THE EFFECTS OF TWELVE QUORUM-SENSING GENE PRODUCTS ON THE EXPRESSION OF *BACABCDE* OPERON IN *BACILLUS SUBTILIS*

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THE EFFECTS OF TWELVE QUORUM-SENSING GENE PRODUCTS ON THE EXPRESSION OF *BACABCDE* OPERON IN *BACILLUS SUBTILIS*

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ABSTRACT

THE EFFECTS OF TWELVE QUORUM-SENSING GENE PRODUCTS ON THE EXPRESSION OF *BACABCDE* OPERON IN *BACILLUS SUBTILIS*

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In *Bacillus subtilis*, genetic competence, sporulation and antibiotic production are controlled by quorum-sensing global regulatory mechanism. Bacilysin, being produced and excreted by certain strains of *Bacillus subtilis,* is a dipeptide antibiotic composed of L-alanine and L-anticapsin. We showed that the biosynthesis of bacilysin is under the control of quorum sensing global regulatory pathway through the action of ComQ/ComX, PhrC (CSF), ComP/ComA in a Spo0K (Opp)-dependent manner. Recently, the *ywfBCDEF* genes of *B. subtilis* 168 were shown to carry biosynthetic core function and renamed as *bacABCDE* operon. The objective of the present study is to elucidate the effects of previously-identified genes *srfA*, *oppA*, *comA*, *phrC*, *phrF*, *phrK*, *comQ* (*comX*), *comP*, *spo0H*, *spo0A*, *abrB* and *codY* on the expression of bacilysin biosynthetic operon *bacABCDE.* In order to monitor the expression of *bac* operon a *B. subtilis* strain, namely OGU1, containing a transcriptional *bacA-lacZ* fusion at *bacA* locus was constructed. Subsequently, each of the above-mentioned genes of cell density signaling was insertionally inactivated by transforming the competent cells of OGU1 with chromosomal DNA of the

corresponding blocked mutant strains. The resulting strains and OGU1 as the control were cultured in PA medium and *bacA*-directed β-galactosidase activities were monitored. *bacA-lacZ* expression was severely impaired in the *srfA*, *oppA*, *comA*, *phrC*, *phrF*, *phrK*, *comQ* (*comX*), *comP*, *spo0H* and *spo0A* disrupted mutants. On the other hand, in the *abrB* single mutant *bacA* expression level increased nearly 2-fold during exponential growth and in the *codY* mutant it severely decreased during the stationary phase.

Keywords: *Bacillus subtilis*; Quorum-sensing; Bacilysin; Transcriptional Fusion

ÖZ

12 ADET HÜCRE SİNYALİ ALGILAMA MEKANİZMASI GEN ÜRÜNÜNÜN *BACILLUS SUBTILIS BACABCDE* OPERON EKSPRESYONU ÜZERİNE ETKİLERİ

Öğülür, İsmail

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Bacillus subtilis'te genetik kompetans, sporulation ve antibiyotik üretimi hücre yoğunluğu sinyali mekanizması ile kontrol edilmektedir. *B. subtilis*'in bazı suşları tarafından sentezlenen basilisin, L-alanin ve L-antikapsinden oluşmuş bir dipeptitdir. Basilisin biyosentezinin, ComQ/ComX, PhrC (CSF), ComP/ComA'nın etkisinde hücre yogunluğu sinyali mekanizmasının kontrolü altında olduğunu ve bunun Spo0K (Opp)'ye bağlı bir biçimde gerçekleştiğini daha önceki çalışmalarımızda göstermiştik. Kısa bir süre önce, *B. subtilis* 168'in *ywfBCDEF* genlerinin basilisin biyosentetik fonksiyonlar taşıdığı gösterilmiş ve ilgili operon *bacABCDE* olarak yeniden adlandırılmıştır. Şimdiki çalışma, daha önce tanımlanan *srfA*, *oppA*, *comA*, *phrC*, *phrF*, *phrK*, *comQ*(*comX*), *comP*, *spo0H*, *spo0A*, *abrB* ve *codY* regülator genlerinin *bacABCDE* basilisin biyosentetik operonunun üzerine transkripsiyon düzeyinde etkilerinin belirlenmesini amaçlamaktadır. *bac* operon ekspresyon düzeyinin ölçülmesi için, *Bacillus subtilis*'e özgü, *bacA* gen lokusu ile füzyon oluşturacak bir *lacZ* geni içeren, *B. subtilis* PY79 *bacA*::*lacZ*::*erm* füzyonu taşıyan suşu oluşturulmuştur. Daha sonra çalışılan hücre yoğunluğu sinyali genleri,

bloke edilmiş uygun mutant suşlar kullanılarak *bacA*::*lacZ*::*erm* füzyonu taşıyan suşun kompetent hücre transformasyonu sonucunda inaktive edilmiştir. Elde edilen suşlar ve füzyonu taşıyan suş (kontrol) PA besiyeri içerisinde büyültülmüş ve *bacA* genine bağlı β-galaktosidaz aktiviteleri ölçülmüştür. *bacA-lacZ* ekspresyonu *srfA*, *oppA*, *comA*, *phrC*, *phrF*, *phrK*, *comQ* (*comX*), *comP*, *spo0H* and *spo0A* inaktif mutantlarda ciddi şekilde azalmıştır. Diğer yandan, *abrB* geni bloke edilmiş mutantta *bacA* ekspresyon düzeyi logaritmik büyüme fazı sırasında yaklaşık iki artmış, *codY* inaktif mutantta ise durağan fazı sırasında ciddi şekilde azalmıştır.

Anahtar kelimeler: *Bacillus subtilis*; Hücre yoğunluğu sinyali; Basilisin; Transkripsiyonel Füzyon

To My Family

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LIST OF ABBREVIATIONS

aa : Amino acid

bp(s) : Base pair(s)

EDTA : Ethylenediaminetetraacetic acid

kb : Kilobase

S. aureus : *Staphylococcus aureus*

lacZ : β-galactosidase

ORF : Open reading frame

E. coli : *Escherichia coli*

EtBr : Ethidium bromide

IPTG : Isopropyl-b D-thiogalactopyranoside

OD : Optical density

ONPG : Ortho-nitrophenyl-b-D-galactopyranoside

CA : Casamino acids

CHAPTER 1

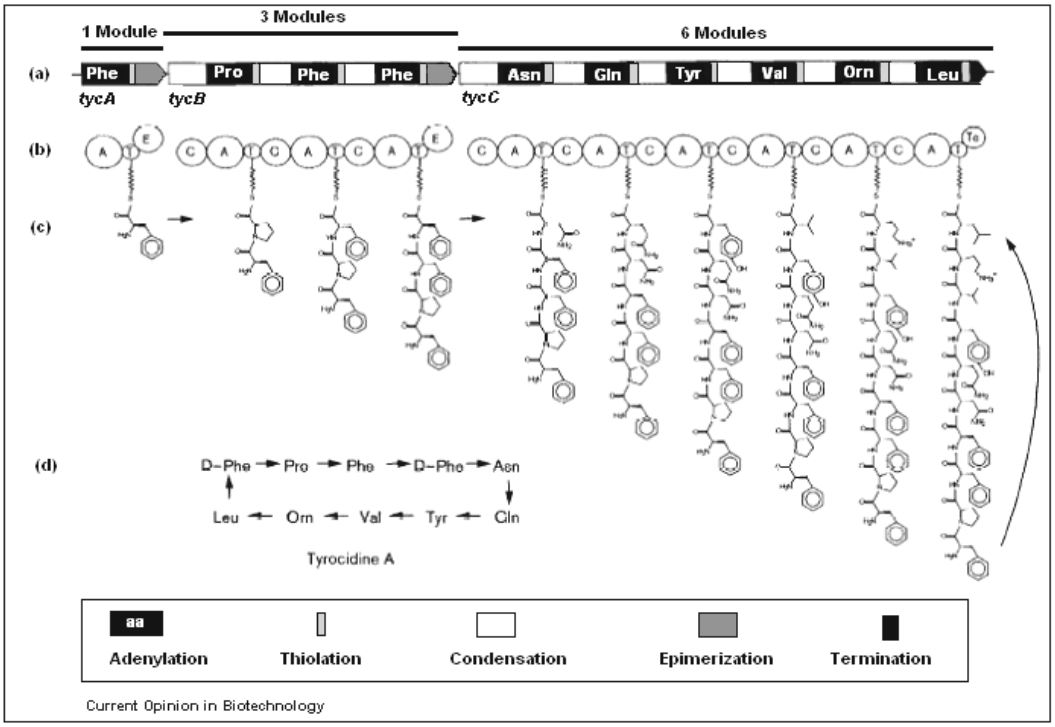
INTRODUCTION

1.1. Nonribosomal Peptide Synthesis

Microbially-produced peptides are among the most biologically active groups of compounds known. Among the microbial peptides, peptide antibiotics are currently the most important ones as they are used for antimicrobial and antitumor therapy, against plant pathogens, as immunosuppressive and cytostatic drugs, for promotion of animal growth and seed germination (Katz and Demain, 1977; Demain, 1980; Demain, 1992; Nakano and Zuber, 1990; Devine, 1995; Boman, 1995; Pichardal *et al.*, 1995; Boman, 1996; Gill *et al.*, 1996).

Two mechanisms have been identified as biosynthetic pathways for the bioactive peptides. The multicyclic lantibiotics, which contain the thioether amino acid lantihionine, for example, are synthesized ribosomally from gene-encoded peptide precursors, which are then modified by complex posttranslational processing (Schnell *et al*., 1988; Zuber *et al*., 1992). On the other hand, a large number of therapeutically useful cyclic and linear peptides synthesized via a template-directed, nucleic-acid-independent nonribosomal mechanism (Weber and Marahiel, 2001). These pharmaceutically important peptides are synthesized as secondary metabolites on large multifunctional enzymes in *Actinomycetes*, *Bacilli* and filamentous fungi, including antibiotics like penicillin and vancomycin, immunosuppressieve agents like cyclosporin A, cytostatic agents like epothilone and antiviral, antitumor, biosurfactant compounds (Mootz and Marahiel, 1997; Mootz and Marahiel, 1999; Schwarzer and Marahiel, 2001; Schwarzer *et al*., 2002; Sieber *et al*., 2002).

These secondary metabolite peptides may be composed of linear, cyclic, or branched peptide chains and contain unique structural features, such as D-amino acids, N-terminally attached fatty acid chains, N- and C-methylated residues, N-formylated residues, heterocyclic elements, and glycosylated amino acids, as well as phosphorylated residues. Moreover, in contrast to proteins produced by ribosomal synthesis, small peptide products contain not only the common 20 amino acids but also hundreds of different building blocks, suggesting a nonribosomal origin of biosynthesis (Marahiel *et al*., 1997; Sieber and Marahiel, 2005; Grünewald and Marahiel, 2006). Also in contrast to the structural diversity of the products, these secondary metabolites share a common mode of synthesis, the so-called "multiple carrier thio-template mechanism". According to this model (Fig. 1.1), peptide bond formation takes place on multienzymes designated as peptide synthetases, on which amino acid substrates are first activated by ATP hydrolysis to the corresponding adenylate. This unstable intermediate is subsequently transferred to another site of the multienzyme where it is bound as a thioester to the cysteamine group of an enzyme-bound 4'- phosphopantetheinyl (4'-PP) cofactor (Marahiel *et al.*, 1997; Mootz *et al.*, 2002; Sieber and Marahiel, 2005; Grünewald and Marahiel, 2006). According to the present multiple carrier model of nonribosomal peptide synthesis, nonribosomal peptide synthetases (NRPSs) are composed of repetitive units called as modules, each about 1.000 – 1.500 amino acids in length, which are capable of incorporating one amino acid constituent at a time into peptide chain (Mootz and Marahiel, 1997; Schwarzer and Marahiel, 2001; Kallow *et al*., 2002). The number and the order of modules within a NRPS match the number and sequence of amino acids incorporated into the peptide that's why these enzymes have also been called "protein templates" (von Döhren *et al*., 1999; Schwarzer and Marahiel, 2001; Weber and Marahiel, 2001).

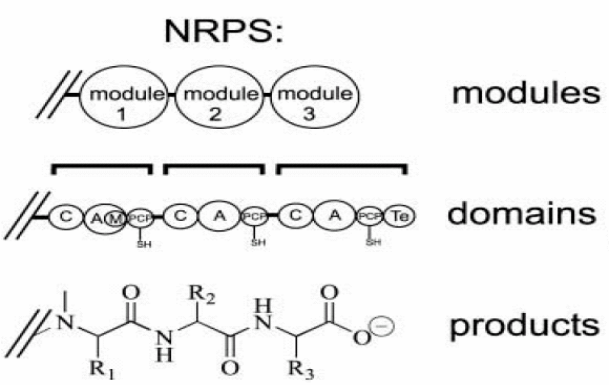


(Taken from: Mootz and Marahiel, 1999).

Figure 1. 1. The multiple carrier thiotemplate mechanism illustrated with the example of tyrocidine A synthesis (Mootz and Marahiel, 1997). Three peptide synthetases; (a) encoded by the genes *tycA*, *tycB*, and *tycC*, act in concert for the stepwise assembly of the cyclic decapeptide. (b) The substrates are recognized and adenylated with the consumption of ATP by the action of the A-domains and subsequently transferred to a thioester linkage on the cofactor 4'-phosphopantetheine (shown as a zigzagged line) of the T-domain, (c) C-domains then catalyze the condensation with the aminoacyl- or peptidyl-moieties on the neighbouring modules. At positions one and four, an epimerization domain converts L-Phe into its stereochemical isomer. (d) A thioesterase-like domain is believed to act as a cyclase to give the final product (Mootz and Marahiel, 1999).

The modules can be subdivided into domains, each responsible for catalyzing the three basic reactions: substrate recognition, activation as acyl adenylate, and covalent binding as thioester. These enzymatic activities are embedded in distinct catalytic domains with highly conserved core motifs within the module (Fig. 1.2) (Linne *et al*., 2001; Schwarzer and Marahiel, 2001). Three domains are necessary as the basic equipment of a NRPS elongation module: an adenylation (A) - domain that

selects the substrate amino acid and activates it as amino acyl adenylate; a peptidyl carrier protein (PCP)-domain that binds the co-factor 4'-phosphopantetheine (4'-PP) to which the activated amino acid is covalently attached; and a condensation (C)-domain that catalyzes peptide bond formation. These domains can therefore be described as the "toolbox" of NRPS (Konz and Marahiel, 1999; Schwarzer and Marahiel, 2001).



(Taken from: Schwarzer and Marahiel, 2001)

Figure 1. 2. From modules to products: the modules of NRPS can be subdivided into domains that catalyze the single enzymatic reactions. The composition of the products is determined by the assembly of active domains found in the corresponding modules (Schwarzer and Marahiel, 2001).

1.2. *Bacillus subtilis*

Bacillus subtilis is the best-studied organisms in the nature and stands after *Escherichia coli* as the second among prokaryotes, in the level of detail at which it is understood. The level of understanding has made the bacterium the central source of physiological gram-positive bacteria analysis, and of bacterial differentiation. *Bacillus* was intensively studied by the mid-20th century because of its role in human, animal and insect infections and the production of many important products

such as antibiotics, it also presents an important source of industrial enzymes, e.g. amylases and proteases, and other useful products (Sonenshein *et al*., 2002).

B. *subtilis* is commonly found in soil, water sources and in association with plants. One of the main characteristics of life in the soil and important implications for the organism's physiology is the tendency to a 'fast or feast' existence (Harwood and Cutting, 1990).

Additionally, *B*. *subtilis* is a chemoorganotroph, so that it is able to maintain a suitable environment containing factors it demands for its growth by simply oxidizing organic compounds belonging to a broad range of family. Moreover, just like many other members of its genus, *B*. *subtilis* is mesophilic and may undergo growth and production of normal-sized colonies within a day when placed at 37^{o}C. Another important characteristic of this organism is its being aerobe and therefore it requires sufficient aeration during growth (Harwood *et al*., 1990).

B. *subtilis* stops growing under nutritional starvation and starts responses to restore grow by increasing metabolic diversity. The responses contain the induction of motility and chemotaxis, and the production of hydrolases (proteases and carbohydrases) and antibiotics. The cells are induced to form chemically, irradiation and desiccation resistant endospores when the responses fail to re-establish growth. The first morphological indication of sporulation is division of the cell into a smaller forespore and a larger mother cell, each with an entire copy of the chromosome. The former is engulfed by the latter and differential expression of their respective genomes, coupled to a complex network of interconnected regulatory pathways and developmental checkpoints, culminates in the programmed death and lysis of the mother cell and release of the mature spore (Stragier and Losick, 1996). *B*. *subtilis* can also differentiate into a physiological state, the competent state, which allows it to undergo genetic transformation in an alternative developmental process (Solomon and Grossman, 1996).

B. subtilis has a genome of 4.2 Mb long (Franguel *et al*., 1999). Its genome sequence was completed in 1997 by an international collaboration (Kunst *et al*., 1997). It is now known that *B. subtilis* uses 275 genes, 25 of which are unknown, in order to grow in rich medium at moderate temperatures and in aerated environment (Kobayashi and Osagura, 2002). Its genome also consists of 17 sigma factors and approximately 250 DNA binding transcriptional regulators. In addition to these, 4106 protein-coding, 86 tRNA, 30 rRNA and 3 small stable RNA genes were located (Ando, 2002; Kobayashi and Osagura, 2002).

In 1947, Burkholder and Giles reported that they isolated many auxotrophic mutants of *B. subtilis*, one of which is a tryptophan requiring strain called BGSC1A1, or *B. subtilis* 168. Subsequently, in 1958, transformable characteristic of this strain was reported and upon this information, *B. subtilis* 168 has become the most useful strain for genetic researches based on this organism (Spizen, 1958; Harwood *et al*., 1990). *B. subtilis* PY79 has also found its place as the wild type strain as being a prototrophic derivative of *Bacillus subtilis* 168.

1.3. Microbially Synthesized Bioactive Peptides by the Genus *Bacillus*

Members of genus *Bacillus* produce a large number of antibiotics as listed in Table 1.1.

B. subtilis is able to produce more than two dozen antibiotics with an amazing variety of structures. They exhibit highly rigid, hydrophobic and/or cyclic structures with unusual constituents like D-amino acids and are generally resistant to hydrolysis by peptidases and proteases. Additionally, cysteine residues are either oxidized to disulphides and/or are modified to characteristic intramolecular C–S (thioether) linkages, and as a result the peptide antibiotics are insensitive to oxidation. The anti-microbially active compounds produced include predominantly peptides that are either ribosomally synthesized and post-translationally modified (lantibiotics and lantibiotic-like peptides) or non- ribosomally generated, as well as a couple of non-peptidic compounds. Recent findings are resumed that show roles for

distinct *B. subtilis* antibiotics beyond the 'pure' anti-microbial action: Non-ribosomally produced lipopeptides are involved in biofilm and swarming development, lantibiotics function as pheromones in quorum-sensing, and a 'killing factor' effectuates programmed cell death in sister cells (Stein, 2005).

Table 1. 1. Bioactive peptides synthesized by *Bacillus* species

Peptide	**Organism**	**Structure**
Bacilysin	*B. subtilis*	Linear
Edeine	*B. brevis* Vm4	Linear
Gramicidin	*B. brevis*	Linear
Iturin	*B. subtilis*	Cyclopeptide
Gramicidin S	*B. braves* ATCC 9999	Cyclopeptide
Tyrocidine	*B. brevis* ATCC 8185	Cyclopeptide
Mycobacillin	*B. subtilis*	Cyclopeptide
Surfactin	*B. subtilis*	Lacton
Polymyxin	*B. polymyxa*	Polypeptide
Bacitracin	*B. licheniformis*	Polypeptide
Bacilysocin	*B. polymyxa*	Phospholipid

1.4. Global Regulation of Gene Expression by Quorum- Sensing

Cell-cell signaling is utilized by many types of cells to regulate gene expression and development. One form of cell-cell signaling involves a regulatory response to cell density signals. This process, sometimes called quorum sensing (Fuqua *et al*., 1994), is typically characterized by regulatory events that are induced as cells grow to high cell density. A variety of chemicals, including acyl homoserine lactones, peptides, and amino acids, are used for microbial cell-cell signaling to regulate many biological processes, including genetic exchange, development, virulence, bioluminescence, and production of antibiotics (Solomon *et al*., 1996). Even though a vast number of diverse quorum-sensing systems exist, they can be divided into two established paradigms that regulate the intraspecific behavior in many bacteria: (i) LuxI/LuxR-type quorum-sensing systems in Gram-negative bacteria responsible for the production of *N*-acyl-L-homoserine lactone autoinducers or type I autoinducers and (ii) oligopeptide/two component-type quorum-sensing circuits in gram-positive bacteria responsible for the production of autoinducer peptides (Lazazzera *et al*., 1997; Perego, 1997; Bacon Shneider *et al*., 2002; Fuqua and Greenberg, 2002; Jian *et al*., 2002). Examples in Gram-negative bacteria, *Pseudomonas aeroginosa* use quorum sensing for biofilm formation and virulence, *Vibrio fischeri* and *Vibrio harveyi* for bioluminescence, *Erwinia carotovora* for antibiotic production, *Argobacterium tumefaciens* for plasmid conjugation, *Rhizobium leguminosarum* for root nodule formation, and so on. In case of Gram-positive bacteria, via quorum sensing phenomenon, *Staphylococcus aureus* and *Enterococcus faecalis* develop virulence, *Streptococcus pneumoniae* and *B. subtilis* show competence and additionally *B. subtilis* gets induced for sporulation (Sturme *et al*., 2002; Taga *et al*., 2003).

Signal transduction in prokaryotes is mainly carried out by the so-called two-component systems consisting of a histidine protein kinase and a response regulator. The kinase acts as a sensor of a specific signal which, upon binding, activates the kinase by inducing autophosphorylation of the protein kinase on a histidine residue. The phosphoryl group is subsequently transferred to a paired response regulator,

thus activating its function, generally of transcription regulation, allowing the cells to respond and adapt to the specific signal (Hoch and Silhavy, 1995).

In *B. subtilis*, several processes are known to be regulated by extracellular peptide signaling, including the initiation of genetic competence, sporulation, production of degradative enzymes and exopolysaccharides, and antibiotic synthesis (Magnuson *et al*., 1994; Solomon *et al*., 1996; Comella and Grossman, 2005). Three types of secreted peptide signaling molecules have been identified: a modified 5- to 10-amino-acid peptide, ComX, that interacts extracellularly with its receptor; lantibiotic peptides (Magnuson *et al*., 1994; Piazza *et al*., 1999; Tortosa *et al*., 2001), such as subtilin, which interact extracellularly with their receptors; and unmodified pentapeptides, known as Phr peptides, that are internalized to inhibit the activity of their target proteins, known as Rap proteins (Lazazzera, 2001; Perego and Brannigan, 2001; Stein, 2005).

B. subtilis encodes a family of 8 Phr peptides (PhrA, PhrC, PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK) and a family of 11 Rap proteins (RapA to RapK) (Table 1.1). Each Phr peptide is encoded in an operon with a Rap protein, and each characterized Phr inhibits the activity of its cotranscribed Rap (Perego and Hoch, 1996; Solomon *et al*., 1996; Kunst *et al*., 1997; Jiang *et al*., 2000; Ogura *et al*., 2003; Hayashi, 2006). The PhrC peptide [also known as competence-and sporulation-stimulating factor (CSF)] also inhibits the activity of an unpaired Rap protein, RapB (Perego, 1997). It is possible that the other unpaired Rap proteins are also inhibited by noncognate Phr peptides (Auchtung *et al*., 2006).

In addition to expression from the upstream *rap* promoter, most *phr* genes are also expressed from a promoter upstream of *phr* that is recognized by RNA polymerase containing the alternative sigma factor, σ^H. This regulation by σ^H causes the level of each *phr* gene to increase as cells transition from exponential growth to stationary phase (Lazezzera *et al*., 1999; McQuade *et al*., 2001).

Table 1. 2. Processes regulated by Rap proteins and Phr peptides in *B. subtilis*. (Auchtung *et al*., 2006).

Rap protein	**Phr peptide**	**Target(s) of Rap**	**Mechanism of Rap**	**Responses regulated by target protein(s)**
RapA	PhrA	Spo0F~P	Stimulates autodephosphorylation	Activates post exponential-phase gene exp. and sporulation indirectly through Spo0A
RapB	PhrC	Spo0F~P	Stimulates autodephosphorylation	Activates post-exponential phase gene exp. and sporulation indirectly through Spo0A
RapC	PhrC	ComA	Inhibits binding of ComA to DNA	Activates exp. of genes involved in production of degradative enzymes, antibiotics, and competence
RapD		Unkown	Unknown	
RapE	PhrE	Spo0F~P	Stimulates autodephosphorylation	Act. post exponential-phase gene exp. and sporulation indirectly through Spo0A
RapF	PhrF	ComA	Inhibits binding of ComA to DNA	Activates exp. of genes involved in production of degradative enzymes, antibiotics, and competence
RapG	PhrG	DegU, ComA	Inhibits binding of DegU to DNA, unknown	Activates expression of genes involved in competence and production of degradative enzymes, and antibiotics
RapH	PhrH	ComA, DegU	Unknown	Activates exp. of genes involved in competence and production of degradative enzymes, and antibiotics
RapI	PhrI	Unknown	Unknown	RapI stimulates gene expression, excision, and transfer of ICEBs1
RapJ		Unknown	Unknown	
RapK	PhrK	ComA	Unknown	Activates exp. of genes involved in production of degradative enzymes, antibiotics, and competence

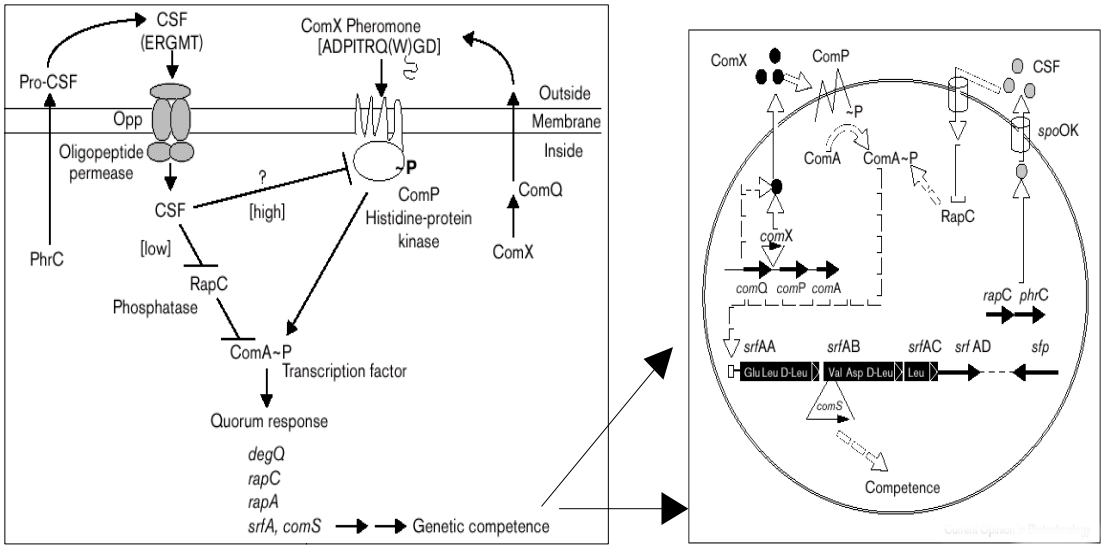
The primary *phr* gene products are pre-Phr peptides that are 38 to 57 amino acids in length. Pre-Phr peptides are exported and cleaved to form the mature Phr pentapeptides (Lazazzera, 2001; Perego and Brannigan, 2001). The oligopeptide permease (Opp), an ATP-binding cassette (ABC) transporter that imports small peptides (Perego *et al*., 1991; Rudner *et al*., 1991), transports the Phr peptides into the cell, where they can inhibit the activities of Rap proteins (Lazazzera *et al*., 1997; Perego, 1997).

Two peptide pheromones, ComX pheromone and CSF (PhrC), accumulate during exponential growth and stimulate the development of genetic competence and expression of several genes involved in other processes (Fig. 1.5). The extracellular and intracellular mechanisms of peptide signaling are represented by ComX pheromone and CSF (PhrC), respectively (Lazazzera and Grossman, 1998).

ComX pheromone is a 10-amino-acid peptide (ADPITRQWGD) with a hydrophobic modification of unknown structure on the tryptophan residue. Two genes, *comQ* and *comX*, are known to be required for ComX pheromone production (Magnuson *et al*., 1994). *comX* encodes the 55-amino-acid precursor of ComX pheromone. *comQ* is located immediately upstream of *comX* in the chromosome (Weinrauch *et al.,* 1991), and *comQ* null mutants do not produce ComX pheromone (Magnuson *et al*., 1994). Production of ComX pheromone requires processing of the 55-amino-acid precursor to 10 amino acids, modification of the tryptophan residue, and export from the cell. ComQ may be involved in the processing and/or modification step (Lazazzera *et al*., 1999). ComX stimulates the activity of the membrane bound receptor histidine kinase ComP. ComP has eight putative membrane-spanning helices and appears to be the direct receptor for ComX pheromone. The hydrophobic modification on the pheromone is required for function and may help to increase the local concentration of ComX pheromone at the membrane where it can interact with and activate ComP. Autophosphorylated ComP donates phosphate to the response regulator ComA . The phosphorylated form of ComA activates expression of several genes, including *comS* (also known as *srfA*), which is the only ComX–ComP–ComA-controlled gene

required for competence development (Lazazzera and Grossman, 1998; Core and Perego, 2003).

CSF (also known as PhrC) is a 5-amino-acid peptide (ERGMT) that contributes to the activation of ComA by inhibiting the activity of the regulator RapC (Solomon *et al*., 1996). Very recently, it was shown that also PhrF and PhrK stimulate ComA's activity by directly inhibiting the activity of their cognate proteins RapF and RapK Bongiorni *et al*., 2005; Auchtung *et al*., 2006). Auchtung and his co-workers also shown that PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression to different magnitudes and that all three peptides are reguired for full expression of ComA-dependent genes.



(Taken from: Mootz and Marahiel, 1997)

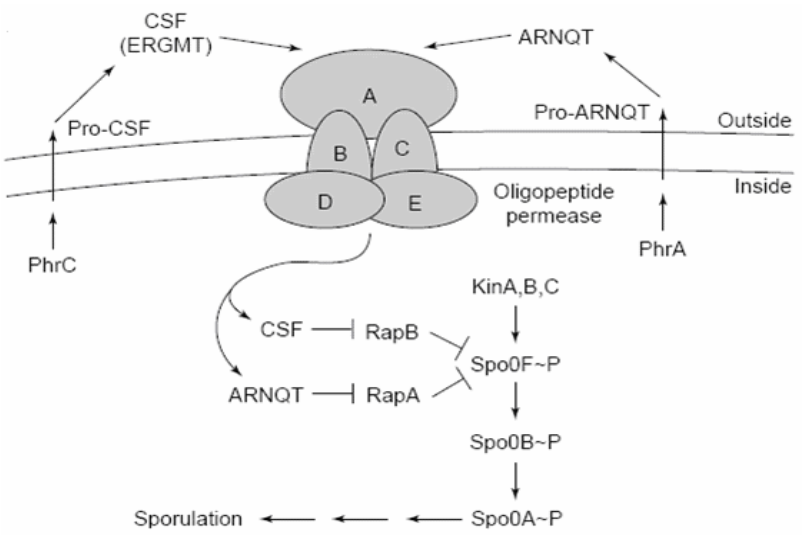
Figure 1. 3. A model for two extracellular signaling peptides mediating the quorum response in *B. subtilis*.

The function of CSF in regulating ComA activity is more complicated than that of ComX pheromone. CSF is a secreted, diffusible peptide (Solomon *et al*., 1996). When CSF reaches a critical concentration, it is transported back into the cell by an oligopeptide permease Opp (also known as Spo0K). It then appears to bind to two different intracellular receptors to modulate the activity of the ComA transcription factor (Fig. 1.5) (Lazezzera and Grossman 1998; Perego, 1997). At low concentrations (1–5 nM), CSF stimulates the activity of ComA apparently by inhibiting the activity of an aspartylphosphate phosphatase, RapC (Fig. 1.5) (Solomon *et al*., 1996). At higher concentrations (>20 nM), CSF interacts with an as yet unidentified receptor, possibly the histidine-protein kinase ComP, to inhibit the expression of ComA-controlled genes (Fig. 1.5) (Lazezzera *et al*., 1997). In addition to these two functions, CSF, at high concentrations, also stimulates sporulation apparently by inhibiting the activity of an alternate aspartyl-phosphate phosphatase, RapB (Solomon *et al*., 1996; Lazezzera *et al*., 1997; Pereggo, 1997).

Production of mature CFS involves several steps, starting with tanscription and translation of *phrC*, the gene encoding the precursor of CSF (Solomon *et al*., 1996). The 40-amino acid primary product of *phrC* has a signal sequence and putative peptidase cleavage sites, indicating that an 11-to 25-amino acid peptide is exported (Perego *et al*, 1996). Lazezzera and coverkers (1999) found that transcription of the *rapC phrC* operon activated by high cell density through ComA~P and that *rapC* and *phrC* regulate their own expression. RapC, by negatively regulating ComA~P, is a part of this homeostatic autoregulatory loop. PhrC (CSF) stimulates ComA activity and positively regulates its own expression. Furthermore, they showed that by mid-exponential phase, CSF is at concentrations that stimulate competence gene expression and that as cells enter stationary phase, the extracellular concentration of CSF reaches levels approaching 100 nM, concentrations that are known to stimulate sporulation and inhibit early competence gene expression.

The ability of CSF to stimulate sporulation involves a similar mechanism to that by which CSF stimulates competence (Fig. 1.7). It requires the phosphatase RapB. RapB dephosphorylates Spo0F~ P (Perego *et al*., 1994) and Spo0F is part of the

phospho-transfer pathway that donates phosphate to the transcription factor Spo0A, which is required for the initiation of sporulation (Grossman, 1995; Hoch, 1993). The phosphatase activity of RapB is inhibited *in vitro* by CSF, indicating that CSF stimulates sporulation *in vivo* by directly inhibiting RapB (Perego, 1997).



(Taken from: Lazazzera *et al*., 1998)

Figure 1. 4. The regulation of sporulation by extracellular peptides. CSF and a peptide, ARNQT, encoded by phrA, are transported into the cell by Opp. Each peptide inhibits the activity of a phosphatase (RapB or RapA) that dephosphorylates Spo0F~P. Spo0F receives phosphate from one of three kinases (KinA,B,C) and donates phosphate to Spo0A through Spo0B (Lazazzera *et al*., 1998).

It is clear that CSF accumulates in culture medium during cell growth and can function in cell–cell signaling. It is also clear that PhrA pentapeptide can function in cell–cell signaling. However, it has been suggested that the active form of modified PhrA, ARNQT, may not normally accumulate to significant levels in culture medium and that PhrA may be involved in cell-autonomous signaling as part of a timing mechanism. At high internal CSF concentrations, CSF inhibits competence and promotes spore development. Specifically, CSF inhibits ComS, reducing

transcription of competence genes and promoting sporulation instead (Lazazzera *et al*., 1997; Solomon, 1995; Solomon *et al*., 1996; Perego and Hoch, 1996; Mirel *et al*., 2000; Stephens, 1998).

The products of *spo0A* and *spo0H* genes, which code for response regulator of the multi-compenent signal transduction system, so called phosphorelay and alternative sigma factor, σ^H, respectively, play a key role in the initiation of sporulation (Burbulys *et al*., 1991; Chibazakura *et al*., 1995; Dubnau *et al*., 1988; Asai *et al*., 1995). A multicomponent phosphorelay consists of five histidine kinases (KinA, KinB, KinC, KinD and KinE) and two phosphorelay proteins (Spo0F and Spo0B) (Perego and Hoch, 2002). Multiple environmental and physiological signals are fed into this system, and under appropriate conditions this leads to phosphorylation of Spo0A, the key sporulation transcription factor (Grossman, 1995; Sonenshein, 2000).

Activity of Spo0A is subject to several auto-stimulatory loops (Strauch *et al*., 1992; Strauch *et al*., 1993; Fujita and Sadaie, 1998). These loops involve transcription of *spo0A* and phosphorylation of Spo0A. Transcription of *spo0A* is directly activated by Spo0A~P and indirectly activated by induced expression of *sigH*. The *sigH* gene encodes an RNA polymerase sigma factor (σ^H) that recognizes an alternative promoter located upstream of *spo0A* (Predich *et al*., 1992) and activates transcription of genes involved in the phosphorylation of Spo0A such as *kinA* and *spo0F*. Furthermore, sigma-H activates transcription of the *spoIIA* operon, which contains the sporulation specific sigma factor, sigma-F (Hoch, 1991).

Once activated by phosphorylation, Spo0A binds to a DNA sequence containing a so-called '0A-box' (Strauch *et al*, 1990), where it exerts its role by acting as a transcriptional activator or repressor. Besides being required for the onset of sporulation, Spo0A is also involved in the transcriptional regulation of various other stationary phase processes. Spo0A influences the expression of 520 *B. subtilis* genes showing that it has indeed a profound effect on the global gene expression pattern of *B. subtilis* (Fawcett *et al*, 2000; Liu *et al*, 2003). Of these 520 genes, 121 are under

the direct control of Spo0A. Several of these encode proteins that themselves are directly or indirectly involved in transcriptional regulation, explaining the global effect of Spo0A on transcription (Molle *et al*., 2003; Castilla-Llorente *et al*., 2006). The levels of Spo0A protein and activity increase gradually during the early stages of sporulation (Fujita and Losick, 2005) and the progressive increase of activated Spo0A explains the temporal fashion by which the low- and high-threshold Spo0A-regulated genes are activated or repressed (Fujita *et al*., 2005).

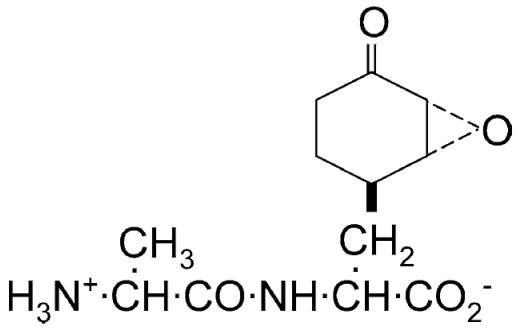
A major role of phosphorylated Spo0A is to repress the expression of *abrB*, a gene encoding a transcriptional regulator that represses various stationary phase processes (Robertson *et al*., 1989). During exponential growth, AbrB represses expression of *sigH*, *kinA* and *abrB* itself (Strauch, 1995). Thus, alleviation of AbrB repression by Spo0A~P at the beginning of the stationary growth phase, stimulates *sigH* and *kinA* expression and therefore *spo0A* transcription and indirectly phosphorylation of Spo0A. In conclusion, the complex autostimulation of *spo0A* could be the basis of the bistable sporulation gene expression (Veening *et al*., 2005).

KinA is the primary kinase in the phosphorelay and is necessary for the phosphorylation of Spo0A (Burbulys *et al*., 1991). It has been demonstrated that the fraction of cells that initiate sporulation is decreased in a *kinA* mutant background (Chung *et al*., 1994). This result suggests that a certain threshold concentration of Spo0A~P is necessary to initiate sporulation and that the activity of the phosphorelay determines the threshold level for autostimulation of Spo0A. This implies that influences on the phosphorelay by external phosphatases could alter the heterogeneous sporulation gene expression, examples are RapA and PhrA (Veening *et al*., 2005). Recently, Castillo-Llorente *et al*. (2008) showed that KinC and, to a minor extent, KinD, are responsible for heterogeneous expression of *spo0A* during logarithmical growth. The low-threshold Spo0A-regulated gene *abrB* has been reported to be expressed at submaximal levels during logarithmical growth of wild-type cells, compared with the levels reached in strains with an inactivated phosphorelay (Perego *et al*., 1988; Trach and Hoch, 1993). Submaximal levels of *abrB* expression during logarithmical growth were also observed in *kinA*, *kinB* or

kinE mutant strains, but the expression levels were higher in *kinC* and *kinD* mutant strains, and maximum *abrB* expression levels were observed in the absence of both KinC and KinD (Trach and Hoch, 1993; LeDeaux *et al*., 1995; Jiang *et al*., 2000). From these results, it was inferred that KinC and KinD would be responsible for generating Spo0A~P to levels causing partial repression of *abrB* transcription during logarithmical growth (Castillo-Llorente *et al*., 2008).

1.5. Dipeptide Antibiotic Bacilysin

The dipeptide bacilysin is one of the simplest peptide antibiotics known (Fig. 1.8) which displays activity against some bacteria and fungi (Kenig and Abraham, 1976; Tschen, 1990). Its proposed amino acid ligase mode of biosynthesis might offer strategies to engineer new derivatives with improved properties (Steinborn *et al*., 2005).



(Taken from: Steinborn *et al*., 2005)

Figure 1. 5. Structure of bacilysin

Bacilysin [L-alanyl-(2.3-epoxycyclohexanone-4)-L-alanine] molecule contains an L-alanine residue at the N terminus and a non-proteinogenic amino acid, L-anticapsin, at the C terminus (Walker and Abraham, 1970). Its antibiotic activity depends on the anticapsin moiety, which becomes released by peptidases (Kenig *et al*., 1976;

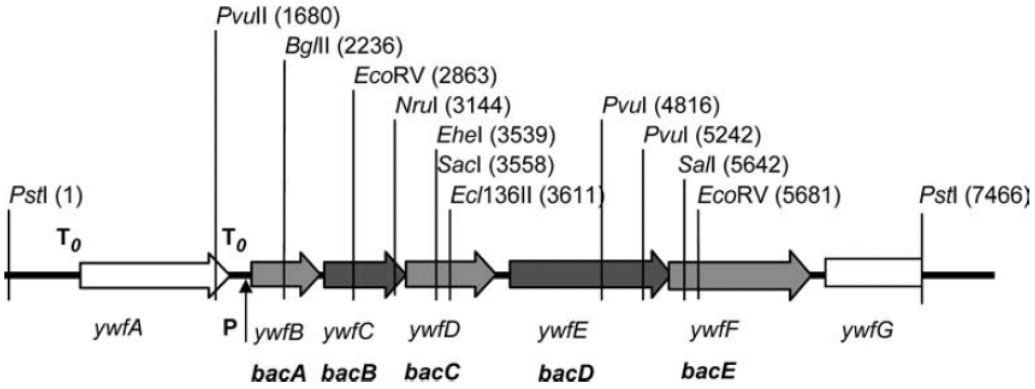
Chmara *et al*., 1982) after bacilysin uptake into susceptible cells by a distinct peptide permease system (Perry and Abraham, 1979; Chmara *et al*., 1981). The intracellular anticapsin then blocks the glucosamine synthetase, and hence, bacterial peptidoglycan or fungal mannoprotein biosynthesis. This leads to cell protoplasting and lysis (Whitnney and Funderburk, 1970; Kenig *et al*., 1976; Chmara *et al*., 1982; Chmara, 1985; Milewski, 1993). Based on its metabolic target, the antibiotic activity of anticapsin becomes specifically antagonized by glucosamine or N-acetylglucosamine (Walton and Rickes, 1962; Kenig and Abraham, 1976).

Biosynthesis of the anticapsin branches from prephenate (Roscoe and Abraham, 1966; Hilton *et al*., 1988). The peptide bound with L-alanine proceeds in a non-ribosomal mode -probably enzymatic- catalysed by an amino acid ligase (bacilysin synthetase) (Sakajoh *et al*., 1987).

Bacilysin production by *B. subtilis* is active when the cells are grown in synthetic medium and becomes repressed and/or inhibited by certain nutrients, like glucose and casamino acid, and temperatures above 30°C (Özcengiz *et al*., 1990; Özcengiz and Alaeddinoglu, 1991; Basalp *et al*., 1992). Its synthesis seems to be under the stringent response (Inaoka *et al*., 2003) as well as under feedback regulation (Özcengiz and Alaeddinoğlu, 1991), and as shown more recently, it was a component of the global quorum-sensing control system (Yazgan *et al*., 2001; Karatas *et al*., 2003). It was definitely shown that the biosynthesis of basilysin is under quorum sensing global regulation through the action of ComQ/ComX, PhrC (CSF), ComP/ComA and in a Spo0K (Opp)-dependent manner in *B. subtilis* (Yazgan *et al.*, 2001; Karatas *et al.,* 2003). The disruption of lipopeptide antibiotic surfactin biosynthetic (*srfA*) operon in the bacilysin producer resulted in a bacilysin-negative phenotype, thus our study verified that the *srfA* operon functions directly in the production of bacilysin (Karatas *et al*., 2003). The loss of bacilysin production in *spo0H* and/or *spo0A*-blocked mutants as well as an increase in the production of bacilysin in *abrB*-disrupted mutants and the suppression of bacilysin-negative phenotype by an *abrB* mutation in *spo0A*-blocked mutants revealed that the transcription of some gene(s) involved in bacilysin formation is under the negative

control of *abrB* gene product which is relieved by Spo0A protein (Karatas *et al*., 2003).

The *ywfBCDEF* genes of *B. subtilis* 168 were shown to carry the biosynthetic core functions and were renamed *bacABCDE* (Fig. 1.9) (Inaoka *et al*., 2003; Steinborn *et al*., 2005). In accordance with the similarity features of the genes *bacABC*, the deduced proteins were good candidates to catalyse the proposed conversion of prephenate to anticapsin (Roscoe and Abraham, 1966; Hilton *et al*., 1988), apparently in three enzymatic steps. *bacDE* (*ywfEF*) have been shown to encode the functions of amino acid ligation and bacilysin immunity respectively (Inaoka *et al*., 2003; Steinborn *et al*., 2005).

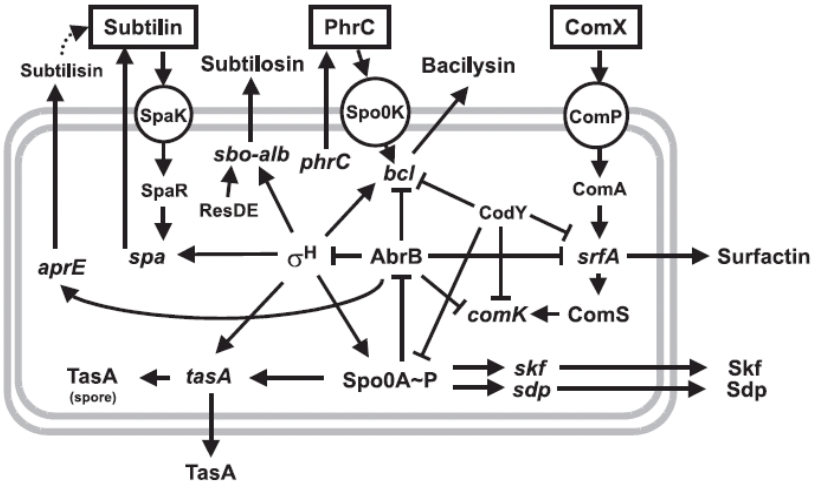


(Taken from: Steinborn *et al*., 2005)

Figure 1. 6. Organization of the bacilysin gene cluster *bacABCDE* relative to open reading frames *ywfABCDEFG* of *Bacillus subtilis* 168. DNA comprises the sequence from 3875148–3867678 bp of the SubtiList database R16.1 (Kunst *et al*., 1997). Proposed terminator (T_0) elements are indicated according the SubtiList database. Sigma A promoter (P) elements_35 (TTGACA) and _10 (TAAAATt) were detected 56 bp and 33 bp upstream of the ATG codon of the *bacA* gene (Steinborn *et al*., 2005).

The study of Inaoka *et al*. (2003) was also showed that guanosine 5'-diphosphate 3'-diphosphate (ppGpp) plays a crucial role in transcription of the *bacABCDE* operon and that the transcription of these genes is dependent upon the level of intracellular GTP which is transmitted as a signal via the CodY-mediated repression system. It was proposed that, bacilysin production in *B. subtilis* is controlled by a dual regulation system composed of the guanine nucleotides ppGpp and GTP (Inaoka *et al*., 2003).

Briefly, bacilysin production is regulated on different levels (Fig. 1.10), negatively by GTP via the transcriptional regulator CodY (Inaoka *et al*., 2003) and AbrB (Karatas *et al*., 2003). Positive regulation occurs by guanosine 5'-diphosphate 3'-diphosphate (ppGpp) (Inaoka *et al*., 2003) and a quorum-sensing mechanism through the peptide pheromone PhrC (Karatas *et al*., 2003).



(Taken from: Stein, 2005)

Figure 1. 7. Regulatory pathways of antibiotic biosynthesis in *B. subtilis*. Survey of the regulatory pathways for the biosynthesis of the *B.subtilis* antibiotics subtilin, subtilosin, bacilysin, surfactin, the killing factor Skf and the spore-associated anti-microbial polypeptide TasA (Stein, 2005).

1.6. The Aim of the Present Study

Under the light of above-mentioned findings related with bacilysin biosynthesis, the present research aimed at the elucidation of the effects of the products of previously-identified genes *srfA*, *oppA*, *comA*, *phrC*, *phrF*, *phrK*, *comQ* (*comX*), *comP*, *spo0H*, *spo0A*, *abrB* and *codY* on the bacilysin biosynthetic operon *bacABCDE* and gaining further insight into the global regulatory mechanisms operating in bacilysin biosynthesis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains and Plasmids

B. subtilis PY79, a prototrophic derivative of standart strain *B. subtilis* 168, was used as bacilysin producer throughout this study. The strains and their genotypes that were used in the study are listed in Table 2.1. *S. aureus* ATCC 9144 was used as the assay organism for bacilysin determinations. *E. coli* DH5α [F' Φd*lac*ZΔ(*lac*ZY A-argF),U169, *sup*E44λ^-, *thi*-1, *gyr*A, *rec*A1, *rel*A1 *end*A1, *hsd*R17] was used as a host for cloning of *B. subtilis* chromosomal DNA. Integrative vector pMutinT3 (Fig. 2.1) was used for the construction of *bacA-lacZ* transcriptional vector. pGEM-T vector (Fig. 2.2) for cloning of PCR products amplified.

2.2. Culture Media

Composition and preparation of culture media are given in the Appendix A.

2.3. Buffers and Solutions

Composition and preparation of buffers and solutions are given in Appendix B.

2.4. Chemicals and Enzymes

The chemicals and enzymes that were used are given in the Appendix C.

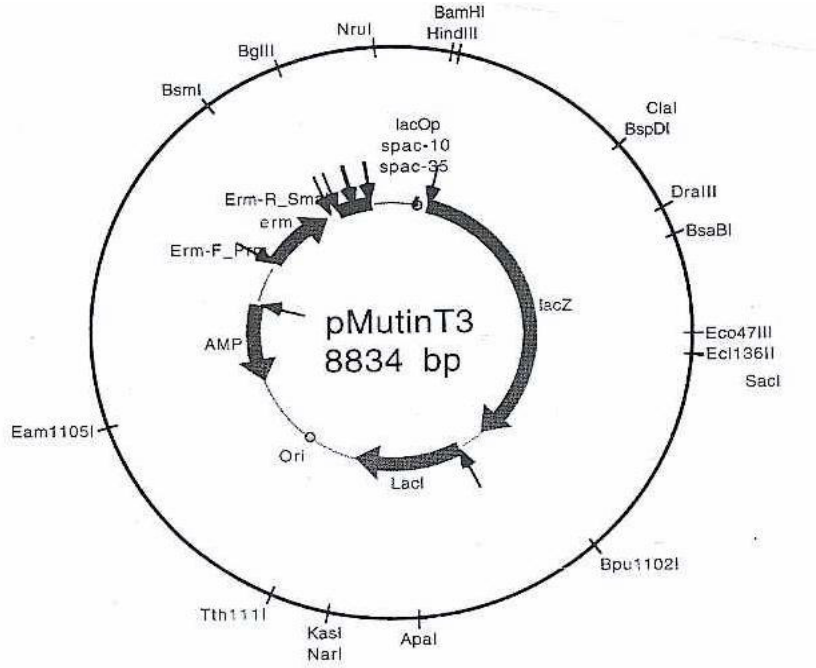


Figure 2. 1. Genomic map of pMUTINT3 vector including the functional genes in the structure as well as the restriction map (Vagner *et al*., 1998).

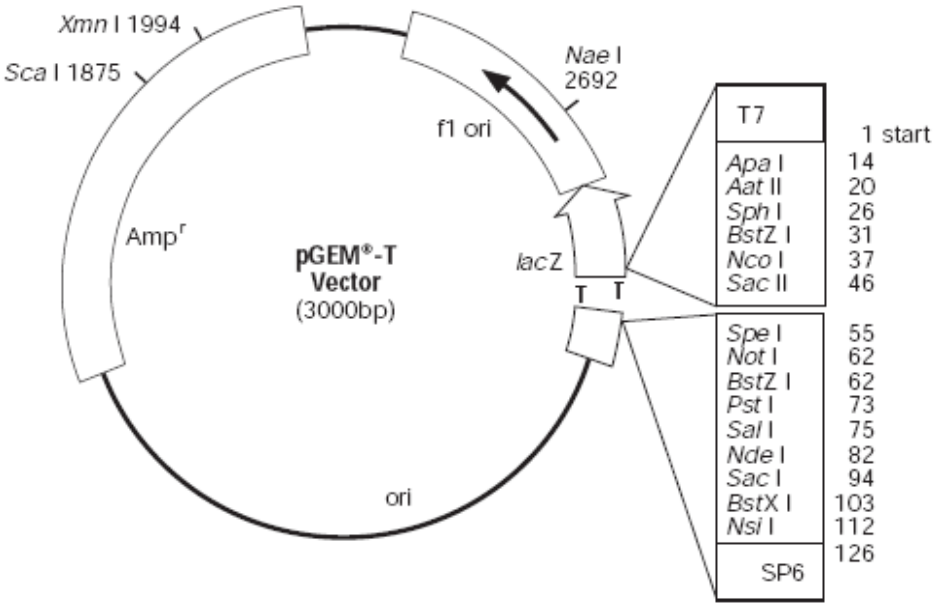


Figure 2. 2. Genomic map of the pGEM-T vector.

Table 2. 1. Bacterial strains and their genotypes used in this study

Strain	Genotype	Source
Bacillus subtilis PY79	Wild type, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman
AK3	*oppA*::*Tn10(spc)*	Our Laboratory
KE10	*srfA*::*erm*	Phillippes University
JRL192	*comA*::*cat*	A. D. Grossman
BD1658	*comP*::*cat*	A. D. Grossman
JMS315	*trpC2 pheA1 comQ*::*spc*	A. D. Grossman
JMS175	*Δspo0A*::*spc*	A. D. Grossman
AG665	*trpC2 pheA1 spo0H*::*cat*	A. D. Grossman
TMH307	*trpC2 unkU*::*spc ΔcodY*	A. D. Grossman
BAL373	*trpC2 pheA1 ΔabrB*::*cat*	A. D. Grossman
JMS751	*phrC*::*erm*	A. D. Grossman
JMA163	*ΔphrF163*::*cat*	A. D. Grossman
CAL7	*ΔphrK7*::*spc*	A. D. Grossman
OGU1	*bacA*::*lacZ*::*erm*	This study
OGU1O	*oppA*::*Tn10(spc) bacA*::*lacZ*::*erm*	This study
OGU1CA	*comA*::*cat bacA*::*lacZ*::*erm*	This study
OGU1CP	*comP*::*cat bacA*::*lacZ*::*erm*	This study
OGU1CQ	*comQ*::*spc bacA*::*lacZ*::*erm*	This study
OGU1S	*Δspo0A*::*spc bacA*::*lacZ*::*erm*	This study
OGU1SH	*spo0H*::*cat bacA*::*lacZ*::*erm*	This study
OGU1C	*ΔcodY*::*spc bacA*::*lacZ*::*erm*	This study
OGU1A	*ΔabrB*::*cat bacA*::*lacZ*::*erm*	This study
OGU1PC	*phrC*::*erm bacA*::*lacZ*::*erm*	This study
OGU1PF	*Δphrf*::*cat bacA*::*lacZ*::*erm*	This study
OGU1PK	*phrk7*::*spc bacA*::*lacZ*::*erm*	This study
OGU1SA	*ΔabrB*::*cat Δspo0A*::*spc bacA*::*lacZ*::*erm*	This study
E. coli DH5α	F' Φd*lacZ*Δ(*lac*ZY A-argF)U169 *sup*E44λ¯*thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17	American Type Culture Collection; Hanahan (1983)

2.5. Maintenance of Bacterial Strains

B. subtilis strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates. *E. coli* and *S. aureus* strains were kept on Luria-Bertani (LB) agar plates. All cultures were stored at 4°C and subcultured monthly. 20 % glycerol stock was prepared for each strain and kept at -80°C.

2.6. DNA Techniques and Manipulations

2.6.1. Plasmid DNA Isolation

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

E. coli plasmid DNA was also prepared by the plasmid miniprep method described by Hopwood *et al*. (1985). Each strain was grown as a patch on selective medium, LB agar containing 100µg/mL ampicillin. About 1 square cm of cell mass was scraped with a sterile toothpick and put into Eppendorf tube containing 100 µL cold STE solution containing 2 mg/mL lysozyme (Appendix B). Each tube was mixed by vortexing to disperse the cells and the toothpick was discarded. The tubes were then incubated on ice for 20 min. 3/5 volume of lysis solution (Appendix B) was added to each tube and vortexed immediately. The mixture was incubated at room temperature for 10 min to lyse the cells and then at 70°C for 10 min to denature DNA. Then, tubes were cooled rapidly in cold water. An equal amount of phenol-chloroform (water saturated, Appendix B) was added, vortexed hard until a homogeneous and milky white mixture was obtained. Finally, the samples were spinned for 5 min at 13,000 rpm to separate phases. 10 µL of supernatant was loaded directly on an agarose gel for electrophoresis.

2.6.2. Chromosomal DNA Isolation

Chromosomal DNA of *B. subtilis* strains was isolated and purified by using a standart procedure devised for *Bacillus* species (Cutting and Horn, 1990).

1.5 mL of overnight culture was centrifuged at 13000 rpm for 5 minutes. The pellet was handled by discarding the supernatant and resuspended in 567 µL of TE buffer (Appendix B) by repeated vortexing. Then, 10 µL of proteinase K (20 mg/mL), 6 µL of RNase (10 mg/mL), 24 µL of lysozyme (100 mg/mL) and 30 µL of 10% SDS were added and the mixture was incubated for 1 h at 37°C water bath. After addition of 100 µL of 5M NaCl solution, the sample was mixed without vortexing until the mucosal white substance become visible. Following, 80 µL of CTAB/NaCl (Appendix B) (prewarmed at 65°C) solution was added and the mixture was incubated for 10 minutes in 65°C water bath. The sample was then extracted with the same volume of freshly prepared phenol/chloroform/isoamyl alcohol (25:24:1) solution and centrifuged at 13000 rpm for 10 minutes. At a later stage, the upper phase was transferred to a new 1.5 mL microfuge tube and 0.7 volume isopropanol was added. After mixing shortly the sample was centrifuged at 13000 rpm for 15 minutes. Later the supernatant was removed and the pellet was washed with 1 mL 70% ethanol centrifuged at 13000 rpm for 5 minutes. Subsequently, the pellet was dried at 37°C for 1 h and dissolved in 10 µL of TE buffer and stored at 4°C. Finally, the isolated DNA was made run on 0.6% agarose gel and the absorbance values at 260 nm and 280 nm were read to determine the concentration and purity of the DNA. In order to calculate the concentration of DNA, the equation used was "concentration (µg/mL) = A_{260} x 50 x dilution factor".

2.6.3. Polymerase Chain Reaction

The oligonucleotide primers were purchased from IONTEK, Co. *bacA* F and *bacA* R are the forward and the reverse primers for the amplification of *bacA* fragment to be cloned, respectively (Table 2.2). pMutinT3 F and pMutinT3 R are the forward and the reverse primers for *erm* resistance gene on pMutinT3 (Table 2.2). In PCR, all

cycles lasted for 1 minute. The denaturation temperature was 94°C and the extention temperature was 72°C. The annealing temperature for the first 5 cycles was 55°C and 60°C for the next 25 cycles. The concentration of chromosomal DNA was 0.01 to 0.001 ng/µL. Primers were used at 1-10 pM (equimolar) and deoxyribonucleoside 5'triphosphates (dNTPs) were used at a final concentration of 2 mM.

Table 2. 2. Sequences and locations of oligonucleotide primers

Primer	Oligonucleotide Sequence	Target Sequence	Location
bacA F	5'-GCC AGG CTT ATG ATT ATA TTG GAT AAT-3'	420 bp long	From 1 bp to 420 bp ORF of *bacA* gene
bacA R	5'-GCG GAT CCG GAT AAA TAT TTT ATT AAA-3'		
pMutinT3 F	5'-TAA GCA GGT TCG TGT TCG TGC-3'	703 bp long	*erm* gene on pMutinT3
pMutinT3 R	5'-GCT GGC AGC TTA AGC AAT TGC-3'		

2.6.4. Agarose Gel Electrophoresis

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus. 0.8% agarose gel was prepared in TAE buffer (Appendix B) and ethidium bromide was added to give a 0.5 µg/mL final concentration in melted agarose gel. Gel loading dye (6X) was added to the samples depending on the sample amount. Electrophoresis was performed at 90 Volts for 45-60 min. The DNA bands were visualized on a shortwave UV transilluminator (UVP) and photographed by using

Vilber Lourmat Gel Imaging System. *Pst*I digested lambda DNA marker (Appendix D) was used to determine the molecular weights of DNA bands.

2.6.5. Gel Extraction

The desired fragments were extracted from the gel by using a Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA). The gel slice containing the DNA band was excised from the gel and weighed. Gel extraction was performed according to the Qiagen's instructions. After recovery, an aliquot was run on agarose gel to assess the yield.

2.6.6. Ligation

Ligation of PCR products to pGEM-T vector was performed as follows: 5 µL 2X ligase buffer, 1 µL (55 ng/µL) pGEM-T vector, 500 ng insert DNA, 1 µL T4 DNA ligase (3 Unit/µL) was mixed and volume was completed to 10 µL with H_2O. Ligation was carried out as overnight incubation at 4^oC. When the pMutinT3 vector was used, 9.5 µL of *bacA* PCR products as insert fragments and 0.5 µL of pMutinT3 vector were mixed in an Eppendorf tube and incubated for 5 min at 65°C. Then, the tube was cooled on ice and spanned down to collect the whole mixture. Following, 2 µL of ligation 10x buffer, 2 µL of Polyethylene glycol (50% PEG 8000), 2 µL of T4 DNA ligase, 4 µL of dH_2O were added into the same Eppendorf tube. Finally, the mixture was again centrifuged for a quick spin and incubated at 4°C for 16 h.

2.6.7. Restriction Enzyme Digestion

Restriction enzymes (*Bam*HI and *Hind*III) were added in a suitable buffer to the DNA to introduce 2 Units per µg of DNA. The mixture was incubated at a temperature appropriate for that restriction enzyme for 3-5 h. The sample was stored at -20^oC when needed.

2.7. Transformation

2.7.1. Preparation of *E. coli* DH5α $CaCl_2$ Competent Cells and Their Transformation

E. coli competent cells were prepared according to the protocol described by Sambrook *et al*. (1989) with slight modifications. In a 250 mL flask, 5–50 mL of LB broth (Appendix A) was inoculated with *E. coli* from a fresh LB agar plate and incubated overnight with shaking at 37°C to obtain a stationary phase culture. 300 µL from this seed culture was inoculated into a fresh flask containing 50 mL LB broth. The culture was incubated for 2–2.5 h at 37°C with vigorous shaking (300 rpm) in an orbital shaker to obtain an exponentially growing culture. Then the culture was split into two sterile pre-chilled 40 mL screwcap centrifuge tubes asepticaly and stored on ice for 10 min. After centrifuging at 4,000 rpm for 10 min at 4°C, supernatants were decanted and each pellet was resuspended in 5 mL of ice-cold 10 mM $CaCl_2$ by vortexing. The cells were spun down at 3,000 rpm for 10 min at 4°C. Finally, supernatants were decanted and each pellet was resuspended gently in 1 mL ice-cold 75 mM $CaCl_2$. The competent cells were stored at – 80°C.

For transformation, competent *E. coli* cells were kept on ice for 15 min. 10 µL of ligation products or 0.5 µg of appropriate plasmid DNA was added to the cells and mixed gently. The mixture was incubated on ice for 30 min. After a heat shock at 42°C for 90 sec, it was incubated on ice for 5 min. 900 µL of LB was added to the mixture and incubated at 37°C for 80 min by gentle agitation (100 rpm). The cells were microfuged at 4,000–5,000 rpm for 10 min and resuspended in 500 µL saline solution. Transformed cells were plated on selective medium containing appropriate antibiotic (100µg/mL ampicillin). For blue-white colony selection, they were plated on LB agar media containing 80 mg/mL X-gal, 0.5 mM IPTG and 100µg/mL ampicillin.

2.7.2. Preparation of *B. subtilis* Component Cells and Transformation

Preparation of *B. subtilis* competent cells and transformation were performed as described by Klein *et al* (1992). HS and LS (Appendix A) mediums were used for the preparation of *B. subtilis* competent cells. 3 mL of overnight culture was prepared in the HS medium by incubation at 37°C and shaking at 250 rpm. 0,5 mL of this overnight inoculum was transformed into 20 mL of LS medium and incubated at 30°C with shaking at 100 rpm until OD_{600} of cultures reached to 0,55. 1 mL of competent cells was transferred into 2 mL Eppendorf tube and 1 µL of DNA was added. Cells were then incubated at 37°C for 2 h with shaking at 250 rpm and were harvested by centrifugation at 5000 rpm for 15 minutes. Cells were resuspended in 100 µL of sterile saline and spread out on selective LB agar plates and incubated at 37°C for 16 h.

2.7.2.1. Induction of MLS Resistance Gene

After the transformation was carried out as explained above and before plating transformed cells onto LB agar plates containing selective antibiotics; the following procedure was used: First of all, 4 µL of 10 mg /mL erythromycin solution was added to a small test tube containing 25 mL LB overlay agar that contains % 0.7 agar in it and was kept molten at 45°C. Then, transformed cells were added to this tube and were poured onto an antibiotic-free LB agar plate (containing 35-40 mL agar). After incubation at 37°C for 1.5 h, a second overlay of LB soft agar containing 40 µL of a 1 mg/mL/25 mg/mL erythromycin/lincomycin mixture was poured onto the first overlay. Colonies were grown out through the surface of the agar plate, after incubation at 37°C for 1-2 days.

2.8. β-galactosidase Assays

Initially, all strains were grown overnight at 37°C in PA medium (Appendix A). Then, they were used to inoculate 100 mL of PA medium to an initial optical density of about 0.1 at 595 nm (OD_{595}). Later, the cultures were incubated at 37°C (250

rpm) for 24 h. Starting from t_0 that was defined as the point at which cells are resuspended, 1 mL of culture was taken as duplicates at each hour for the later execution of β-galactosidase assay. For the measurement of growth at OD_{595}, the necessary amount of cultures was taken as dilutions. The following procedure was applied to the 1 mL of cultures:

After each sampling, the culture was centrifuged at 13000 rpm for 5 min and the supernatant was discarded. Then, the pellet was washed with 500 µL of ice-cold 25 mM Tris-Cl (pH 7.4) by centrifugation. Following, the removal of the supernatant, the pellet was washed in 0.5 ml of ice old 25 mM TrisHCl (pH 7.4) for 5 min at 13000 rpm in a microfuge. Then supernatant were discarded and cells were put on ice.

Following the removal of the supernatant, the pellet was resuspended in 640 µL of Z-buffer via vortexing and 160 µL of lysozyme was added. Later, the solution was vortexed for a second and incubated at 37°C for 5 min. Subsequently, the samples were taken on ice and 8 µL of 10% Triton-X100 was added. After vortexing for a while, the extracts were stored on ice.

β-galactosidase assay was continued by prewarming the extracts in 30°C water bath for 5 min. Subsequent to this, 200 µL of ONPG solution was added and the solution was controlly watched for a yellowish color formation. Following the complete settlement of yellowish color, the reaction was stopped by adding 0.4 mL of 1 M Na_2CO_3 and reaction time was recorded. Reaction time refers to a period that was started by adding ONPG and continued until the settlement of the yellow color. At last, the samples were centrifuged at 13000 rpm for 5 min and the supernatant was taken to measure A_{420} and A_{550} of it. Calculations for *β*-galactosidase activity were carried out according to the formulation below (Miller, 1972) and graphs for β-galactosidase activities were drawn.

$$\text{Miller units} = \frac{A_{420} - (1.75 \times A_{550})}{\text{Reaction time (min)} \times OD_{595}} \times 1000$$

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Construction of *bacA* Insertional Plasmid

To examine the expression of *bacA* operon, we constructed a *B. subtilis* strain, OGU1 containing a transcriptional *bacA-lacZ* fusion at *bacA* locus. For this purpose, we used the *B. subtilis* integration vector pMUTIN T3. Upon the integration of pMUTIN T3 vector into the target gene, *lacZ* reporter gene becomes transcriptionally fused to the target gene, allowing its expression profile to be monitored.

For integration of pMUTIN T3 vector into *B. subtilis* PY79 *bacA* gene locus, initially a 420 bp long fragment, 5'- *bacA* gene fragment was amplified by PCR using the chromosomal DNA of wild type strain *B. subtilis* PY79 as a template. The primers *bacA* F and *bacA* R (Table 2.2) used for the amplification of *bacA* fragment contained the extra residues including a *BamH*I or *Hind*III site at their 5' ends, respectively. Thus, a total of 437 bp PCR product was obtained by PCR (Fig. 3.1).

437 bp *bacA* PCR fragment was purified from the gel and ligated into pGEM-T vector. After transformation of *E. coli* DH5α cells with the ligation product, white colonies containing recombinant pGEM-T were selected on X-gal/IPTG plus ampicillin-containing agar plates. pGEM-T carrying 437 bp insert were isolated from a recombinant colony and digested with *BamH*I and *Hind*III to release the cloned insert DNA (Figure 3.2). Cloning into pGEM-T vector was also verified by

PCR amplification of the gene using putative recombinant plasmid as the template (Fig. 3.2).

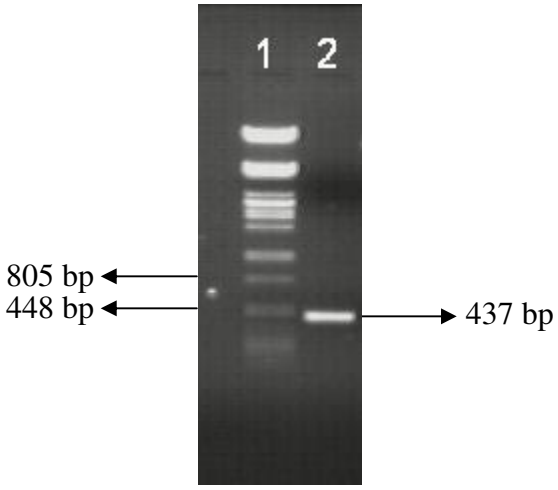


Figure 3. 1. 437 bp *bacA* PCR product obtained with primers *bacA* F and *bacA* R designed for *bacA* gene. Lane 1: Lambda DNA/*Pst*I size marker (Appendix C), Lane 2: *bacA* gene (437 bp).

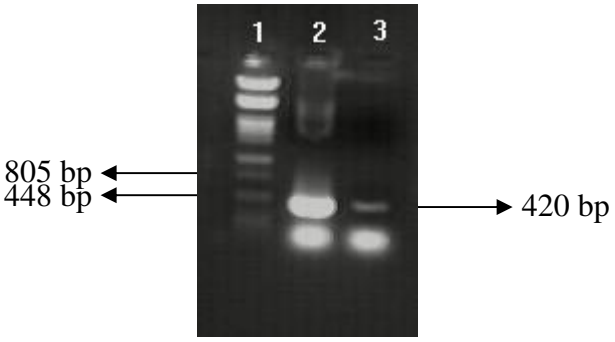


Figure 3. 2. Double-digested recombinant pGEM-T plasmid carrying *bacA* gene and PCR product using recombinant pGEM-T as a template. Lane 1: Marker (Lambda DNA/*Pst*I), Lane 2: 437 bp PCR product of *bacA* gene by using putative recombinant plasmid as the template, Lane 3: Recombinant pGEM-T digested with *BamH*I and *Hind*III restriction endonucleases.

Recombinant pGEM-T plasmid was isolated from the transformant *E. coli* cells for cloning of *bacA* gene into the integrative plasmid pMUTIN T3 vector. The recombinant pGEM-T and pMUTIN T3 vectors were digested with *BamH*I and *Hind*III restriction enzymes and the digests were run in an agarose gel. Linearized pMUTIN T3 and *bacA* fragment released from pGEM-T vector were extracted from the gel and ligated. The ligation product was used to transform *E. coli* DH5α cells and putative recombinant colonies were selected on 100 $\mu g.mL^{-1}$ ampicillin-containing agar plate. The recombinant pMUTIN T3 plasmid of an expected size (9254 bp) was isolated from the transformant (Fig. 3.3) and the cloning of 420 bp *bacA* PCR fragment into the pMUTIN T3 vector was verified with *BamH*I and *Hind*III double digestion (Fig. 3.4).

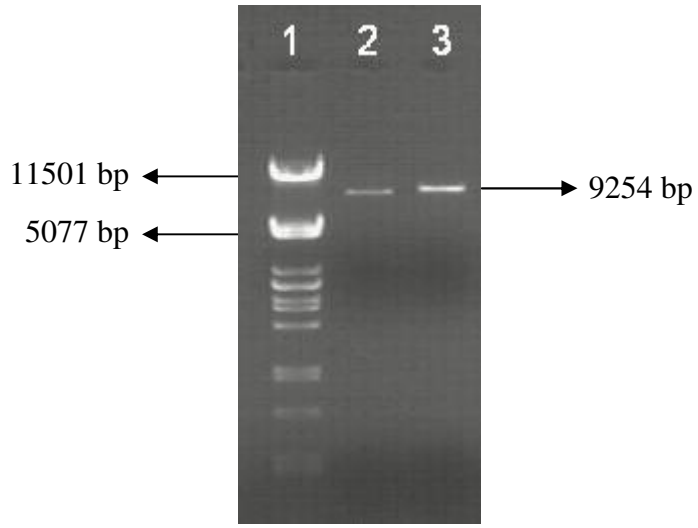


Figure 3. 3. pMutinT3 carrying the *bacA* gene fragment. Lane 1: Marker (Lambda DNA/*Pst*I), Lane 2: pMUTIN T3 vector (8834 bp), Lane 3: Recombinant pMUTIN T3 carrying *bacA* gene fragment.

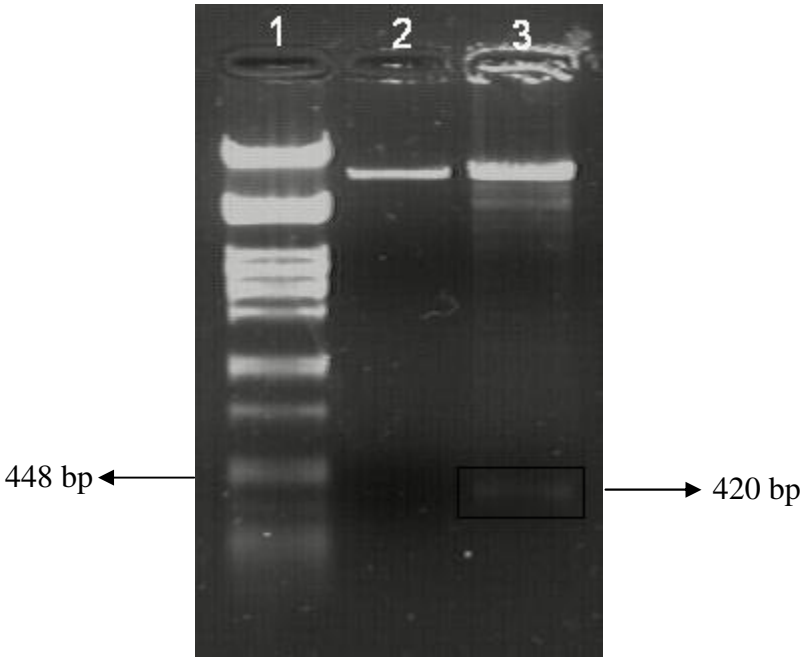


Figure 3. 4. Verification of cloning of the *bacA* gene into pMUTIN T3 by double digestion with *BamH*I and *Hind*III restriction enzymes. Lane 1: Marker (Lambda DNA/*Pst*I), Lane 2: pMUTIN T3 vector, Lane 3: Digestion products.

Thus, it was concluded that the plasmid constructed was a desired recombinant containing the 420 bp *bacA* insert fragment in its structure. The structure of the resulting recombinant pMUTIN T3 plasmid vector is shown in Fig. 3.5.

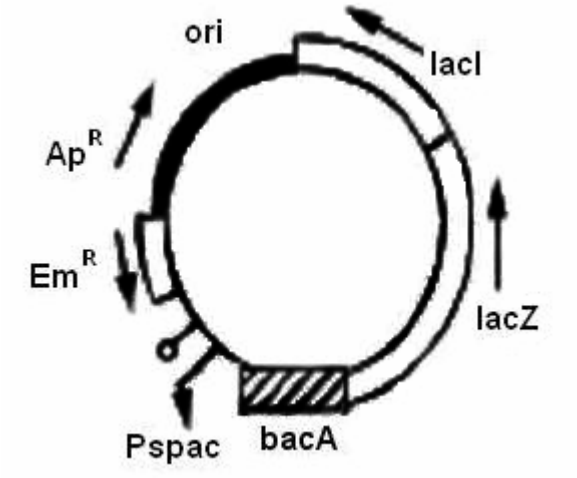


Figure 3. 5. *bacA*::*lacZ* fusion construct in pMUTIN T3.

3.2. Construction of *bacA*::*lacZ* Transcriptional Fusion in *B. subtilis*

For permanent construction of *bacA*::*lacZ* transcriptional fusion in *B. subtilis*, the recombinant pMUTIN T3 plasmid DNA was used to transform *B. subtilis* PY79 to erythromycin resistance gene (Erm^R) by integration through a single cross-over event at *bacA* locus (Campbell-like insertion). ColE1 origin of replication on pMUTIN T3 enables replication in *E. coli* but not in *B. subtilis.* The transformants would arise only by recombination between cloned *bacA* fragment and its counterpart in the chromosome. Therefore, integration of plasmid into *bacA* gene locus by single crossover resulted in *bacA*::*lacZ* transcriptional fusion.

However, the selection for erythromycin resistance on LB plates containing erythromycin (1µg/mL) could not be readily achieved due to the generation of the spontaneous erythromycin resistant *B. subtilis* PY79 cells. To overcome this problem, the selection method for Macrolide-Lincosamide-Streptogramin B-resistance (MLS^R) conferred by *erm* gene was used (Section 2.7.2.1). The blue colony containing recombinant pMUTIN T3 was selected on X-gal/IPTG plus eryhtomycin and lincomycin-containing agar plate. Chromosomal DNA of the resultant MLS^R transformant was isolated and screened with PCR analysis by using

primers specific to *erm* resistance gene on pMUTIN T3 in order to verify the insertion of the recombinant into the chromosome. As seen in Fig. 3.6 (lane 4), 703 bp *erm* gene was amplified by using the chromosomal DNA of MLS^R *B. subtilis* transformant as a template. It was concluded that the recombinant plasmid integrated into the chromosome by Campbell recombination and the corresponding MLS^R *B. subtilis* PY79 transformant was designated as OGU1.

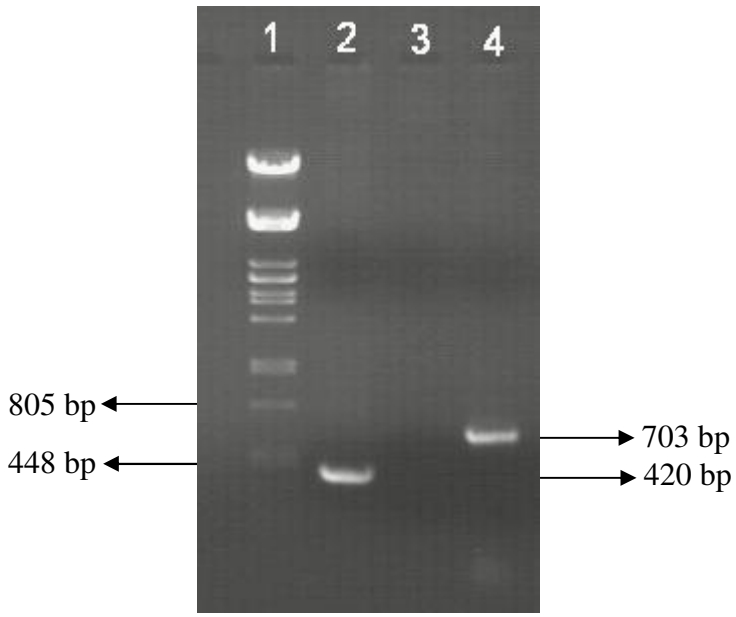


Figure 3. 6. Verification of the construction of *bacA*::*lacZ*::*erm* strain (OGU1) via PCR. Lane 1: Marker (Lambda DNA/*Pst*I), Lane 2: PCR product of *bacA* gene, Lane 3: Negative control via PCR by using chromosomal DNA of *B. subtilis* PY79, Lane 4: PCR product for *erm* gene on pMUTIN T3 by using the chromosomal DNA of MLS^R resistant mutant (OGU1).

3.3. Expression of Transcriptional *bacA-lacZ* Fusion in PA Medium

To examine the expression of *bacA,* OGU1 and wild type *B. subtilis* PY79 cells were grown in PA medium at 37°C and the β-galactosidase activity of cells was measured at various stages of growth. As seen in Fig. 3.8, in the PA medium,

expression of bacA-*lacZ* fusion was relatively constant during exponential growth (detected as 20-30 Miller units), but increased the transition between exponential and stationary phase and reached to its maximal level (detected as 130 Miller units) upon entry into stationary as consistent with the measured bacilysin activity (Fig. 3.7) which accumulated at the beginning of the stationary phase during growth in PA medium.

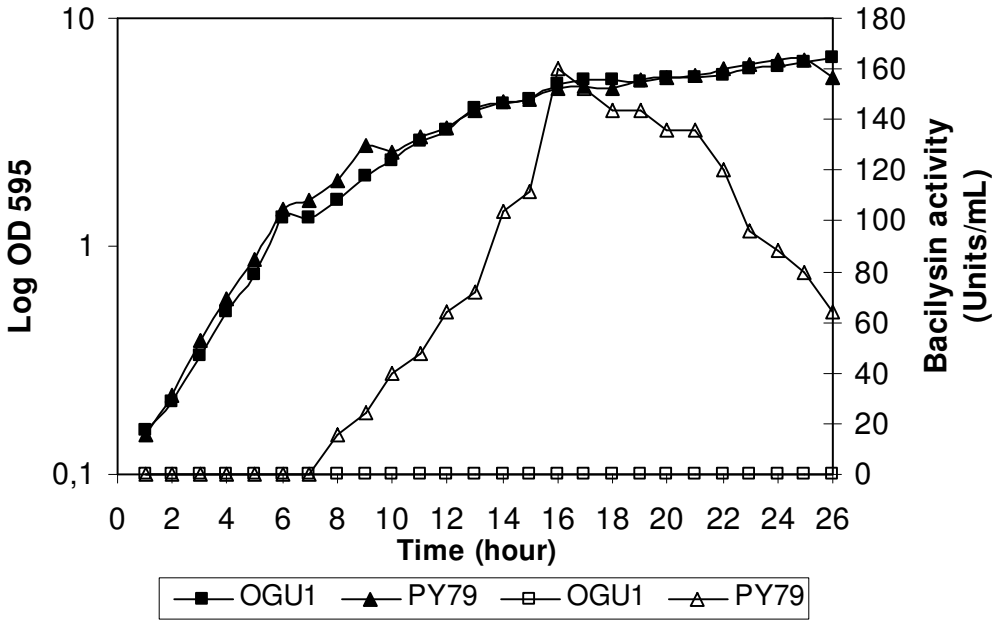


Figure 3. 7. Growth and bacilysin activity of *B. subtilis* PY79 and OGU1 (*bacA*::*lacZ*::*erm*) strains.

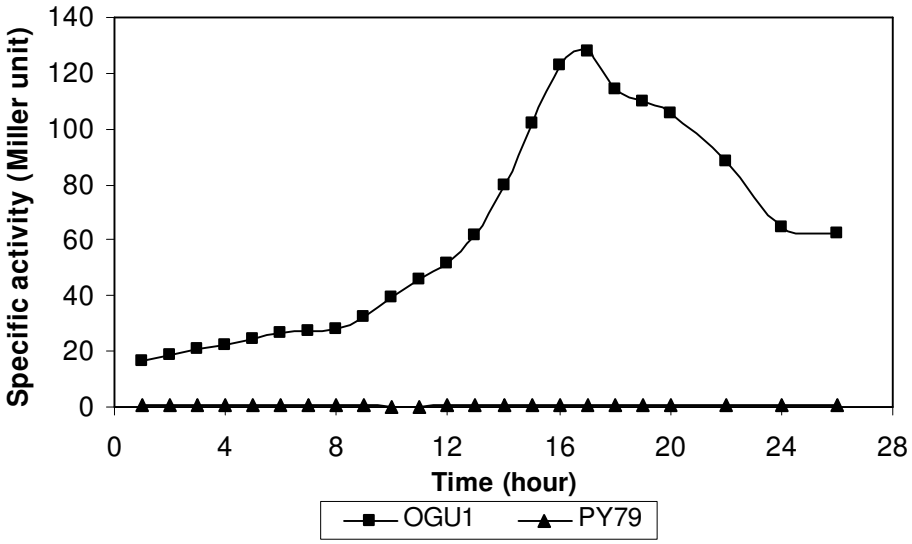


Figure 3. 8. Expresion of transcriptional *bacA-lacZ* fusion in PA medium.

3.4. Insertional Inactivations of Quorum Sensing Genes and Their Effects on the Expression of *bacABCDE* operon in *B. subtilis*

3.4.1. Insertional Inactivation of *srfA* Gene and Its Effect on the Expression of *bacABCDE* Operon in *B. subtilis*

srfA operon covers a DNA region of about 29 kb containing four modular open reading frames (ORFs); ORF1 (*srfA*-A),ORF2 (*srfA*-B), ORF3 (*srfA*-C) and ORF4 (*srfA*-D). The first three of these ORFs encode the three subunits of surfactin synthetase and each is able to act as free distinct enzyme. These subunits, together, catalyse the formation of heptapeptide portion (Glu-Leu-Leu-Val-Asp-DLeu-Leu) of the lipopeptide antibiotic surfactin (Nakano *et al*., 1991; Fuma *et al*., 1993; Nakano *et al*., 1988; Vollenbroich *et al*., 1994). The studies of Nakano *et al*. (1991) provided the evidence that 5' half of the *srfA* operon is required for three processes; surfactin biosynthesis, competence development and sporulation, whereas the 3' half of *srfA* operon is required for only sporulation and surfactin biosynthesis. Recent studies which revealed that the *srfA* operon contains the competence regulatory gene *comS*

as another ORF residing within and out of frame with *srfAB* (Hamoen *et al*., 1995; van Sinderen *et al*., 1993) elucidated the function of *srfA* operon in the competence development.

To analyze the effects of *srfA* null mutation, insertional inactivation of *srfA* gene in OGU1 (*bacA*::*lacZ*::*erm*) was generated by transforming the competent cells of *ΔsrfA* $ComS^+$ MLS^R mutant strain of PY79 (constructed by transforming the competent cells of PY79 with chromosomal DNA of KE10) with chromosomal DNA of the OGU1 strain with the selection for Erm^R (1µg/ml) and Ln^R (25µg/ml). The transformants were then screened on X-gal containing agar plate. One of the blue transformants was selected and named as OGU1RF (*srfA*::*erm bacA*::*lacZ*::*erm*).

The resulting strain (OGU1RF) and OGU1 as the control were cultured in PA medium and the β-galactosidase activities of the cells were measured at intervals in various stages of growth (Fig. 3.9).

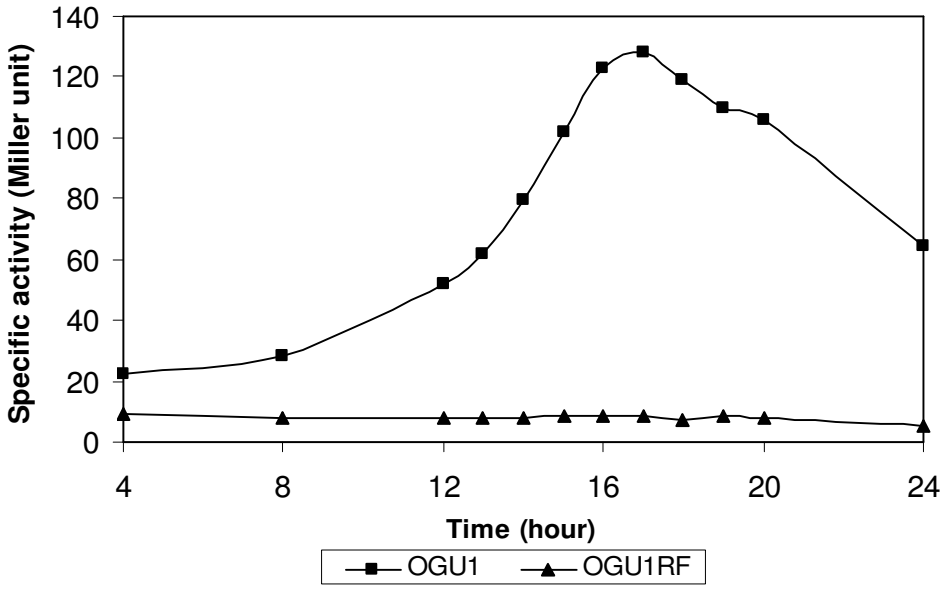


Figure 3. 9. β-galactosidase activities of OGU1 and OGU1RF in PA medium.

As can be seen in Fig. 3.9, the disruption of *srfA* gene completely abolished *bacA-lacZ* expression, suggesting that *bacABCDE* operon expression depends on a functional surfactin operon. Surfactin biosynthesis has been shown to be linked to initiation of sporulation and competence development via two pheromone ComX- and CSF-dependent pathways (Magnuson *et al*., 1994; Solomon *et al*., 1996; Lazazzera, 1997).

3.4.2. Insertional Inactivation of *oppA* Gene and Its Effect on the Expression of *bacABCDE* Operon in *Bacillus subtilis*

B. subtilis opp operon encodes five proteins—OppA, OppB, OppC, OppD, and OppF. OppA binds to the peptide substrate and facilitates its uptake by interacting with and delivering the peptide to the OppBCDF complex (Perego *et al*., 1991). *opp* was first identified as a sporulation locus, named *spo0K*, because null mutations caused decreased sporulation efficiency (Rudner *et al*., 1991; Perego *et al*., 1991).

Insertional inactivation of *oppA* gene in OGU1 (*bacA*::*lacZ*::*erm*) was generated by transforming the competent cells of OGU1 with the chromosomal DNA of the *oppA*::*Tn10(spc)* mutant strain AK3 with selection for Erm^R (1µg/ml) and Spc^R (100µg/ml). The transformants were then screened on an agar plate. One of the transformants was selected and named as OGU1O (*oppA*::*Tn10(spc) bacA*::*lacZ*::*erm*).

The resulting strain (OGU1O) and OGU1 as the control were cultured in PA medium and the β-galactosidase activities of the cells were measured at intervals in various stages of growth (Fig. 3.10).

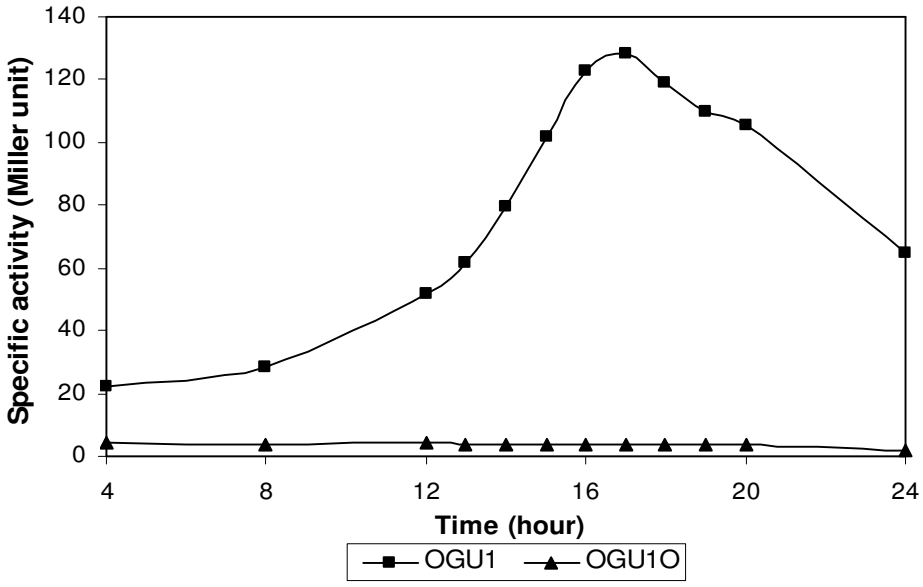


Figure 3. 10. β-galactosidase activities of OGU1 and OGU1O in PA medium.

As shown in Fig. 3.10, *bacA-lacZ* expression was severely impaired in the *oppA* blocked mutant, indicating that *oppA* gene product regulates bacilysin biosynthesis by affecting the expression of *bac* operon. The *B. subtilis* Opp oligopeptide permease contributes to the regulation of sporulation, competence, and also bacilysin biosynthesis, at least in part, by importing specific signaling peptides derived from *phr* gene products (Lazazzera and Grossman, 1998; Lazazzera, 2001; Yazgan *et al*., 2001; Perego and Brannigan, 2001; Pottathil and Lazazzera, 2003). Very recently, it was shown that ComA-dependent gene expression is stimulated by Phr peptides including PhrC, PhrF and PhrK and that all three peptides are required for full expression of ComA-dependent genes (Auchtung *et al*., 2006) and the involvement of ComA and PhrC in bacilysin biosynthesis was shown (Yazgan *et all*., 2001). Therefore, Opp-imported pheromones PhrF and PhrK besides PhrC (CSF) are thought to have functions in the expression of *bacA* operon. To test this possibility, we examined the expression of *bacA-lacZ* fusion in the PhrC, PhrF and Phr K null mutant backgrounds, as shown in the subsequent sections.

3.4.3. The Effects of Phr Peptides on the Expression of *bacABCDE* Operon in *B. subtilis*: Insertional Inactivation of *phrC*, *phrF* and *phrK* Genes

Insertional inactivation of *phrC* gene in OGU1 (*bacA*::*lacZ*::*erm*) was generated by transforming the competent cells of *phrC*::*erm* PY79 (constructed by transforming the competent cells of PY79 with chromosomal DNA of JMS751) with chromosomal DNA of the OGU1 strain with the selection for Erm^R (1µg/ml) and Ln^R (25µg/ml). The transformants were then screened on X-gal containing agar plate. One of the blue transformants was selected and named as OGU1PC (*phrC*::*erm bacA*::*lacZ*::*erm*).

Insertional inactivation of *phrF* gene in OGU1 (*bacA*::*lacZ*::*erm*) was generated by transforming the competent cells of OGU1 with chromosomal DNA of the *ΔphrF163*::*cat* mutant strain JMA163 with the selection for Erm^R (1µg/ml) and Cm^R (5 µg/ml). The transformants were then screened on an agar plate. One of the transformants was selected and named as OGU1PF (*ΔphrF163*::*cat bacA*::*lacZ*::*erm*). OGU1PK (*ΔphrK7*::*spc bacA*::*lacZ*::*erm*) strain was generated in the same way by using the chromosomal DNA of CAL7 strain as the donor.

The resulting strains (OGU1PC, OGU1PF and OGU1PK) and OGU1 as the control were cultured in PA medium and the β-galactosidase activities of the cells were measured at intervals in various stages of growth (Fig. 3.11, 3.12, 3.13).

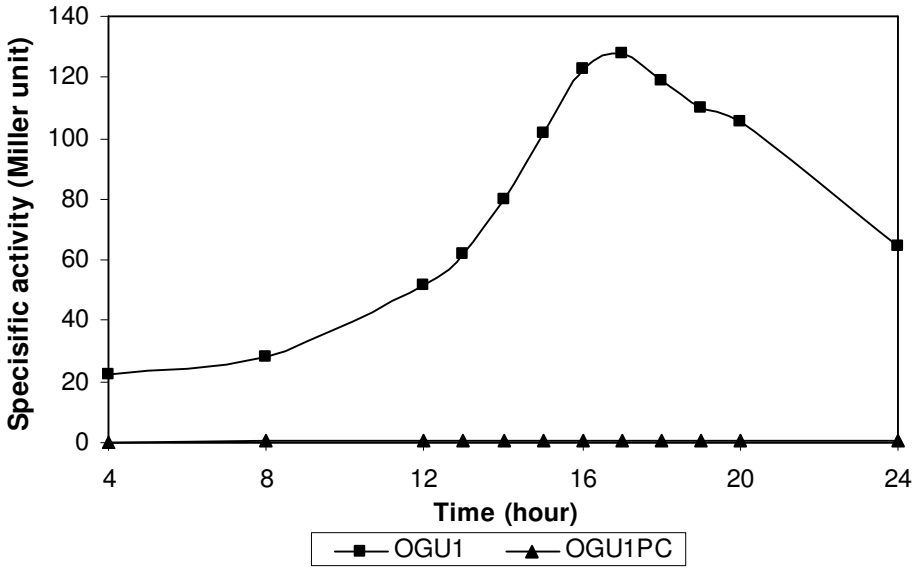


Figure 3. 11. β-galactosidase activities of OGU1 and OGU1PC in PA medium.

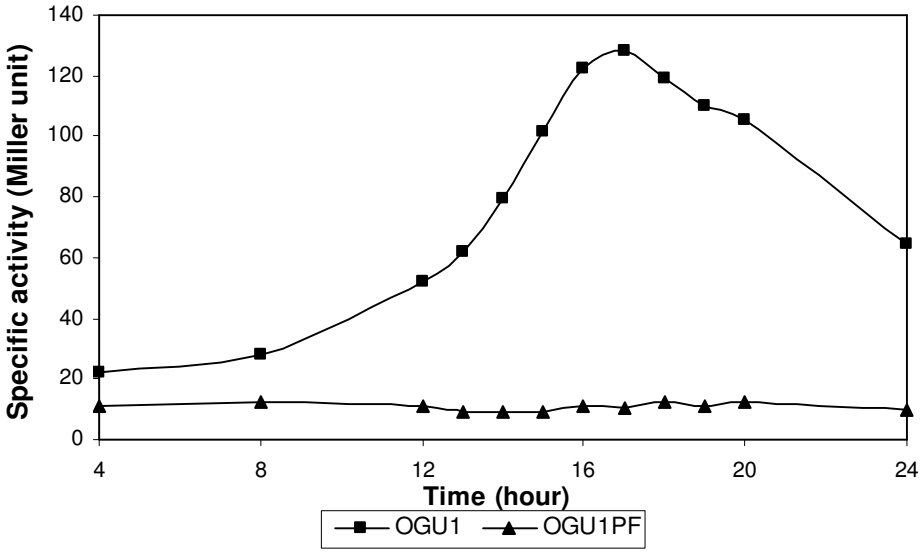


Figure 3. 12. β-galactosidase activities of OGU1 and OGU1PF in PA medium.

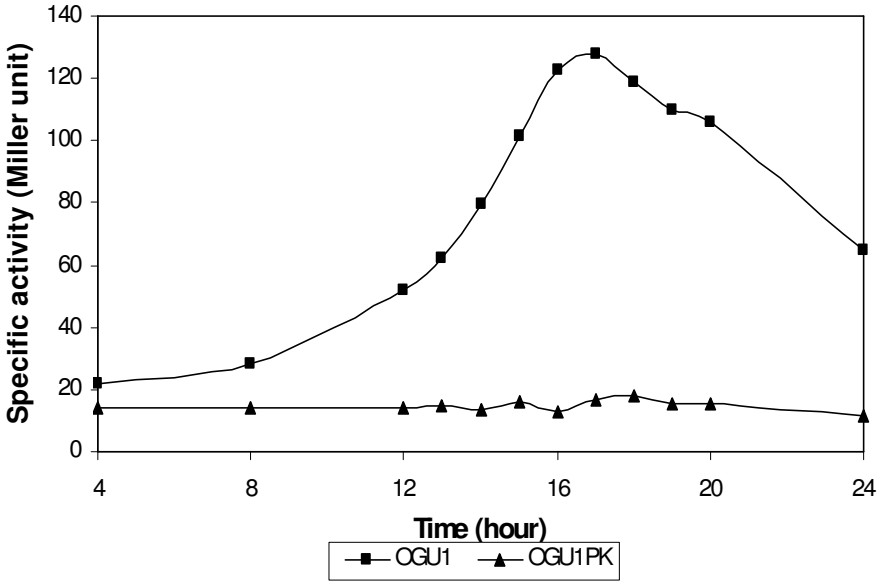


Figure 3. 13. β-galactosidase activities of OGU1 and OGU1PK in PA medium.

As shown in Fig 3.11, Fig. 3.12 and Fig. 3.13, the deletion of *phrC*, *phrF* and *phrK* genes impaired *bacA-lacZ* expression, indicating that the Phr C, F and K peptides are required for the expression of *bacABCDE* operon, most likely by enhancing the full activation of transcriptional factor ComA~P as mentioned before.

Besides the Phr peptides internalized by Opp, activation of ComA directly enhanced by a membrane-bound histidine protein kinase, ComP via donating phosphate to its couple ComA in response to stimulating by signal peptide ComX which is processed and secreted by ComQ (Ansaldi *et al*., 2002; Magnuson *et al*., 1994; Solomn *et al*., 1996). On the other hand, most of the *phr* genes have sigma H promoters upstream of the gene and internal to the cognate *rap* gene. Thereby, alternative sigma factor, σ^H enhances the level of each *phr* gene to increase as cells transition from exponential growth to stationary phase (Auchtung *et al*., 2006).

3.4.4. The Effects of *comA*, *comP*, *comQ* (*comX*) and *spo0H* Null Mutations on the Expression of *bacABCDE* Operon in *B. subtilis*

Possible involvement of ComQ(ComX), ComP/ComA and *spo0H* in the expression of *bacA-lacZ* was next investigated.

Insertional inactivation of *comA* gene in OGU1 (*bacA*::*lacZ*::*erm*) was generated by transforming the competent cells of OGU1 with chromosomal DNA of the *comA*::*cat* mutant strain JRL192 with the selection for Erm^R (1 µg/ml) and Cm^R (5 µg/ml). The transformants were then screened on an agar plate. One of the transformants was selected and named as OGU1CA (*comA*::*cat bacA*::*lacZ*::*erm*). OGU1CP (*comP*::*cat bacA*::*lacZ*::*erm*), OGU1CQ (*comQ*::*spc bacA*::*lacZ*::*erm*) and OGU1SH (*spo0H*::*cat bacA*::*lacZ*::*erm*) strains were generated in the same way by transforming OGU1 with the chromosomal DNA of BD1658, JMS315 and AG665 strains, respectively.

The resulting strains (OGU1CA, OGU1CP, OGU1CQ and OGU1SH) and OGU1 as the control were cultured in PA medium and the β-galactosidase activities of the cells were measured at intervals in various stages of growth (Fig. 3.14, 3.15, 3.16 and 3.17).

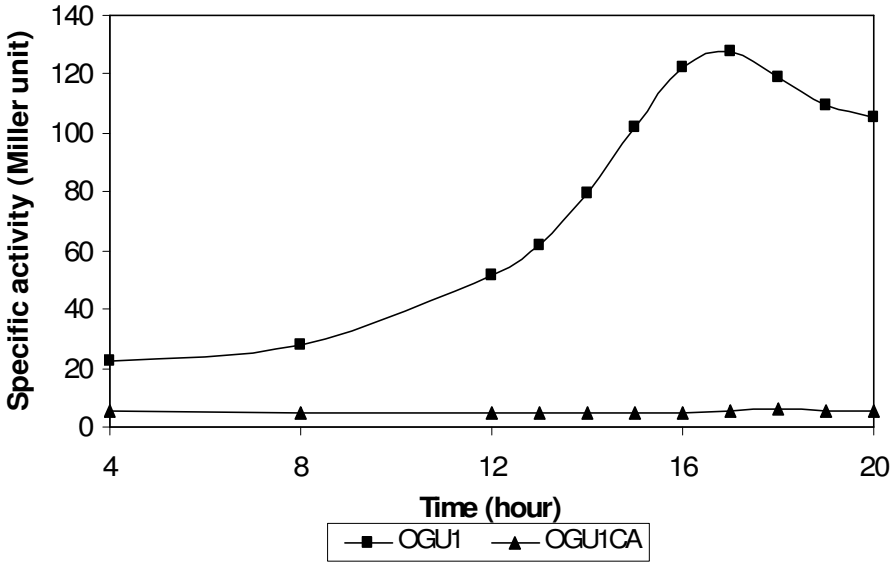


Figure 3. 14. β-galactosidase activities of OGU1 and OGU1CA in PA medium.

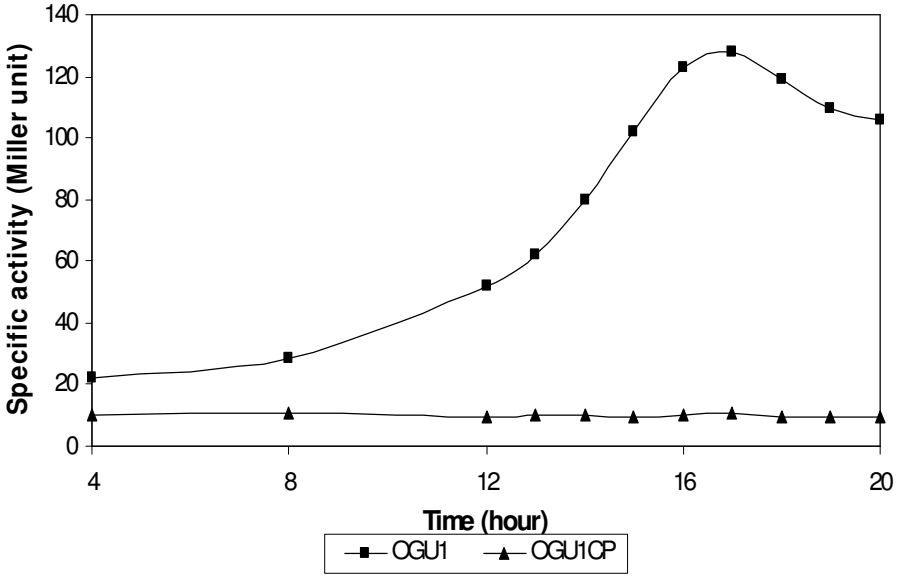


Figure 3. 15. β-galactosidase activities of OGU1 and OGU1CP in PA medium.

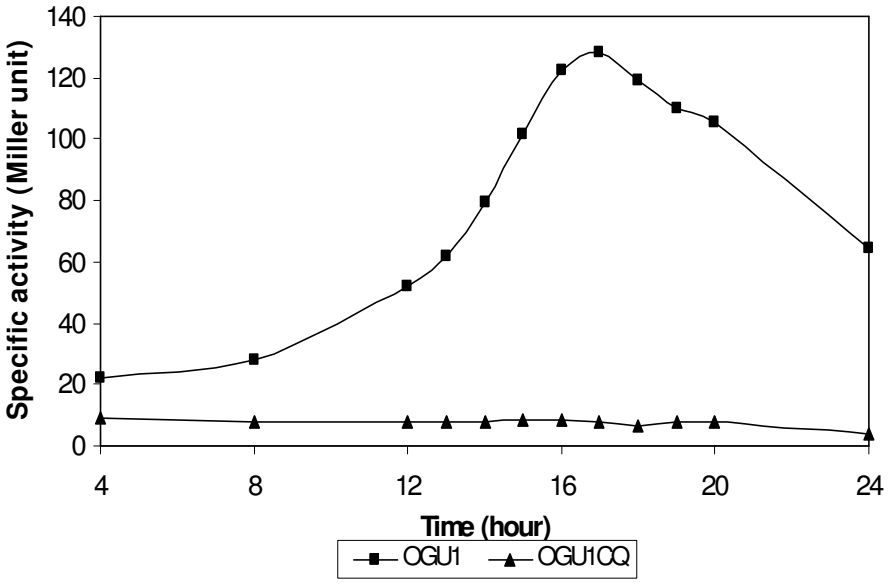


Figure 3. 16. β-galactosidase activities of OGU1 and OGU1CQ in PA medium.

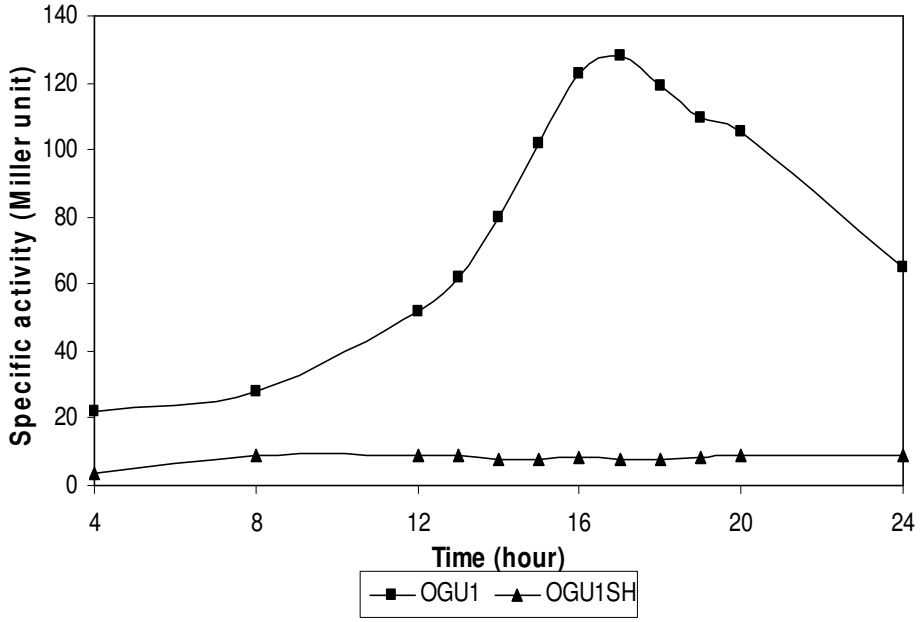


Figure 3.17. β-galactosidase activities of OGU1 and OGU1SH in PA medium.

As shown in Fig. 3.14, Fig 3.15, Fig. 3.16 and Fig. 3.17, β–galactosidase assays coupled to *bac* operon activity revealed that the deletion of *comA*, *comP*, *comQ and spo0H* genes completely abolished *bacA-lacZ* expression, indicating that these gene products regulate bacilysin biosynthesis by affecting the expression of *bac* operon. If we consider that ComP and ComX are required for phosphorylation and activation of ComA and σ^H is required for an increased level of *phr* gene during transition state, thereby enhancing the full activation of ComA, it is reasonable to suggest that the transcription of *bac* operon is ComA~P-dependent.

In *B. subtilis*, production of and resistance to antibiotics are known to be regulated by *spo0-abrB* global-control system (Ito *et al*., 1971). Consistently, it has been previously shown that bacilysin biosynthesis is under the negative control of AbrB transition state regulator which is relieved by Spo0A protein (Karataş *et al*., 2003). Therefore, the possible involvement of *spo0A-abrB* gene products in the regulation of *bacA* expression was also investigated, as explained in the next section.

3.4.5. The Effects *spo0A* and *abrB* Null Mutations on the Expression of *bacABCDE* Operon in *B. subtilis*

To analyze the effects of *spo0A* and *abrB* null mutations, *spo0A* and *abrB* genes were deleted in the transcriptional *bacA–lacZ* fusion bearing strain OGU1 by transforming the competent cells of OGU1 with chromosomal DNA from the *B. subtilis* strains AG504 (Δ*spo0A*::*cat*) and BAL373 (Δ*abrB*::*cat*) to generate OGU1S (*Δspo0A*::*spc bacA*::*lacZ*::*erm*) and OGU1A (*ΔabrB*::*cat bacA*::*lacZ*::*erm*) strains, respectively. On the other hand, we also analyzed the expression of *bacA-lacZ* fusion in Δ*spo0A*-Δ*abrB* double mutant genetic background since a *spo0A* mutant has a pleiotropic phenotype and contains higher levels of the AbrB protein than do wild–type strains due to repression of *abrB* expression by Spo0A. *spo0A-abrB* double mutant strain OGU1SA (*Δspo0A*::*spc ΔabrB*::*cat bacA*::*lacZ*::*erm*) was constructed by transforming the competent cells of OGU1S with chromosomal DNA of the *ΔabrB*::*cat* mutant strain BAL373 with selection for Erm^R (1μg/ml), Cm^R (5 μg/ml) and Spc^R (100μg/ml) on agar plate.

The resulting strains (OGU1S, OGU1SA and OGU1A) and the wild-type strain OGU1 were grown in PA medium at 37°C and β-galactosidase activities were measured at intervals in various stages of growth (Fig. 3.18, 3.20 and 3.22).

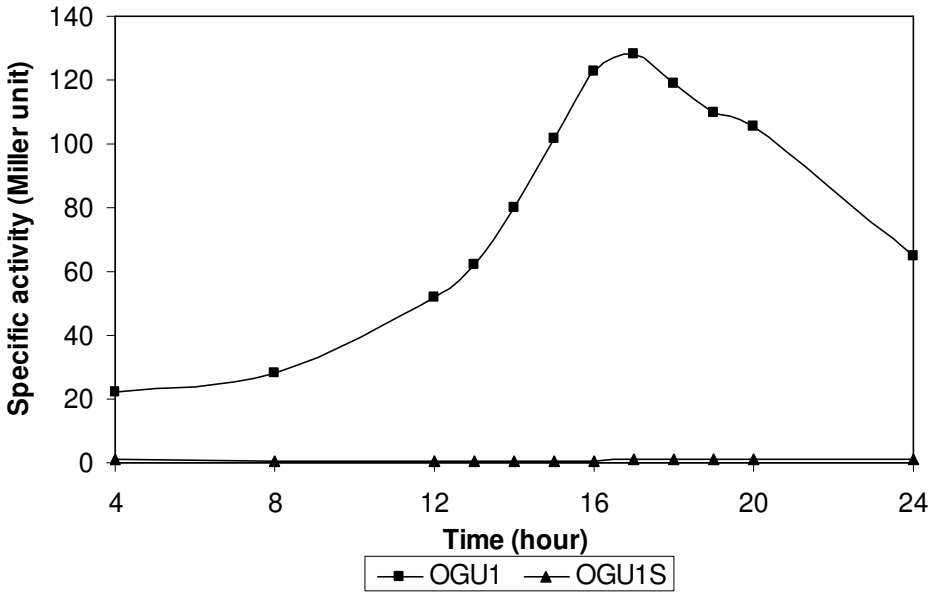


Figure 3. 18. β-galactosidase activities of OGU1 and OGU1S in PA medium.

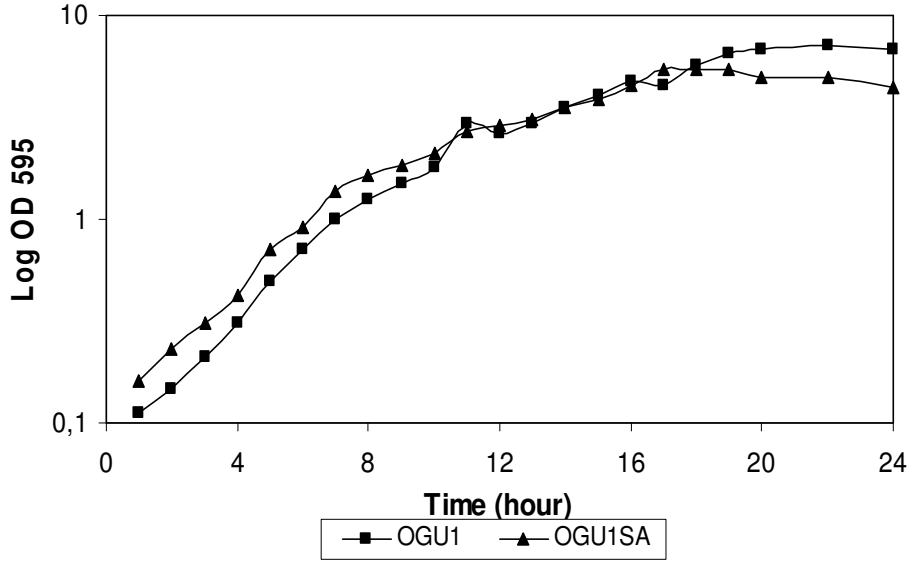


Figure 3. 19. Growth of OGU1 (*bacA*::*lacZ*::*erm*) and OGU1SA (*Δspo0A*::*spc ΔabrB*::*cat bacA*::*lacZ*::*erm*) in PA medium.

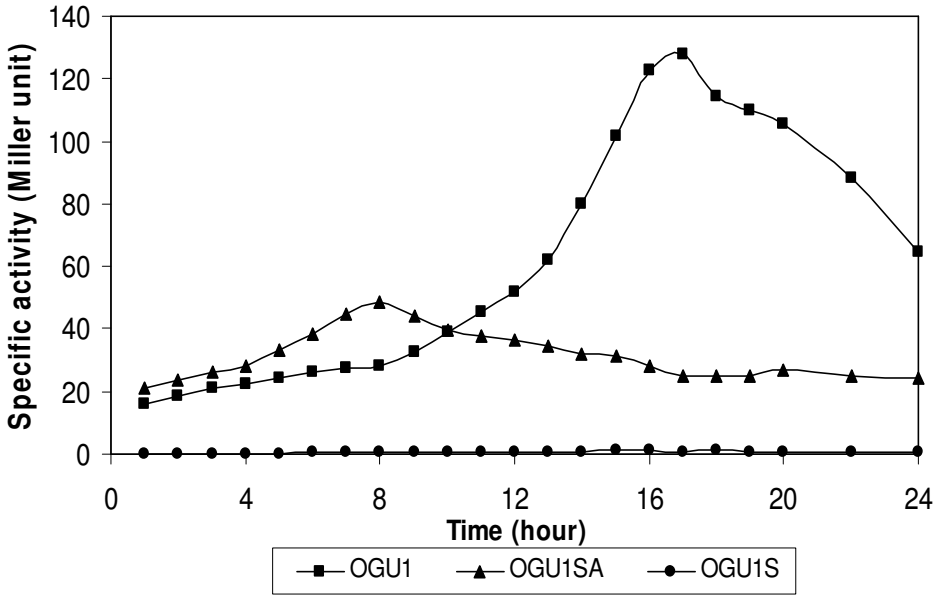


Figure 3. 20. β-galactosidase activities of OGU1, OGU1SH and OGU1S in PA medium.

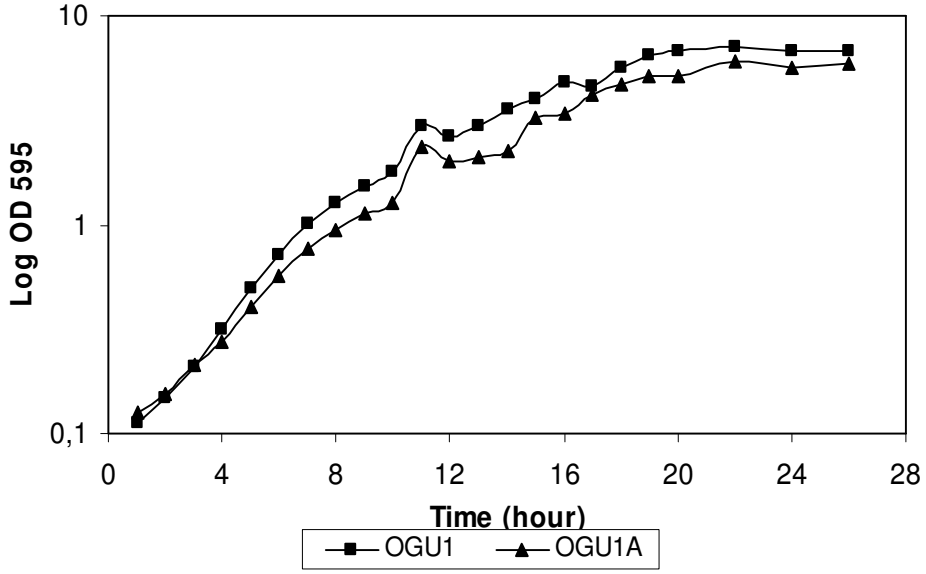


Figure 3. 21. Growth of OGU1 (*bacA*::*lacZ*::*erm*) and OGU1A (*ΔabrB::cat bacA::lacZ::erm*) in PA medium.

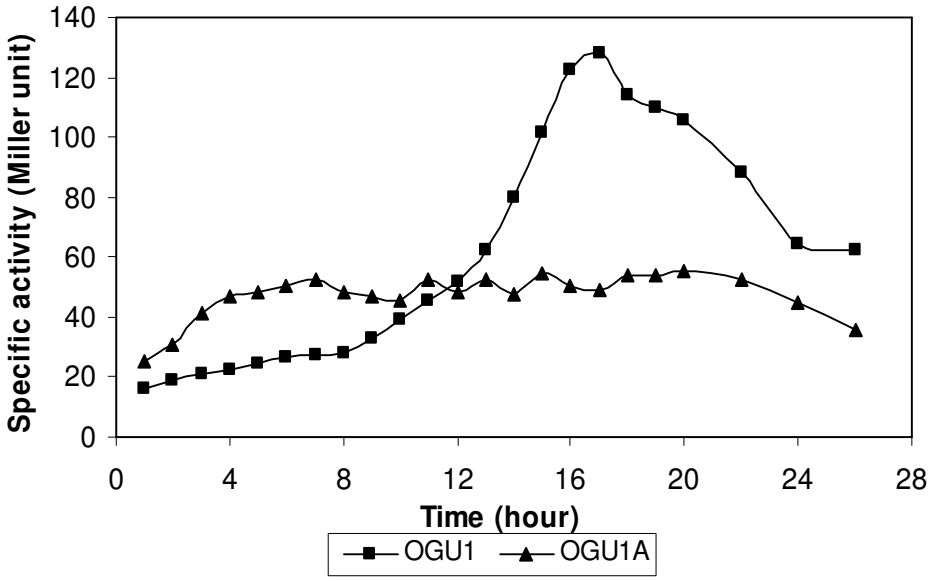


Figure 3. 22. β-galactosidase activities of OGU1 and OGU1A in PA medium.

As can be seen in Fig. 3.18, the disruption of *spo0A* gene completely abolished *bacA-lacZ* expression and this defect was relieved by a null mutation in *abrB* during exponential growth, but partially relieved during stationary phase (Fig. 3.20). On the other hand, almost the same *bacA*-expression profile was detected in *abrB* single mutant in which *bacA* expression level increased nearly 2-fold relative to the wild type-level during exponential growth, but continued with the same expression level throughout the stationary phase without peaking (Fig. 3.22). As based on these findings, there was strong evidence of direct repression by AbrB of the transcription of *bac* operon during exponential phase, while AbrB activity is required for the induction as well as maximum *bac* expression occurs at the onset of stationary phase. This finding is not suprising, since AbrB is known to interact directly with the promoter regions of several genes that are normally induced in the transition from exponential growth to stationary phase (Philips and Strauch, 2002).

3.4.6. Insertional Inactivation of *codY* Gene and Its Effect on the Expression of *bacABCDE* Operon in *Bacillus subtilis*

CodY protein was demonstrated to play a key role in the initiation of sporulation and genetic competence by detecting intracellular GTP. Being a novel GTP-binding protein, it represses early statonary genes and since a *codY*-null mutant was able to sporulate under conditions of nutrient excess, *codY* appears a critical factor that normally prevents sporulation under such conditions (Ratnayake-Lecamwasam *et al*., 2001; Inaoka and Ochi, 2002). Inaoka *et al*. (2003) reported that the disruption of *codY* gene induced transcription of *bac* biosynthetic genes.

In order to cross check the effect of *codY* gene on the expression of *bac* operon, *codY* was insertionally inactivated in OGU1 (*bacA*::*lacZ*::*erm*) by transforming the competent cells of *trpC2 unkU::spc ΔcodY* mutant strain of PY79 (constructed by transforming the competent cells of PY79 with chromosomal DNA of TMH307) with chromosomal DNA of the OGU1 strain with the selection for Erm^R (1µg/ml) and Spc^R (100µg/ml). The transformants were then screened on X-gal containing

agar plate. One of the blue transformants was selected and designated as OGU1C (*unkU*::*spc ΔcodY bacA*::*lacZ*::*erm*).

The resulting strain OGU1C and the wild-type strain OGU1 as the control were grown in PA medium at 37°C and β-galactosidase activities were measured at intervals in various stages of growth (Fig. 3.24).

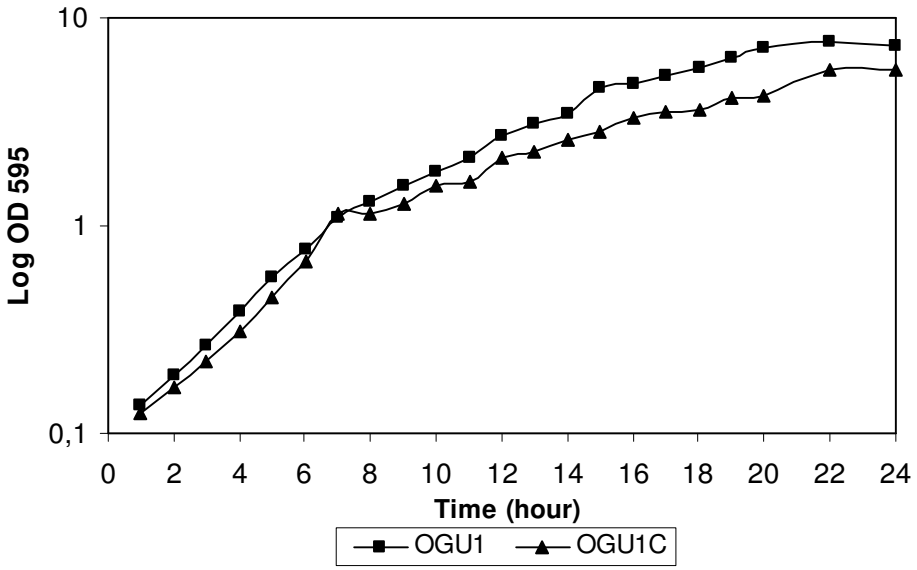


Figure 3. 23. Growth of OGU1 (*bacA*::*lacZ*::*erm*) and OGU1C (*unkU*::*spc ΔcodY bacA*::*lacZ*::*erm*) in PA medium.

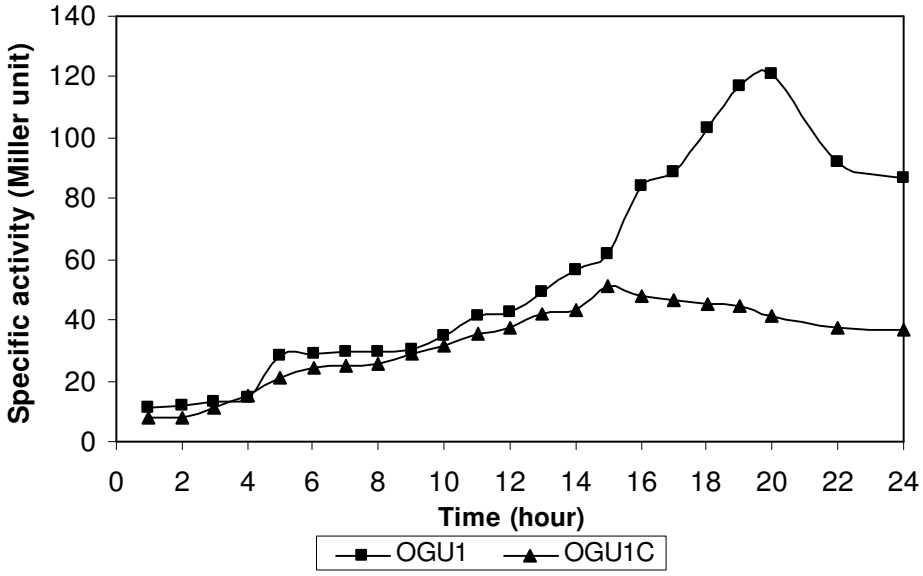


Figure 3. 24. β-galactosidase activities of OGU1 and OGU1C in PA medium.

As shown in Fig. 3.24, *bacA-lacZ* expression was not eliminated, but severely decreased in *codY* blocked mutant during the stationary phase, indicating that *codY* gene product may be required for optimum expression of bacilysin biosynthetic genes. However, this finding was inconsistent with that of Inaoka *et al*. (2003) who reported a 2-3 fold increase in the expression of bacilysin biosynthetic genes upon the disruption of *codY*.

It is known that there is a nutritional repression system mediated by CodY regulatory protein which interacts with the *srfA* and *comK* promoter regions in *B. subtilis*, resulting the repression of the expression of many genes, particularly in rich medium conditions (Serror and Sonenshein,1996). In order to check whether *bac* operon expression is subjected to CodY-dependent nutritional repression, effect of amino acid addition on *bacA-lacZ* expression was examined by supplementing PA medium with casamino acids (CA) at 0.1% final concentration.

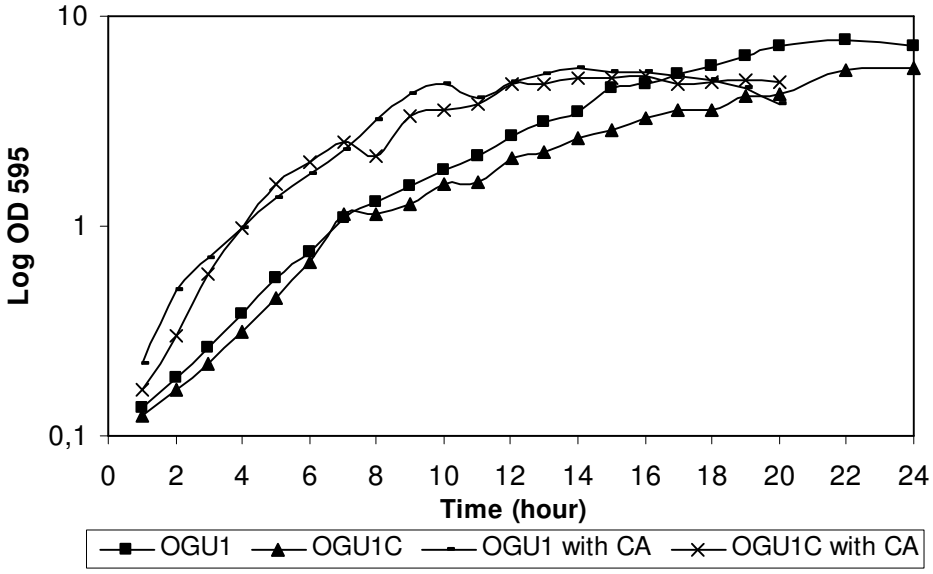


Figure 3. 25. Growth of OGU1 (*bacA*::*lacZ*::*erm*), OGU1 with CA, OGU1C (*unkU*::*spc ΔcodY bacA*::*lacZ*::*erm*) and OGU1C with CA in PA medium.

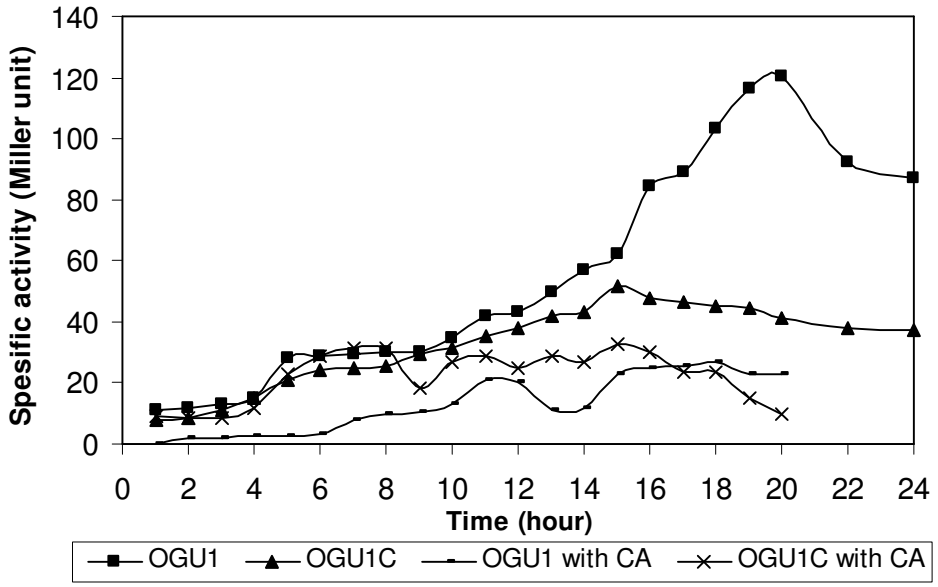


Figure 3. 26. β-galactosidase activities of OGU1, OGU1 with CA, OGU1C and OGU1C with CA in PA medium.

As seen in Figures 3.25 and 3.26, casamino acids significantly improved the growth in OGU1 (*bacA*::*lacZ*::*erm*) and OGU1C (*unkU*::*spc ΔcodY bacA*::*lacZ*::*erm*), but significantly suppressed the expression of *bac* operon. However, the disruption of *codY* totally relieved the effect of casamino asids during the logarithmic growth, and allowed a higher level of expression during the stationary phase. It follows that the suppressive effect of the excess amino acids is not mediated by *codY* and it is unlikely that ppGpp and GTP play a critical role in bacilysin production, as claimed by Inaoka *et al*. (2003).

CHAPTER 4

CONCLUSION

- The expression of the *bacA-lacZ* fusion (OGU1) constructed in this study was relatively constant during the exponential growth, but increased at the transition between exponential and stationary phases and reached to maximal level upon entry into stationary phase in PA medium.

- To understand whether the products of quorum-sensing genes affect bacilysin biosynthesis through *bacABCDE* operon directly, the genes were insertionally inactivated by transforming the competent cells of OGU1 with chromosomal DNA of the corresponding blocked mutant strains and *bacA*-directed β-galactosidase activities were subsequently analyzed.

- The disruption of *srfA* gene completely abolished *bacA-lacZ* expression, suggesting that *bacABCDE* operon expression depends on a functional surfactin operon.

- *bacA-lacZ* expression was severely impaired in the *oppA* blocked mutant, indicating that *oppA* gene product regulates bacilysin biosynthesis by affecting the expression of the *bac* operon.

- The deletion of *phrC, phrF* and *phrK* genes impaired *bacA-lacZ* expression. Therefore, the pheromone peptides Phr C, F and K are required for the expression of

bacABCDE operon, most likely by enhancing the full activation of transcriptional factor ComA~P.

- The deletion of *comA*, *comP*, *comQ* and *spo0H* genes severely impaired *bacA-lacZ* expression, indicating that these gene products regulate bacilysin biosynthesis by affecting the expression of *bac* operon. If we consider that ComP and ComX (ComQ) are required for phosphorylation and activation of ComA and σ^H is required for increased level of *phr* gene during transition state, thereby enhancing the full activation of ComA, it is reasonable to accept that the transcription of *bac* operon could be ComA~P-dependent.

- Disruption of *spo0A* gene resulted in complete elimination of *bacA-lacZ* expression and this defect was relieved by a null mutation in *abrB* during exponential growth which was partially relieved during the stationary phase. On the other hand, the same *bacA*-expression profile was also detected in *abrB* single mutant in which *bacA* expression level increased two-fold as compared to that of wild type during exponential growth, but continued with the same expression level throughout the stationary phase without peaking. Based on these findings, there was strong evidence that the transcription of *bac* operon is directly repressed by AbrB during exponential phase while AbrB activity is required for the induction and maximum *bac* expression at the onset of stationary phase.

- bacA-lacZ expression severely decreased in *codY*-blocked mutant during the stationary phase, but not in the exponential growth phase. The expression was also greately suppressed upon amino acid supplementation to the medium in wild-type strain, however, codY disruption totally relieved this suppression during the logarithmic growth. It was therefore concluded that the CodY repressor which plays a key role in the initation of sporulation and genetic competence do not play a negative role in bacilysin biosynthesis.

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APPENDIX A

COMPOTIONS AND PREPERATION OF CULTURE MEDIA

Perry and Abraham (PA) Medium (pH 7.4)

KH_2PO_4	1 g/L
KCl	0.2 g/L
$MgSO_4.7H_2O$*	0.5 g/L
Glutamate.Na.H_2O	4 g/L
Sucrose*	10 g/L
Ferric citrate**	0.15 g/L
Trace elements**	1 ml
$CoCl_2.6H_2O$	0.0001 g/L
Ammonium molybdate	0.0001 g/L
$MnCl_2.4H_2O$	0.001 g/L
$ZnSO_4.7H_2O$	0.0001 g/L
$CuSO_4.5H_2O$	0.00001 g/L

*Autoclave separately

**Filter sterilization

Luria Bertani (LB) Medium (1000ml)

Tryptone	10 g/L
Yeast Extract	5 g/L
NaCl	5 g/L

Distilled H_2O was added up to 1000ml and then autoclaved for 15 minutes.

Luria Bertani (LB) Agar Medium (1000 ml)

Tryptone	10 g/L
Yeast Extract	5 g/L
$NaCl_2$	5 g/L
Agar	15 g/L

Bioassay Medium (pH 7.1)

$Na_2HPO_4.2H_2O$	3.3 g/L
KH_2PO_4	1 g/L
NaCl	1 g/L
Glucose*	10 g/L
$MgSO_4.7H_2O$*	0.7 g/L
Na_3.citrate.$2H_2O$	0.5 g/L
Glutamic acid.Na.H_2O**	2.4 g/L
13 amino acid***	0.0125 g/L (each)
$FeSO_4.7H_2O$**	0.01 g/L
Yeast Extract****	0.05 g/L
Agar****	

*Autoclave glucose and $MgSO_4.7H_2O$ together, separately

**Filter Sterilization

***Arginine, cystidine, glycine, histidine, leucine, methionine, phenylalanine, proline, threonine, tryptophane, tyrosine, valine, alanine (all in L-form)

****Autoclave separately

10X-S-base

$(NH_4)_2SO_4$	20 g/L
$K_2HPO_4.3H_2O$	140 g/L
KH_2PO_4	60 g/L
Na_3.citrate.$2H_2O$	10 g/L

Autoclave together and cool down to 50°C and supplement with 1 ml sterile 1 M $MgSO_4$.

HS medium (30 ml)

10X-S-base	3 ml
Glucose (50%)	300 µl
Yeast Extract (10%)	300 µl
Casaminoacid (2%)	300 µl
Arg (8%) + His (0.4%)	3 ml
Tryptophan (0.5%)	300 µl
Phenylalanine (0.3%)	450 µl

Complete up to 30 ml with sterile distilled H_2O and store at cold room (+4°C) up to one week at most.

LS Medium (20 ml)

10X-S-Base	2 ml
Glucose	200 µl
Tryptophan	200 µl
Phenylalanine	30 µl
Casaminoacid	100 µl
Beef Extract	200 µl
Spermine (50mM)	200 µl

$MgCl_2$ (1M) (filter steriled) 50 µl

Freshly prepare and complete up to 20 ml with sterile distilled H_2O.

2xYT Medium (1000ml)

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g
Agar	15 g (Add before autoclaving for solid 2xYT medium)

APPENDIX B

COMPOSITIONS OF BUFFERS AND SOLUTIONS

TE Buffer (pH 8)

Tris base (2 moles)	10 mM
EDTA	1 mM

CTAB/NaCl Solution (10 % CTAB/ 0.7 M NaCl)

4.1 g of NaCl was dissolved in 80 mL of dH2O. Then, 10 g of CTAB (hexadecyl trimethyl ammonium bromide) was added and dissolved with vigorously shaking and gentle heating up to 65 ° C. Final volume was made up to 100 mL with dH_2O.

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

Phenol	500 g
Chloroform	500 mL
Distilled water	400 mL

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

SET Buffer

25 g/L Sucrose
0.05 M Tris-HCl (pH 8.0)

0.5 M EDTA (pH 8.0)

Sterilized at 121 ^{o}C under 1.1 Kg/cm^{2} pressure for 15 min.

STE Buffer

10.3% Sucrose
25 mM EDTA pH: 8.0
25 mM Tris-HCl pH: 8.0
2 mg/mL Lyzozyme

TAE Buffer (50X)

Tris base (2 moles)	242 g
Glacial acetic acid (57.1 ml)	57.1 ml
EDTA (100mL 0.5M)	100 ml (0.5 M, pH 8.0)

Add Distilled H_2O up to 1L and adjust pH to 8 by HCl

Low Melting Agarose Gel (1%)

Agarose	0.5 g
TAE buffer (1X)	50 ml

Add 1.5µl EtBr (final concentration: 0.5 µg/ml) before pouring the gel into tray.

Physiological Sodium Chloride Solution (0.85%) (1000 ml)

$NaCl_2$ 8.5 g
Dissolve in 1000 ml distilled water and autoclave.

Tris-Cl Solution (25mM, 1000 ml, pH 7.4)

Tris (hydromethyl)aminomethane 3.03 g
Dissolve in 1000ml distilled water and adjust pH to 7.4 with HCl (1 M)

Z Buffer (500 ml, pH 7.0)

$Na_2HPO_4.7H_2O$	60mM, 5.33 g
NaH_2PO_4	40 mM, 3.12 g
KCl_2	10 mM, 0.373 g
$MgSO_4.7H_2O$	1 mM, 0.123 g

All mixed and dissolved within 500ml distilled water and the pH was adjusted to a value of 7.0.

β-mercaptoethanol	final concentration: 270 µl / 100 ml (add to Z buffer on the day on use of)
Lysozyme	final concentration: 2.5 mg/ml
ONPG	final concentration: 4.0 mg/ml

APPENDIX C

CHEMICALS AND ENZYMES

Chemicals	Supplier
Agar Bacteriological	Merck
Agarose	Prona
$AgNO_3$	Merck
Ammonium persulfate	AppliChem
Arginine	Sigma
$CaCl_2.2H_2O$	Merck
Casamino acid	AppliChem
Cloramphenicol	Sigma
Chloroform	Merck
CTAB	Sigma
dNTPs	MBI Fermentas
Dimethylformamide	Merck
EDTA	AppliChem
Ethanol	Botafarma
Ethidium bromide	Sigma
Formaldehyde	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
HCl	Merck
Histidine	Sigma

IPTG	Sigma
Isoamyl alcohol	Merck
Isopropanol	Merck
KH_2PO_4	Merck
K_2HPO_4	Merck
KCl	Merck
Luria Broth	Q-Biogene
Methanol	Merck
Mercaptoethanol	Sigma
$MgCl_2.6H_2O$	Merck
$MgSO_4.7H_2O$	Merck
$MnCl_2.4H_2O$	Merck
$MnSO_4.H_2O$	Merck
Na_2CO_3	Merck
$Na_2SO_4.10H_2O$	Merck
Na_3citrate.$2H_2O$	Merck
N-acetylglucosamine	Sigma
NaCl	Merck
NaOH	Merck
NH_4Cl	Merck
2-Nitrophyl β-D-galacto pyranoside (ONPG)	Sigma
Nutrient Broth	Merck
PEG 6000	Merck
Phenol	Merck
Phenol-chloroform-isoamylalcohol	Sigma
Phenylalanin	Sigma
Potassium acetate	Merck
SDS	Merck
Spermidine	Sigma
Sucrose	Merck
Tris-base	Merck
Triton-X100	Sigma

Tryptone	Difco
Tryptophane	Sigma
X-Gal	MBI Fermentas
Yeast Extract	Oxoid

Enzymes

*Bam*HI	MBI Fermentas
*Hin*dIII	MBI Fermentas
Lysozyme	AppliChem
Proteinase K	Sigma
RNAse A	Sigma
*Sac*I	MBI Fermentas
*Xba*I	MBI Fermentas
Taq DNA Polymerase	MBI Fermentas
T4 DNA Ligase	MBI Fermentas

APPENDIX D

MARKERS

Marker	Supplier
Lambda DNA/*Pst*I Marker	MBI Fermentas