gene cluster was repressed throughout the incubation in the *ccaR* mutant. The most drastic decline in the expression profile was

detected in the *lat* gene with a 2212-, 1718- and 1291-fold lower level of expression throughout the time course, it was followed by *cmcI, cefD, blp, cefF* genes that displayed as much as 1081-, 299-, 225-, 639-fold less expression in the mutant strain.

- Time-dependent expression analyses of *ccaR, lat, cmcI, cefD* and *blp* genes in the recombinant C11 strain gave data highly consistent with those obtained from qRT-PCR based expression analyses of them in the *ccaR* disrupted strain. In C11 strain, *ccaR* expression exerted a significant increase of about 4.8- and 5-fold at T_{24} and T_{30}. The significant expression levels at T_{24} and T_{30} was maintained by *lat, cmcI, cefD* and *blp* genes with 4.3-, 5.1-, 2.7- and 4.3-fold relative increases, respectively. Intriguingly, *pcbR* expression increased up to 3.2-fold at T_{15}.

- EMSA experiments were conducted in order to support the data obtained from qRT-PCR analyses regarding regulation by CcaR of cephamycin C gene cluster. As a result of EMSA, *ccaR*, *lat* promoters and *cefD-cmcI* bidirectional promoter on the cephamycin C gene cluster were found to be the targets for CcaR binding, confirming previous reports (Santamarta *et al*., 2011). On the other hand, the regulation of *cefF* by CcaR was not clarified by gel retardation assay in our experimental conditions. Among the alternative upstream regions of the genes on the cephamycin C gene cluster tested for binding of CcaR protein in EMSA experiments, only the promoter of *blp* showed a faint mobility shift with CcaR.

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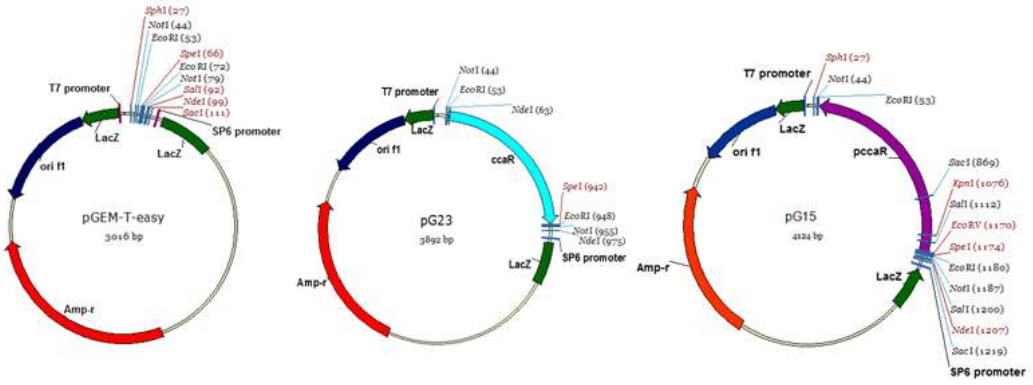
APPENDIX A

MAPS OF PLASMID VECTORS AND MARKERS

A.1. Plasmid vectors



(a) **(b)** **(c)**

Figure A.1. 1. Structure of plasmid vectors **(a)** pGEM-T easy: *E. coli* cloning vector, **(b)** pG23: *ccaR* carrying pGEM-Teasy vector, **(c)** pG15: Recombinant pGEM-Teasy that carries *ccaR* with its own promoter (p*ccaR*).

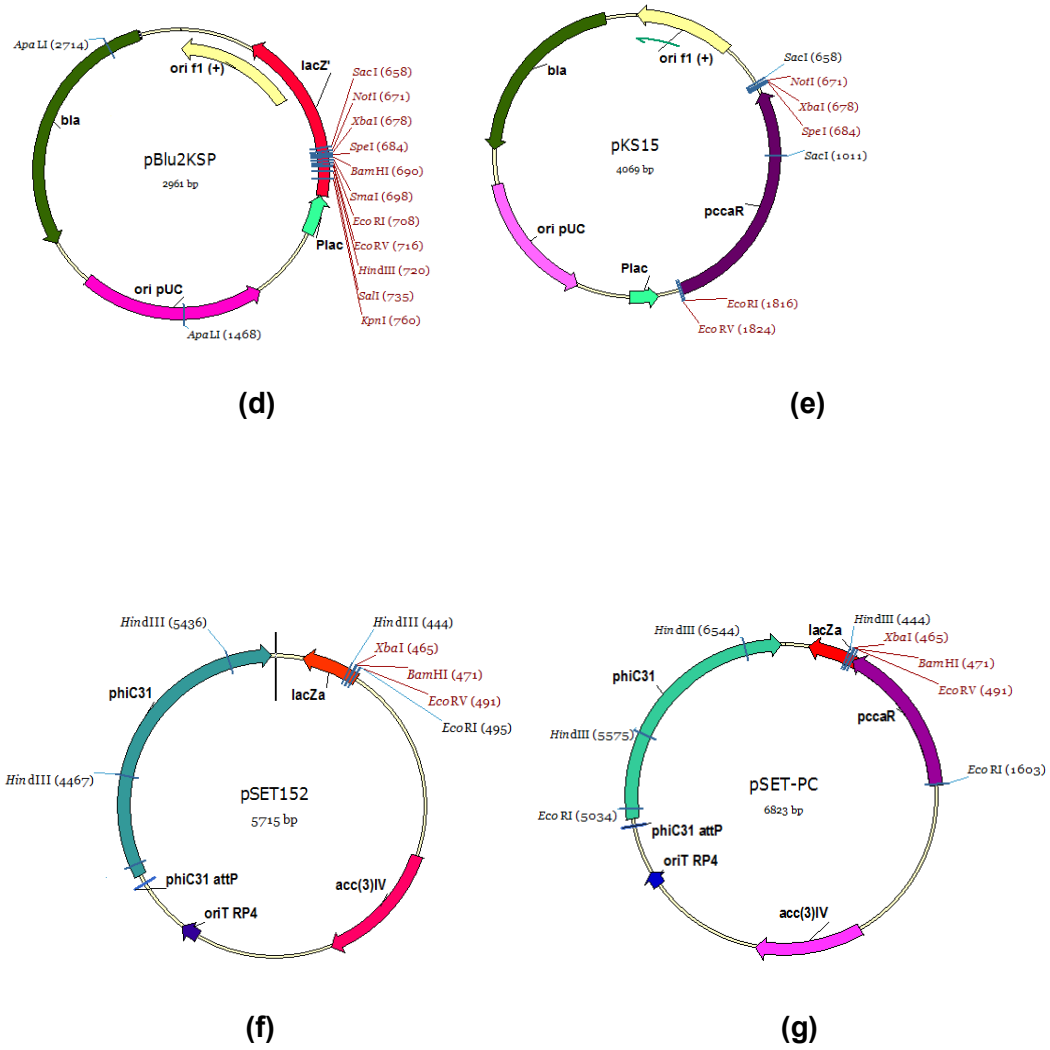


Figure A.1. 2. Structure of plasmid vectors **(d)** pBlueskriptIIKS+: *E. coli* cloning vector, **(e)** pKS15: Recombinant pGEM-T easy that carries p*ccaR*, **(f)** pSET152: *E. coli/Streptomyces* bifuncitional integration vector, **(g)** pSET-PC: p*ccaR* carrying pSET152.

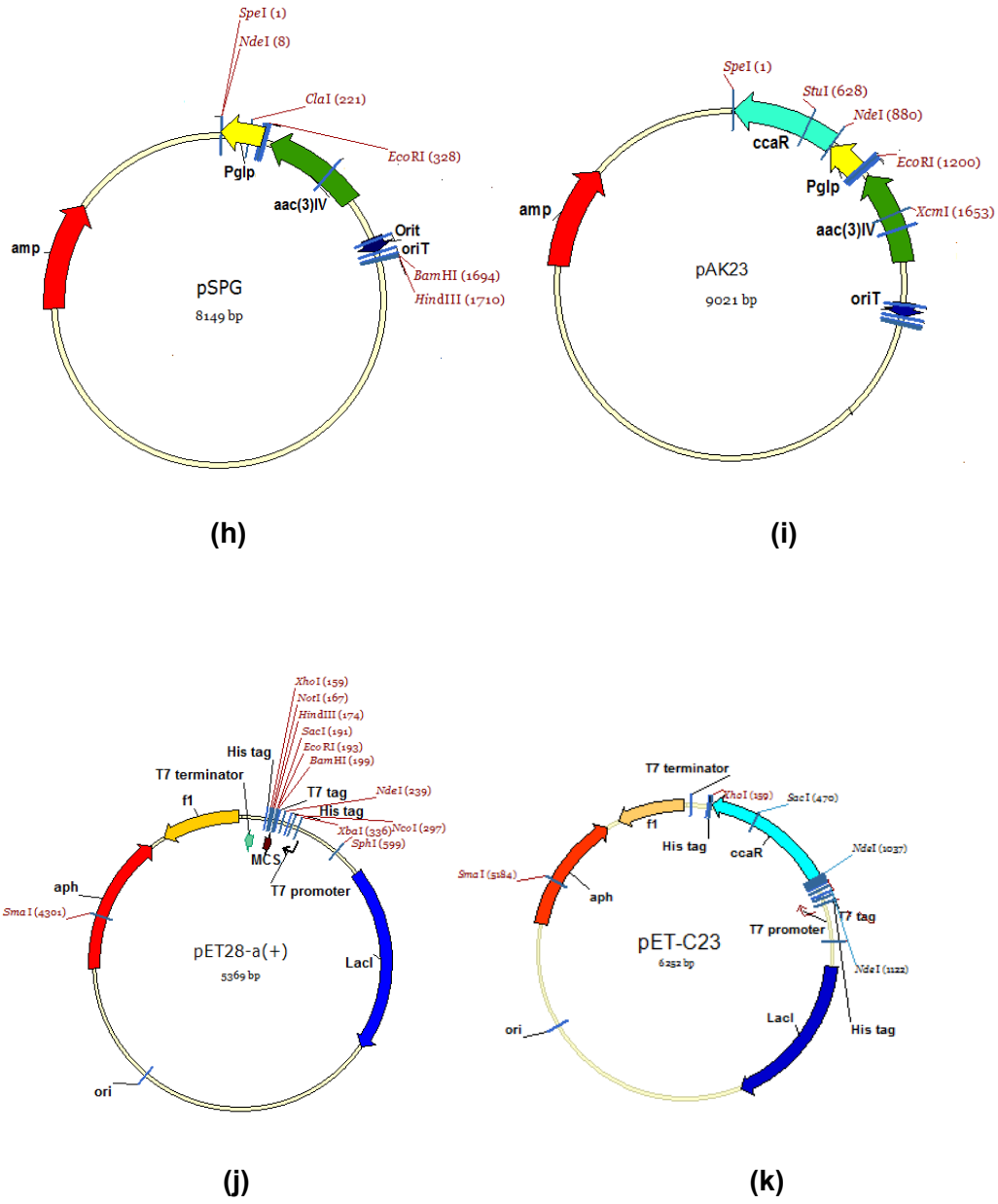
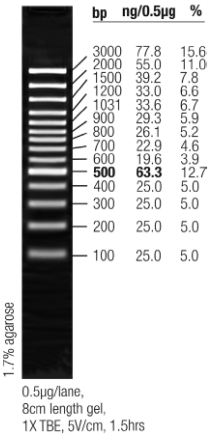
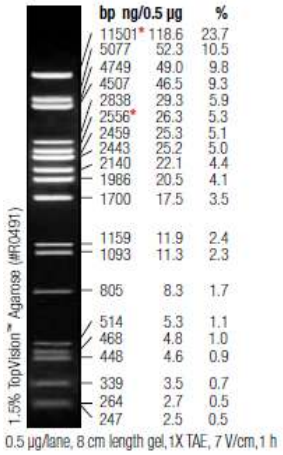


Figure A.1. 3. Structure of plasmid vectors **(h) pSPG:** *Streptomyces* expression vector, **(i) pAK23:** *ccaR* carrying pSPG, **(j) pET28a(+):** *E. coli* His-Tag expression vector, **(k) pET-C23:** *ccaR* carrying pET28a.

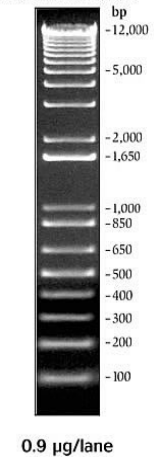
A.2. Markers



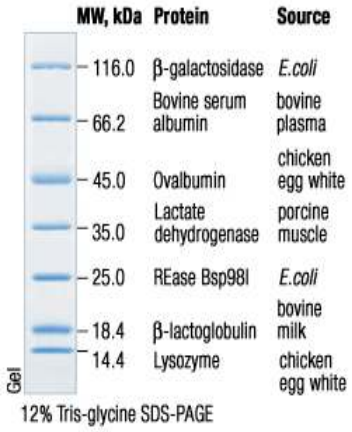
(a)



(b)



(c)



(d)

A.2. 1. Markers used in the present study. **(a)** GeneRuler 100 bp DNA ladder plus, **(b)** Lambda DNA/PstI marker 24, **(c)** 1 kb plus DNA ladder, **(d)** Unstained protein molecular weight marker.

APPENDIX B

NUCLEOTIDE SEQUENCE OF *CCAR* AND LIST OF PRIMERS

t tcccacagcc
ttcccaccca cccgtcccga ctcgccgtga agccccgggt tcttccgggt tcaccgaggc
tgtcccaaat cgtccatgcc ttgagggtcc cgctgcgtga tcgaaccgta acccttggaa
tttctgtgga ttaagcgtaa acatgggtgc cgacaccaag gattacgccg aagccatgtc
cacccctctc ggcgagggcg tggttccttc acaaggggga ccgcc
ggagggagcatATGaa cacctggaat
gatgtgacga tccggctcct ggggccggtg acactcgtga aaggttccgt accgataccc
atccgcgggc agcgacagcg gcgattcctc gcctcattag cgctgcgacc gggccaggtc
atctccaagg aagcgatcat cgaagactcc tgggacgggg agccaccact gaccgtttcg
ggccagttgc agacgtcggc ctggatgatc cggaccgcgc tggcggaggc ggggctgccc
cgcgacgccc tcggctccca cgaccgcggc tacgaactgc gcgtcctgcc ggactccatc
gacctcttcg tcttccggga ggccgtgcgc gccgtgcggg acctgcacgc acgcggtcag
caccaggagg cgtccgaacg gctcgacacg gcgctcgccc tgtggaaggg gcccgccttc
gcggatgtga cctccagtcg gctgcggctg cggggcgaga ccctggagga ggagcggacc
gccgcggtcg agctgcgcgc cctgatcgat gtcggcctcg gctactacgg ggacgcgatc
acccggctgt cggagctcgt cgatcacgac ccgttccgtg aggacctgta tgtgagcctg
atgaaggcct actacgcgga gggccgccag gccgacgcga tccaggtctt ccaccgcgcg
aaggacatcc tgcgggagca gatcggcatc agccccggcg agcggatgac aagggtcatg
caggccatcc tgcgtcagga cgagcaggtc ctgcgggtcg gtaccccggc ctgaaaccgc
gcgcgatacg ggaatgtttg tcgacgtttc cctgaaccaa cgctgaagaa acgttcttct
tctcacaacg gcgggga

Figure B. 1. Nucleotide sequence of *ccaR* with its own promoter (GeneBank accession number: AF073897) and the location of the primers.

Table B. 1. Sequences of primers used for *ccaR* amplification and for verification of recombination via PCR

Primer name	Sequence	Tm	Amplicon size (bp)	Annealing
ccaR_F	5'_ggagggag**catATG**aacacctggaatgatgtg_3'	80.6	876	62
ccaR_R	5'_tccccgccgttgtgagaaga_3'	72.4		
pccaR_F	5'_ttcccacagccttcccacccacccgtcccgactcgc_3'	89.4	1108	63
ccaR_R	5'_tccccgccgttgtgagaaga_3'	68.4		
pSET_F_Alp	5'_tagtcctgtcgggtttcgccac_3'	67.6	1602	57
ccaR_R	5'_tccccgccgttgtgagaaga_3'	68.4		
pSPG_R	5'_tgcctttgctcggttgatcc_3'	65.6	1409	55
ccaR_R	5'_tccccgccgttgtgagaaga_3'	68.4		

Table B. 2. Primers designed for RT-PCR and Q-RT-PCR analysis of the genes in the cephamycin C cluster

Gene name Accession #	Primer name	Primer sequence	Tm	size (bp)	Location
cefE M32324	CEFE-FORW CEFE_REV	5´ ACCTCGCCCGTCCCCACCA 3´ 5´ GTCCAGATCCGCTCGAAGTCACCG 3´	65.3 67.8	161	393-414; 556-534
cefF AF073896	CEFF_FOR CEFF_REV	5´ GGATGGCCCCGCACTACGACCTG 3´ 5´ CGCGCCGCACATCACGACG 3´	69.6 65.3	149	463-482; 638-617
cmcH AF073896	CMCH_FOR CMCH_REV	5´ CGTGGGCGCGTTCTACCTCGTCG 3´ 5´ CCATCAGCTTGCCCGCGTCGTTC 3´	69.6 67.8	164	393-414; 556-534
cmcJ AF073896	CMCJ_FOR CMCJ_REV	5´ CGCTTCAGCCGGTTCCAGAT 3´ 5´ CCATCAGCTTGCCCGCGTCGTTC 3´	61.4 62.1	176	463-482; 638-617
ccaR Z81324	CCAR_FQ2 CCAR_RQ2	5' CACCTGGAATGATGTGACGA 3' 5' GCTTCCTTGGAGATGACCTG 3'	60 62	144	6-25; 149-130
pcbR U877786	PCBR_FQ PCBR_RQ	5' GTGGGGCTCGGCTATTGGGGTTAC 3' 5' CCAGGCGCCGAGGAAGGTGT 3'	78 68	111	100-123; 210-191
pbpA AF001743	PBPA_FQ PBPA_RQ	5' GGCGCTGCTGCTCGTCAT 3' 5' TGTCGCGCAGGGTGAGGA 3'	60 60	116	21-38; 137-120
orf10 Z81324	ORF11_FQ ORF11_RQ	5' TGCGCTGGCTGGGGGTCTC 3' 5' CGCAGGGGCAGCCGTGAAT 3'	66 64	133	143-161; 275-257
pcd AF073895	PCD_FQ PCD_RQ	5' CAGCAATCAGTGGTACCGACGAGA 3' 5' CGCGCAGGCCGAACAGAT 3'	74 60	138	11-34; 148-131
cmcT AF073895	CMCT_FQ CMCT_RQ	5' GGCGGTCATGCTGCTGGTCT 3' 5' CGCTGTCGGGGGTGATGG 3'	66 62	137	1035-1054; 1171-1154
pcbC U877786	PCBC_FQ PCBC_RQ	5' CGCGGCTCGGGCTTCTTCTAC 3' 5' GGATCGCCAGGTCGTGCTTCTC 3'	70 72	111	100-123; 210-191
lat M64834	LAT_QF LAT_QR	5' TCACCCAGAAGCGGTATCTC 3' 5' CGTACGGCACCGAATAAAGA 3'	62 60	159	161-180; 319-290
cefD	CEFD_QF CEFD_QR	5' GCTGTGGCAGGCGCGGGAGAG 3' 5' GCAGTGACGACGCGACGAGGTTGA 3'	74 78	110	180-200; 289-266
pcbAB M64834	PCBAB_QF PCBAB_QR	5' CACGCTCGGCATCTGGAAGG 3' 5' GTGGTGCCGGTTGGTGACGA 3'	66 66	124	987-1006; 1110-1090
bla Z54190	BLA_QF BLA_QR	5' CATCTGGAGCGGCGGGTCA 3' 5' CGGATGGCGGCGTCACAGA 3'	64 64	116	340-358; 455-437
blp Z81324	BLP_QF BLP_QR	5' CGCAGGGCCACTTCTTCTTCAAC 3' 5' GCCTCCGTCATGCCCGTCTG 3'	72 68	130	230-252; 359-340
cmcI AF0073896	CMCI_QF CMCI_QR	5' CTGTTCCGGGGTCTGGGTGAG 3' 5' GGAGAAGTCCGAGTAGCCGAGGTC 3'	70 78	123	40-60; 123-100
hrdB M90411	HRDB_F HRDB_R	5´ CGCGGCATGCTCTTCCT 3´ 5´ AGGTGGCGTACGTGGAGAAC 3´	57.6 61.4	109	1225-1241; 1343-1314

Table B. 3. Primers designed for analysis of co-transcriptional profiles of genes in the cephamycin C cluster

Gene name Accession #	Primer name	Primer sequence	Tm	Amplicon size (bp)	Location
cefF-cmcH AF073897	FH_F FH_R	5' CGTCGAGCGTCTTCTTCCTGCG 3' 5' GGTTCCTGGGTGCCCTCGTATCC 3'	74.2 74.0	421	788-809; 151-173
cmcJ-cefF AF073897	JF_F JF_R	5' CTCAAGGACCGCGAGAACTACT 3' 5' CATCCCGGTCACGCACTC 3'	65.7 68.1	516	604-625; 73-90
cmcI-cmcJ AF073897	IJ_F IJ_R	5' GGCGGTCGACCATCTCCTC 3' 5' CCATCTCGAAGCCCATCCC 3'	69.5 69.0	446	513-531; 181-199
cmcT-pbpA AF073897 AJ001743	TPBPA_F TPBPA_R	5´ GCATCGCCAACGTCAACATC 3´ 5´ GCATGGTCAGGGTGAGGAAGTA 3´	59.4 62.1	1491	1352-1371; 2842-2821
cmcT-pbp2 AF073897 AJ001743	TPBP2_F TPBP2_R	5´ CTCGCCGTCGTTCTCACCCTCAC 3´ 5' GTCTCCGCCTCCTCAGCCTTTTCC 3'	67.8 67.8	823	1336-1358; 2158-2135
cefD-cefE M32324	CEFDE_FORW CEFDE_REV	5´ GGGCCGCACGCTCCTCAC 3´ 5´ ACTTCAGCTCGGTGTCGGTCAGA 3´	65.1 64.2	455	957-974; 1411-1389
cefE-pcd AJ001743	CEFEP_FORW CEFEP_REV	5´ TCGGGGGCAACTACGTGAACATCC 3´ 5´ CCAGCGGGTGCCAGGTCTCCA 3´	66.1 67.6	522	893-916; 1414-1394
pcd-cmcT AF073895	PCDCTA_FOR PCDCTA_REV	5´ CCATCCGGCTGAACAACGA 3´ 5´ GCAGCGCCACGGTCATCA 3´	58.8 60.5	819	1247-1265; 2065-2048
ccaR-orf11 Z81324	CCARORF11F CCARORF11R	5' CGGCGAGCGGATGACAAG 3' 5' CAGGACAGTGCGGCGGTGA 3'	71.3 74.0	1416	711-728; 2126-2108
cmcH-ccaR AF730897	CMCHCCAR_F CMCHCCAR_R	5´ ACGGCGACGGGTTCATCAA 3´ 5´ CAGGAGCCGGATCGTCACAT3´	58.8 61.4	1064	1454-2472; 2517-2498
pcbAB-pcbC	PCBABCF PCBABCR	5' GCTGTTCCTCATCGACCCGTA 3' 5' CCGTGGTTCGTGGCGTAGA 3'	68.4 69.5	559	317-297; 45-73
lat-pcbAB M64834	LATAB_F LATAB_R	5' GGCGGCGACAACCACCTCAG 3' 5' GCAACGCTCGCTCGACACTCC 3'	74.4 73.7	861	742-761;
blp-lat M64834	BLPLAT_F BLPLAT_R	5' GCGGCTGGCCACCTACAACAAGAC 3' 5' CGGCGCCGAGGCAAAGAAGG 3'	74.9 76.6	672	56-76
orf11-blp AF730897	OBLP_F OBLP_R	5' TGCTGACGGCAACGGGAAATC3' 5' CCGTCTGGGTCTTGTTGTAGGTGG 3'	73.3 71.3	898	315-338; 191-211
ccaR-orf AF730897	CCAO_F CCAO_R	5' GGCGAGCGGATGACAAGGG 3' 5' CCGGACGGGCCAACAACTG 3'	70.5 69.2	673	813-832; 424-447

Table B. 4. Sequences of primers used in EMSA

Primer name	Sequence	Probe size (bp)	Definition/Accession number
F1 R1	GAATAGCCGGTTCCTGAGC ATCATTCCAGGTGTTCATGG	950	Intergenic region between *cmcH* and *ccaR*/ AF730897
F2 R2	GGTAGGGAGGGGAGAGTCC TCGTCACATCATTCCAGGTG	300	Upstream of *ccaR* gene/AF730897
F4 R4	GTGGTGAGAGCAGGTGACG ACGGCGGACGGGCGGACA	109	Upstream of *bla* gene/Z54190
F5 R5	GGCGATGACGAGCAGCAG GGCGTCTGCAACCAGGTG	1209	Upstream of *pbpA* gene/AJ001743/AF073895
F6 R6	GTCCTCGCAGCGGAACTC GCGTGGACTTCTCCCAGTAG	496	Upstream of *cmcT* gene/ AJ001743/AF073895
F7 R7	CTGATTGCTGCTGTGACCAT CGCACCTCCAGTGTGTTCT	206	Upstream of *pcd* gene/ AJ001743
F8 R8	CCTTGTCCCTCAGACACCTG TCTCGGCGAACTTCTACACC	243	Upstream of *cefE* gene/M32324
F9 R9	CGGTGTTGAGGTTGACGAC TTGCCCTCTTCCTTGAGTGT	859	Intergenic region between *cefD* and *cmcI*/ AH006362/M32324
F10 R10	CACGGCCCCGAGGACGAC GAGGTAGAAGACCCCCATCC	402	Upstream of *cefF* gene/ AH006362
F11 R11	GCACGTCGAGCGTCTTCT GTCATGTCCCGGCTTGAAT	290	Upstream of *cmcH* gene/AF073897
F12 R12	GGATGACAAGGGTCATGCAG CAGGGCCCCTTTGAGTACC	431	Upstream of *orf11* gene/Z81324/AF073897
F13 R13	ACCTGGGTGTGCTGGATCT CATGTGAAGGAACCCCTTGT	387	Upstream of *blp* gene/ Z81324/AF073897
F14 R14	CTGAGCAACAGCTCGTCA CCCATGGGTGAGAACTCCT	212	Upstream of *lat* gene/M64834
F15 R15	TCATGTACACGGAGCACCAG GACATCATTCGTGGGCTCTC	309	Upstream of *pcbAB* gene/ M64834/U12015
F17 F17	GCCATAGTTGTCGGAATGC CTACTCCAATCGCGGTGAAC	228	Upstream of *pcbC* gene/Petrich *et al.*, 1992
F18 R18	GCTGTTCCTCATCGACCCGTA CCGTGGTTCGTGGCGTAGA	559	Upstream of *pcbR* gene/U87786

APPENDIX C

COMPOSITION AND PREPARATION OF CULTURE MEDIA

C.1. Liquid Media

Luria Broth (LB)	**g/l**
Luria Broth	25

Sterilized at 121 °C for 15 min

Trypticase Soy Broth (TSB)	**g/l**
Trypticase Soy Broth	30

Sterilized at 121 °C for 15 min

Starch Asparagine (SA) Medium (Aharonowitz and Demain, 1979)

600 ml of dH_2O was boiled and the following components were added to the water by continuous stirring:

	g/l
Starch	10
MOPS	21
K_2HPO_4	4.4

After complete solubilization of the components, the solution was cooled to RT, pH is adjusted to 6.8 and volume is completed to 800 ml.

Sterilized at 121 °C for 15 min

After sterilization, the following sterile components were added to the medium:

	ml/l
L-Asparagine (10 g/l)	200
$MgSO_4$. 7 H_2O (0.6 g/ml)	2
Trace element solution*	1

***Trace element solution**	g/l
$FeSO_4.7H_2O$	1
$MnCl_2.4H_2O$	1
$ZnSO_4.7H_2O$	1
$CaCl_2.3H_2O$	1.3

C.2. Solid Media

LB Agar	g/l
Luria Broth	25
Agar	15

Sterilized at 121 °C for 15 min

TSA	g/l
Tryptic Soy Broth	30
Agar	20

Sterilized at 121°C for 15 min

Mannitol Soya flour (MS) Agar (Hobbs *et al*., 1989)	g/l
Soya flour	20
Agar	20
Mannitol	20

Sterilized at 121 °C for 15 min (autoclaved twice)

APPENDIX D

BUFFERS AND SOLUTIONS

D.1. Plasmid and Chromosomal DNA Isolation

SET buffer

NaCl	75 mM
EDTA (pH 8.0)	25 mM
Tris-HCl (pH 7.5)	20 mM

TE buffer

Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1mM

STE buffer

Sucrose	0.3 M
Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	25 mM

Lysis Solution

NaOH	0.3 M
SDS	2 %

Phenol-chloroform solution (water saturated) (Hintermann, 1981)

Phenol	500 g
Chloroform	500 ml
Distilled water	400 ml

The solution was stored at RT, protected from light.

D.2. Agarose Gel Electrophoresis

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

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

Loading Buffer (6X)

Bromophenol blue	0.25 %
Xylene cyanol FF	0.25 %
Sucrose in water	40 %

Ethidium Bromide Solution

Ethidium Bromide (10 mg/ml)	100 µl/l
TAE Buffer (1X)	1 l

D.3. Selection of Recombinant Colonies

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

X-Gal	20 mg
Dimethylformamide	1 ml

The solution was stored at -20 °C protected from light.

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
dH_2O	1 ml

The solution was filter sterilized and stored at -20 °C.

D.4. Buffers used for *E. coli* competent cell preparation

Buffer 1

Potassium acetate	30 mM
$RuCl_2$	100 mM
$CaCl_2$	10 mM
Glycerol (87 %)	8.6 ml

The volume was completed to 50 ml with dH_2O and filter sterilized.

Buffer 2

MOPS	10 mM
$RuCl_2$	10 mM
$CaCl_2$	75 mM
Glycerol (87 %)	8.6 ml

Adjusted pH 6.5 with 0.2 M KOH. The volume was completed to 50 ml with dH_2O and filter sterilized.

D.5. DNA quantification for growth determination

Diphenylamine Reagent

Diphenylamine	1.5 g
Glacial acetic acid	100 ml

Cover with aliminium foil to protect from light.

Add the followings just prior to use,

Concentrated H_2SO_4	1.5 ml/100ml
Acetaldehyde (1.6 %)	0.1 ml/20 ml

Aqueous Acetaldehyde (1.6 %)

Acetaldehyde	1 ml
dH_2O	49 ml

1 N $HClO_4$

$HClO_4$ (80 %)	10.87ml/100ml
$HClO_4$ (60 %)	16.74ml/100ml

D.6. Bioassay/HPLC Analysis

1 M MOPS	**g/l**
MOPS	20.9 g
dH_2O	1000 ml

pH is adjusted to 6.8 with NaOH, filtered and protected from light.

Sodium acetate solution	**g/l**
Sodium acetate	4.1

pH is adjusted to 6.0 with acetic acid, filtered.

Sodium dihydrogen phosphate solution	**g/l**
$NaH_2PO_4.H_2O$	15

pH is adjusted to 4.0 with 50 % phosphoric acid, filtered.

Mobil phase

Sodium dihydrogen phosphate solution (pH 4.0)	95 %
Methanol (HPLC grade)	5 %

D.7. Protein Purification

LEW Buffer

Urea	8 M
NaH_2PO_4	50 mM
NaCl	300 mM

pH 8.0

Equilibration Buffer

LEW Buffer supplemented with 10 mM imidazole, pH 8.0

Washing Buffer

LEW Buffer supplemented with 20 mM imidazole, pH 8.0

Elution Buffer

LEW Buffer supplemented with 250 mM imidazole, pH 8.0

Dialysis Buffer

Urea	2.5 M
NaH_2PO_4	50 mM
NaCl	300 mM

pH 8.0

D.8. Polyacrylamide Gel Electrophoresis

Bradford Reagent (5X)

Coomassie Brillant Blue G250 100 mg

dissolved in 50 ml of ethanol (95 %). The solution was mixed with 100 ml of 85 % phosphoric acid made up to 1 l with dH_2O. The reagent was filtered through Watman No. 1 filter paper.

Tris HCl (1.5 M)

Tris base	54.45 g
dH_2O	150 ml

pH is adjusted to 8.8 with HCl, completed to 300 ml with dH_2O and stored at 4 °C.

Tris HCl (0.5 M)

Tris base 6 g

dH_2O	60 ml

pH is adjusted to 6.8 with HCl, completed to 100 ml with dH_2O and stored at 4 °C.

Acrylamide/Bis (30:1)

Acrylamide	146 g
N.N'-Methylene-bis Acrylamide	4 g
dH_2O	500 ml

Filtered and stored at 4 °C. Protected form light.

Coomassie R-250 Staining

Coomassie blue R-250	0.25 g
Methanol	125 ml
Glacial Acetic acid	25 ml
dH_2O	100 ml

Fixation Solution

Ethanol	40 ml
Glacial Acetic Acid	10 ml
dH_2O	50 ml

Destaining Solution

Methanol	100 ml
Glacial Acetic acid	100 ml
dH_2O	800 ml

Running Buffer (5X) (For SDS-PAGE)

Tris base	15 g
Glycine	72 g
SDS	5 g
dH_2O	1 l

Stored at 4 °C.

Sample Buffer (4X)

dH_2O	3 ml
Tris HCl (0.5 M)	1 ml
Glycerol	1.6 ml
SDS (10 %)	0.4 ml
β-mercaptoethanol	0.4 ml
Bromophenol blue (0.5%, w/v)	0.4 ml

D.9. EMSA

Binding Buffer

HEPES	80 mM
KCl	200 mM
$MgCl_2$	20 mM
$MnCl_2$	0.5 mM
Glycerol	40 %
Tris-HCl (pH 7.5)	16 mM
1 µg poly[dI-dC] or Salmon Sperm DNA (1µg/µl)	

Sample Buffer

TBE (0.25X) (For Native PAGE)	60 %
Glycerol	40 %
Bromophenol Blue (w/v)	0.2 %

Tris-Borate Buffer (TBE) (1 X) (For Native PAGE) (Sambrook *et al*., 1989)

Tris Base	90 mM
Boric Acid	90 mM
EDTA (0.5 M, pH 8.3)	4 ml/l
Distilled water added to	1000 ml
pH 8.0-8.3	

D.10. PREPARATION OF ANTIBIOTIC STOCKS

	Stok Concentration	Final Concentration
Ampicillin	100 mg/ ml dH_2O	100 µg/ml
Kanamycin	50 mg/ml dH_2O	25 or 50 µg/ml
Chloramphenicol	25 mg/ml ethanol	25 µg/ml
Penicillin G	40 mg/ml dH_2O	40 µg/ml
Apramycin	50 mg/ml dH_2O	50 µg/ml

APPENDIX E

CHEMICALS AND THEIR SUPPLIERS

E.1. Chemicals

Acetaldehyde	Sigma
Acrylamide	Sigma
Agar	Merck
Agarose	Prona
Ammonium persulphate	AppliChem
Ampicillin	Sigma
Apramycin	Sigma
Boric acid	Merck
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
β-mercaptoethanol	Sigma
$CaCl_2.2H_2O$	Merck
Cephalosporin C	Sigma
Chloroform	AppliChem
Chloramphenicol	Sigma
Coomassie Brillant Blue G-250	Merck
Coomassie Brillant Blue R-250	Sigma
DEPC	AppliChem
Dimethylformamide	Merck
Diphenylamine	Sigma

DMSO	Sigma
EDTA	Sigma
Ethanol	Botafarma
Ethidium bromide	Sigma
$FeSO_4.7H_2O$	Merck
Glacial Acetic Acid	Merck
Glycerol	Merck
Glycine	Merck
HEPES	Merck
Herring sperm DNA	Sigma
HCl	Sigma
$HClO_4$	Merck
H_2SO_4	Merck
Imidazole	Merck
IPTG	Sigma
Isoamylalcohol	AppliChem
Isopropanol	Merck
K-acetate	Merck
Kanamycin	Sigma
KCl	Merck
K_2HPO_4	Meck
KH_2PO_4	Merck
L-Asparagine	Sigma
Luria Broth	Sigma
Mannitol	Merck
Methanol (HPLC grade)	Sigma
$MgCl_2$	Merck
$MgSO_4.7H_2O$	Merck
$MnCl_2.4H_2O$	Merck
MOPS	Sigma
Na-acetate	Merck
NaCl	Sigma
NaH_2PO_4	Merck
Nalidixic acid	Sigma

NaOH	Merck
NN' Methylene Bisacrylamide	Sigma
Orto-phosphoric acid	Merck
Penicillin G	Sigma
Phenol	Amresco
Phenol (water-saturated)	AppliChem
Phenol-choloroform	Amresco
Poly[d(I-c)]	Roche
Random primers	Invitrogen
$RuCl_2$	Merck
Salmon Sperm DNA	Sigma
SDS	Merck
Soybean flour	Commercial
Starch	Merck
Sucrose	Merck
TEMED	AppliChem
Tris-HCl	Merck
Tryptic Soy Broth	Oxoid
Urea	Sigma
X-Gal	Fermentas
Xylene cyanol FF	Sigma
$ZnSO_4.7H_2O$	Merck

E.2. Enzymes

Lysozyme	Sigma
Proteinase K	Sigma
T4 DNA Ligase	Promega
*EcoR*I	Roche
*Hind*III	Roche
*Xba*I	Roche
*Not*I	Roche
*Sac*I	Roche
*Nde*I	Fermentas
*Spe*I	Fermentas

Go Taq DNA polymerase	Promega
Taq polymerase	Fermentas
Phusion DNA polymerase	NEB
T4 ligase	Fermentas
T4 ligase of pGEM-T easy	Promega

E.3. Size Markers

*Pst*I digested /Lambda DNA ladder	Fermentas
O'GeneRuler 100 bp DNA ladder	Fermentas
1 kb DNA plus ladder	Invitrogen
Lambda DNA/*EcoR*I-*Hind*III	Fermentas

E.4. Kits

Plasmid isolation Kit (Midi)	Qiagen
Plasmid Isolation Kit (Mini)	Fermentas
Gel Elution Kit	GeneMark
RNeasy mini isolation kit	Qiagen
RNA protect solution	Qiagen
RNAase Free DNAase set	Qiagen
DNA-free™ kit	Ambion
Phase lock gel heavy tubes	5-Prime
Superscript™ One-Step RT-PCR kit	Invitrogen
SuperScript™ III Reverse Transcriptase kit	Invitrogen
SYBR Green I Ex-Taq Mix	Takara
SYBR Green I Nucleic Acid Gel Stain	Invitrogen
LightCyler® Capillaries	Roche
Protino®Ni-TED 2000 packed columns	Macherey-Nagel

CURRICULUM VITAE

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EDUCATION

Degree	Institution	Year of Graduation
M. Sc.	METU, Biology	2005
B. Sc.	Ondokuz Mayıs University, Biology Education	2002

WORK EXPERIENCE

Year	Place	Enrollment
2002-2011	METU, Biology	Research Assistant

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

Thesis:

M. Sc. Thesis: Coleoptera-specific (Cry3Aa) delta-endotoxin biosynthesis by a local isolate of *Bt* subsp. *tenebrionis*, gene cloning and characterization

Research Articles:

1. Kurt A, Özcengiz, G (2011) Increased production of Coleoptera-specific delta-endotoxin by homologous expression of *cry*3Aa11 in *Bacillus thuringiensis* Mm2. *Turk. J. Biol.* **35**: 585-592.

2. Santamarta I, López-García MT, Kurt A, Nárdiz N, Álvarez-Álvarez R, Pérez-Redondo R, R. Pérez-Redondo, Martín JF, Liras P (2011) Characterization of DNA-binding sequences for CcaR in the cephamycin–clavulanic acid supercluster of *Streptomyces clavuligerus. Mol. Microbiol*. **81** (4): 968-981.

3. Kurt A, Özkan M, Özcengiz G (2005) Inorganic phosphate has a crucial effect on Cry3Aa delta-endotoxin production. *Lett. Appl. Microbiol.* **41**: 303-308.

4. Kurt A, Özkan M, Sezen K, Demirbağ Z, Özcengiz G (2005) Cry3Aa11: a new rank of Cry3Aa δ-endotoxin from a local isolate of *Bacillus thuringiensis*. *Biotechnol. Lett.* **27**: 1117-1121.

International Congress Presentations:

Poster presentations:

1. Mutlu A, Kurt A, Özcengiz G. (2011) Increasing clavulanic acid production both in wild type and industrial *Streptomyces clavuligerus* strains by amplification of *claR* gene. Horizons in Molecular Biology, 8th International PhD Student Symposium (14-17 September, 2011, Göttingen, Germany). Abstract Book, p93.

2. Kurt A, Liras P, Özcengiz G. (2011) Monitoring the regulatory effect of *ccaR* gene on the cephamycin C gene cluster by qPCR analyses. Presented in "4th FEMS Congress of European Microbiologists" (June 26-30, 2011, Genova, SWITZERLAND). In CD.

3. Kurt A, Vanli G, Adakli Z, Yegen Y, Durak İ, Özcengiz G. (2009) Metabolic engineering for enhanced clavulanic acid production by an industrial strain of

Streptomyces clavuligerus. Biology of Streptomycetes (October, 7-11, 2009 Münster, Germany) Abstract Book, p. 161.

4. Kurt A, Vanli G, Adakli Z, Yegen Y, Durak İ, Liras P, Özcengiz G. (2009) Integration of a regulatory gene into the chromosome of an industrial *Streptomyces clavuligerus* strain for enhanced clavulanic acid biosynthesis. BIOTECH METU 2009, International Symposium on Biotechnology: Developments and Trends (September, 27-30, 2009 Ankara, Turkey) Abstract Book, p73.

5. Kurt A, Santamarta I, Liras P, Martin JF, Özcengiz G. (2009) Transcriptional analysis of the effect of *ccaR* regulatory gene on cephamycin C biosynthesis by *Streptomyces clavuligerus*. BIOTECH METU 2009, International Symposium on Biotechnology: Developments and Trends (September, 27-30, 2009 Ankara, Turkey) Abstract Book, p74.

6. Okay S, Ünsaldı E, Taşkın B, Kurt A, Piret J, Liras P, Özcengiz G. (2009) Metabolic engineering of aspartate pathway for increased production of cephamycin C in *Streptomyces clavuligerus*. BIOTECH METU 2009, International Symposium on Biotechnology: Developments and Trends (September, 27-30, 2009 Ankara, Turkey) Abstract Book, p99.

7. Kurt A, Özcengiz G. (2008) Homologous expression of *cry*3Aa11 in *Bacillus thuringiensis* Mm2 increases the production of Coleoptera-specific delta-endotoxin. IUMS-XII. International Congress of Bacteriology and Applied Microbiology (5-9 August 2008, İstanbul). Abstract Book, p. 77.

8. Kurt A, Özkan M, Özcengiz G. (2006) The effects of inorganic phosphate and sugar concentrations on Cry3Aa11 biosynthesis by *Bacillus thuringiensis* Mm2. 2nd FEMS Congress of European Microbiologists (July 4-8, 2006 Madrid, Spain) Abstract Book, p121.

National Congress Presentations:

Oral presentations:

1. Kurt A, Özcengiz G. (2007) *cry3Aa11* Geninin Çoklu Kopyasının *Bacillus thuringiensis* Mm2' nin Toksin Üretimi Üzerine Etkisi. XV. Ulusal Biyoteknoloji Kongresi (28-31 Ekim 2007, Antalya). Bildiri Kitabı, sf. 313-316.

2. Kurt A, Ozkan M, Ozcengiz G. (2005) *Bacillus thuringiensis tenebrionis* Mm2' ya ait *cry*3Aa11 geninin klonlanması, karakterizasyonu ve *Bacillus subtilis* 168' de

ifade edilmesi. XIV. Ulusal Biyoteknoloji Kongresi (31 Ağustos-2 Eylül 2005, Eskişehir) Bildiri ve Poster Özetleri Kitabı, sf. 78-81.

Poster presentations:

1. Kurt A., Özkan M. ve Özcengiz G. (2005) *Bacillus thuringiensis* subsp. *tenebrionis*' in yerel bir izolatında kınkanatlılara karşı aktif (Cry3Aa11) delta-endotoksin biyosentezinin optimizasyonu. XIV. Ulusal Biyoteknoloji Kongresi (31 Ağustos-2 Eylül 2005, Eskişehir) Bildiri ve Poster Özetleri Kitabı sf. 565.

2. Özcan O, Kurt A, Aras A, Özkan M, Özcengiz G. (2005) Yenilenebilir atıkların Lepidoptera-, Diptera- ve Kınakanatlılar- özgü kristal protein üretimi için substrat olarak kullanılması ve üretime etkileri. XIV. Ulusal Biyoteknoloji Kongresi (31 Ağustos-2 Eylül 2005, Eskişehir) Bildiri ve Poster Özetleri Kitabı sf. 310-316.

PROJECTS:

1. TBAG 109T962 (2010-2012): Transcriptional and translational analysis of the effect of *ccaR* regulatory gene in Cephamycin C biosynthesis by *Streptomyces clavuligerus* (Kurt A, Özcengiz G.).

2. TEYDEB-3080871 (1501-Sanayi Ar-Ge Projeleri Destekleme Programı) **(2008-2010):** Enhancement of clavulanic acid production by using recombinant DNA techniques (Kurt A, Vanlı G, Özcengiz G, Durak İ.).

3. TBAG 106T535 (2007-2009): Genome and proteome wide analysis of transcriptional regulator *yvfI* Gene activity and dipeptide antibiotics bacilysin biosynthesis in *B.subtilis* (Karatas A, Özcengiz G, Köroğlu TE, İrigül Ö, Kurt A, Öğülür İ).

4. TBAG-2413 (104T023) (2004-2006): Improvement of larvicidal activity and spectrum of *Bacillus thuringiensis* subspecies *tenebrionis* by genetic manipulations (Kurt A, Okay S, Özkan M, Özcengiz, G.).

5. METU-08-11-DPT.2002K120510-BTEK-11 (2003-2005): Studies on Coleoptera-specific (*cry*3Aa) delta endotoxin production by a local isolate of *Bacillus thrungiensis* subspecies *tenebrionis* (Kurt A, Özkan M, Özcengiz G.).

RESEARCH ACTIVITY:

Studies on transcriptional analysis of cephamycin C gene cluster in a *ccaR* minus mutant of *S. clavuligerus*, Institute of Biotechnology (INBIOTEC) Leon, Spain; for one year starting from January 2008 (supervised by Dr. Paloma Liras).

AWARDS/GRANTS:

1. B. Sc. education First Honor degree in Biology Education, Third Honor degree in Education Faculty, Ondokuz Mayıs University, June 2001

2. TÜBİTAK International Scientific Publication Awards, 2005, 2011

3. METU Scientific Publication Award, 2005

4. ÖYP Grant for one year research in INBIOTEC, Leon Spain, 2008