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STANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

**IDENTIFICATION OF NEW GENES RELATED
WITH BACILYSIN BIOSYNTHESIS BY Tn-10
MUTAGENESIS METHOD IN *BACILLUS SUBTILIS***

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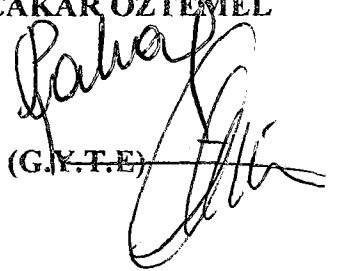
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***BACILLUS SUBTILIS*'TE BACILYSIN BİYOSENTEZİ İLE
İLGİLİ YENİ GENLERİN Tn-10 TRANSPON
MUTAJENEZ METODU İLE TANIMLANMASI**

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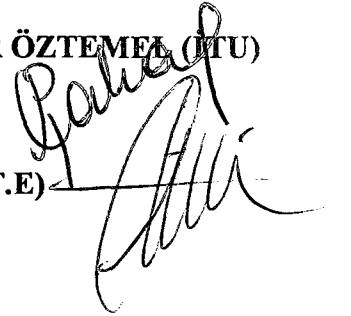
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ABBREVIATIONS

BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
ddH ₂ O	: Double distilled water
DNA	: Deoxyribonucleic acid
DSM	: Difco's Sporulation Medium
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidium bromide
IPTG	: Isopropyl-β-D-thiogalactopyranoside
kb	: Kilobase
LB- broth	: Luria Bertani broth
LMP agarose	: Low melting point agarose
NCBI	: National Center for Biotechnology Information
OD	: Optical density
ORF	: Open Reading Frame
PA	: Perry and Abraham Medium
PCR	: Polymerase chain reaction
TAE	: Tris acetate EDTA
Tris	: Hydroxymethyl aminomethane

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IDENTIFICATION OF NEW GENES RELEATED WITH BACILYSIN BIOSYNTHESIS BY TN-10 MUTAGENESIS METHOD in *BACILLUS SUBTILIS*

SUMMARY

The members of the species *Bacillus* produce a wide variety of secondary metabolites with antimetabolic and pharmacological activities. Most of these metabolites are small peptides that have unusual components and chemical bonds and are synthesized nonribosomally by multifunctional enzyme complex called peptide synthetases. Bacilysin, being produced and excreted by certain strains of *Bacillus subtilis*, is a dipeptide antibiotic composed of L-alanine and L-anticapsin. Biosynthesis of dipeptide antibiotic bacilysin is under the quorum sensing global regulation through the action of ComQ/ComX, PhrC (CSF), ComP/ComA and in a Spo0K (Opp)-dependent manner in *B. subtilis*. Additionally, the disruption of *srfA* operon in the bacilysin producer resulted with the bacilysin-negative phenotype, thus, this study verified that the *srfA* operon functions directly in the production of bacilysin. 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. Very recently, the *yvfBCDEF* genes of *B. subtilis* 168 were shown to carry biosynthetic core function genes renamed as *bacABCDE* and *bacD* gene was proved to encode the function of amino acid ligation. In addition to these genes, we have already identified another gene, namely *yvfI* (unknown; similar to transcriptional regulator) by employing Tn-10 mutagenesis method.

Under the light of this findings, in this present study, we focused on cloning and identification of new gene(s) involved in the biosynthesis of bacilysin. For this purpose, Transposon Tn10 mutagenesis was employed to disrupt the genes related with bacilysin biosynthesis in *Bacillus subtilis* PY79. Blocked mutants were phenotypically selected from the transposon library through bacilysin bioassay and three identical bacilysin nonproducer mutants [*Bac*::Tn10(*ori-spc*)] were selected. The disrupted gene(s) from these mutants were cloned, sequenced and characterized. This study resulted with the identification of a novel gene *yvfI* (unknown, similar to transcriptional regulator) involved in bacilysin biosynthesis. Next, a deletion vector for distrupction of *yvfI* gene in *Bacillus subtilis* PY79 was constructed for further verification of the effect of *yvfI* gene on bacilysin biosynthesis. The introduction of ($\Delta yvfI::spc$) mutation into PY79 resulted in the elimination of bacilysin biosynthesis.

Keywords: Bacilysin, peptide antibiotics, Tn10 mutagenesis, *yvfI*, *Bacillus subtilis*

BACILLUS SUBTILIS’TE BACILYSIN BİYOSENTEZİ İLE İLGİLİ YENİ GENLERİN TN-10 TRANSPOZON MUTAJENEZ METODU İLE TANIMLANMASI

ÖZET

Bacillus türleri antimetabolik ve farmakolojik aktiviteye sahip çok çeşitli ikincil metabolitler üretirler. Bu metabolitlerin çoğu, olağan dışı bileşenlere ve kimyasal bağlara sahip küçük peptidlerdir olup peptid sentetaz adı verilen multifonksiyonel enzim kompleksleri tarafından ribozomal olmayan bir biçimde sentezlenirler. *Bacillus subtilis*’ in bazı suşları tarafından sentezlenen bacilysin L-alanin ve L-anticapsin’ den oluşmuş bir dipeptiddir. Bacilysin biyosentezinin, ComQ/ComX, PhrC (CSF), ComP/ComA’ nın etkisinde quorum sensing mekanizmasının kontrolü altında olduğunu ve bu biyosentezin Spo0K (Opp)’ ye bağlı bir biçimde gerçekleştiği gösterilmiştir. Ayrıca, bacilysin üreticilerindeki *srfa* operonunun bozulmasıyla bacilysin–negatif bir fenotipin oluşması *srfa* operonunun doğrudan bacilysin üretimi ile ilgili olduğuna işaret etmiştir. Aynı çalışma içerisinde sporlanma genlerinden *spo0H* ve/veya *spo0A* faktörlerinin bloke edildiği mutantlarda bacilysin üretiminin kaybolması, aynı zamanda *abrB* genleri bozulmuş mutantlarda bacilysin üretiminin artması ve ayrıca *spo0A* faktörünün bloke edildiği ve *abrB* geninin bozulduğu mutantlarda bacilysin–negatif fenotipin ortadan kalkması, bacilysin üretiminde görev alan bazı genlerin transkripsiyonlarının *abrB* gen ürünün negatif kontrolü altında olduğunu ve SpoOA proteini ile desteklendiğini ortaya çıkardı. Oldukça yakın bir zaman öncesinde, *B. subtilis* 168’in *ywfBCDEF* genlerinin bacilysin biyosentetik fonksiyonlar taşıdığı gösterilmiş ve *bacABCDE* olarak yeniden adlandırılmıştır. Bu operon içerisinde bulunan *bacD* geninin ise amino asid ligasyonundan sorumlu ilgili bacilysin sentetaz olduğu doğrulanmıştır.

Bu bulguların devamı olarak, bu çalışmada, bacilysin biyosentezi ile ilgili yeni genlerin klonlanması ve aydınlatılması üzerine yoğunlaşmıştır. Bu amaçla, Transpozon Tn10 mutajenez metodu uygulanmıştır. Bloke olmuş mutantlar, transpozon kütüphanesinden biyoassay yolu ile fenotipik olarak seçilmiş ve üç benzer bacilysin üretemeyen mutant [Bac⁻::Tn10(*ori-spc*)] izole edilmiştir. Bu mutantlarda bloke edilmiş genler klonlanmış ve nukleotid dizileri analiz edilmiştir. Bu çalışma, *B. subtilis* genomunda henüz kimse tarafından tanımlanmamış yeni bir gen olan *yvfI* geninin (bilinmeyen: transkripsiyonel düzenleyicilere benzer) biyosentezle ilgisini ortaya çıkarmamızla sonuçlanmıştır. Bunu takiben, *yvfI* geninin bacilysin biyosentezi üzerindeki rolünün doğrulanması amacı ile, *Bacillus subtilis* PY79’da *yvfI* genini bloke edecek bir delesyon vektörü oluşturulmuştur. ($\Delta yvfI::spc$) mutasyonunun PY79’a uygulanması bacilysin biyosentezinin eliminasyonu ile sonuçlanmıştır.

Anahtar kelimeler: Bacilysin, peptid antibiyotikler, Tn10 mutasyonu, *yvfI*, *Bacillus subtilis*

1. Introduction

1.1. Nonribosomal System of Peptide Biosynthesis

Several hundred peptides of different structural types are known, in which their composition cannot only originate from ribosomal system, the latter being restricted to the 20 protein amino acids and their possible modification products (Kleinkauf and Von Döhren, 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). A large number of therapeutically useful cyclic and linear peptides are synthesized via a template-directed, nucleic-acid-independent nonribosomal mechanism (Strohl, 1997; Cane, 1997 and 1999; Du and Shen, 2001; Weber and Marahiel, 2001; Weinig *et al.*, 2003; Clair *et al.*, 2004). The hypothesis of nonribosomal machinery was confirmed by biochemical studies on cell-free extracts from several peptide antibiotic producers. Cell-free production of a particular antibiotic could be restored even in the presence of RNase or ribosome inhibitors (Gevers *et al.*, 1968).

These pharmaceutically important peptide compounds are synthesized nonribosomally as secondary metabolites on large multifunctional enzymes in *Actinomyces*, *Bacilli* and filamentous fungi, including antibiotics like penicillin and vancomycin, immunosuppressive 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).

The physiological role in the producer organism is still a speculating issue and may be correlated to signaling for the coordination of growth and differentiation, induction of iron uptake on iron starvation, defense against competitor

microorganisms triggering the excretion of antimicrobials, or invasion processes directed by the production of host-selective toxins (Tamehiro *et al.*, 2002; Kallow *et al.*, 2002; Sharma and Johri, 2003).

The structural diversity of these peptides is remarkably large, which is due to incorporation of a large number of unusual, nonproteinogenic residues. This protein template driven peptide synthesis is not restricted to the set of proteinogenic amino acids, but can recruit a large number of unusual residues. The constituents incorporated into the products are -L, -D and β forms of amino acids, hydroxy and carboxy acids, which can be N-methylated, acylated, reduced or epimerized. Furthermore, the peptide backbone of assembled product can be linear, cyclic or branched cyclic and involved in heterocyclic ring formation (Mootz and Marahiel, 1997; Mootz and Marahiel, 1999; Von Dohren *et al.*, 1999).

Despite this structural diversity, these peptides are almost synthesized by large, multifunctional protein complexes named nonribosomal peptide synthetases (NRPS), which use a multiple-carrier thiotemplate mechanism in a stepwise assembly from the amino acid monomers (Kleinkauf and von Döhren, 1990; Stein and Vater, 1996; Marahiel *et al.*, 1997; Konz and Marahiel, 1999; Von Dohren *et al.*, 1999; Weber and Marahiel, 2001; Schwarzer and Marahiel, 2001; Schwarzer *et al.*, 2002). According to the present multiple carrier model of nonribosomal peptide synthesis, peptide synthetases are composed of repetitive units (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. This is why these enzymes have also been called “protein templates” (von Döhren *et al.*, 1999; Schwarzer and Marahiel, 2001; Weber and Marahiel, 2001).

The building blocks of NRPS, modules, can be subdivided into domains, each responsible for catalyzing the 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 (Linne *et al.*, 2001; Schwarzer and Marahiel, 2001). A basic or minimal module consists of an amino acid activating (adenylation) domain, an acyl carrier (thiolation or peptidyl carrier, PCP) domain and a condensation domain, which is

responsible for peptide bond formation. These domains can therefore be described as the “toolbox” of NRPS (Marahiel *et al.*, 1997; Konz and Marahiel, 1999; von Döhren *et al.*, 1999; Schwarzer and Marahiel, 2001).

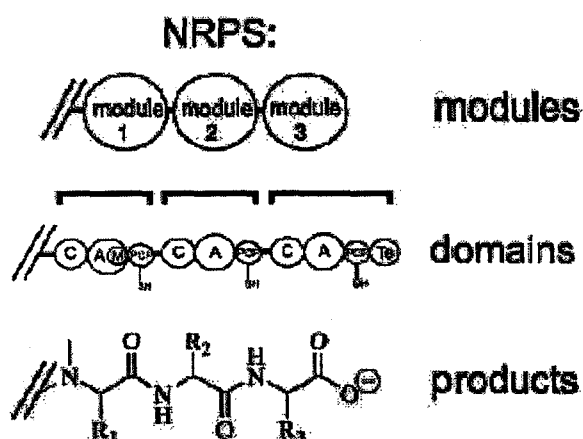


Figure 1.1: From modules to products: the modules of NRPS can be subdivided into domains that catalyze the single enzymatic reactions. The composition of the products is determined by the assembly of active domains found in the corresponding modules (Schwarzer and Marahiel, 2001).

Within the last decade, a large number of genes encoding NRPS of bacterial and fungal domains have been cloned, sequenced and characterized. In bacterial systems, the genes encoding for several NRPS involved in the synthesis of a specific peptide are typically organized in operons that can span regions of 6-45 kb. The single NRPS in these systems can be comprised of 1-8 modules. In contrast to bacterial systems, the fungal protein templates are encoded by large, single genes (Marahiel *et al.*, 1997; Konz and Marahiel, 1999).

1.1.1. Adenylation Domain (A)

The adenylation domain is the most important domain of each module as it recognizes and activates the appropriate residue as its acyl adenylate, using ATP to power the reaction, analogous to the activation of amino acids as aminoacyl tRNAs during ribosomal protein synthesis. Adenylation domains of NRPS belong to a large superfamily of adenylate forming enzymes that includes insect luciferases and acyl and aryl CoA synthetases. Despite the diversity in their origin, all of these enzymes share a homologous region of 556 amino acids that contains a set of highly conserved signature sequences. In general these enzymes have a unique subdomain structure; a highly conserved region links the large amino-terminal and carboxy-

terminal subdomains. Adenylate formation involves a rotational movement of subdomains, possibly stabilizing the adenylate structure (Turgay *et al.*, 1992; Conti *et al.*, 1996; Conti *et al.*, 1997; Mootz and Marahiel, 1997; Dieckmann *et al.*, 1999; Weber and Marahiel, 2001).

The first step in peptide synthesis is selection of the substrate residue at the binding pocket of the A domain. It is possible that a large part of the NRPS code operates using a lock and key process dependent on the substrate specificity of the adenylation domain itself (von Döhren *et al.*, 1999). Specificity-determining region of this enzyme family to a stretch of about 100 amino acid residues between two highly conserved core motifs, A4 and A5 (Stachelhaus *et al.*, 1999). Sequences of nonribosomal peptides are predicted from translated sequences of respective synthetases themselves. The “contact-residue approach” attempts to define a code for amino acid selection, using a set of amino acid residues found within the A domain itself, analogous to three anticodon nucleotides on the tRNA chain used in ribosomal protein synthesis (Stachelhaus *et al.*, 1999).

A striking difference between the ribosomal and nonribosomal systems is the application of an accurate proof-reading mechanism for ribosomal protein synthesis, nonribosomal synthesis in general shows less stringent substrate selection and incorporation (Stachelhaus *et al.*, 1999). As a consequence of the multiple-carrier thiotemplate mechanism with an A domain for each residue incorporated into the peptide product, a relaxed substrate selectivity for some of the positions within the product can be observed in contrast to ribosomal system, where accuracy of amino acid incorporation is highly controlled (Silvian *et al.*, 1999). For example, the cyclic decapeptide tyrocidine consists of a mixture of four compounds that vary in two positions, and for the undecapeptide immunosuppressive agent cyclosporin about 30 variants are known (Ruttenberg and Mach, 1966; Traber, 1997). For the producing microorganisms, this variability is of evolutionary value: it allows the production of a series of bioactive compounds, covering a broader spectrum of effects using just one set of machinery (Weber and Marahiel, 2001).

1.1.2. Thiolation Domain – Peptidyl Carrier Protein (PCP)

In most NRPS modules, the A domain is followed by a thiolation domain (T) which is ca. 100 amino acids in length. It is also designated as the “Peptidyl Carrier

Protein" (PCP), showing analogy to the acyl carrier protein of fatty acid and polyketide synthetases (Konz and Marahiel, 1999). PCPs bind the monomers and intermediates of the growing peptide chain as thioesters through the thiol moiety of their prosthetic group 4'-phosphopantetheine (4'PP) and facilitate their directed transport through the NRPS assembly line. This cofactor is covalently tethered to an invariant serine residue within the conserved region, CoreT (GG [H, D] S [L, I]) (Marahiel *et al.*, 1997; Weber *et al.*, 2001). Conversion of each PCP from the inactive apo form into active holo form is performed by a special class of CoASH-binding 4'PP transferases in a posttranslational reaction, referred as the priming step, which uses CoA as the source of 4'PP (Lambalot *et al.*, 1996; Reuter *et al.*, 1999).

1.1.3. Condensation Domain (C)

The C domain, about 450 residues in length, is the site of the peptide-bond formation and chain translocation in nonribosomal peptide synthesis. This domain catalyzes the peptide bond formation between two adjacent modules: an upstream peptidyl-S-PCP donor is attacked by a downstream aminoacyl-S-PCP acceptor nucleophile. Recently, it has been demonstrated that C domains have high substrate selectivity for the incoming aminoacyl-S-PCP nucleophile acceptor, but they are less specific for the incoming peptidyl-S-PCP electrophile donor. The amino acid selective acceptor site was found to be responsible for preventing internal mis-initiation and to control the timing of substrate epimerization in NRPS. Evidently, the C domain seems to discriminate against size and stereochemistry of the monomeric amino-acyl acceptor (Lambalot *et al.*, 1996; Reuter *et al.*, 1999; Weber and Marahiel, 2001).

However, the C domains share a highly conserved core sequence C3, His-His-X-X-Asp-Gly with a class of well-studied acyl transferases. This signature motif has been identified to be critical for amide-bond formation. Although the importance of the C domain in the elongation reaction has been demonstrated by the deletion and the mutational experiments (Stachelhaus *et al.*, 1998), the catalytic mechanism of peptide-bond formation is unknown and no structural information is currently available for this central domain (Weber and Marahiel, 2001).

1.1.4. Thioesterase Domain (TE)

In most bacterial systems, a fourth domain, the termination/thioesterase domain, about 250 residues in length, has been found to be essential for product release

(Schneider and Marahiel, 1998). The TE-domain is located at the extreme C-terminal module of the corresponding biosynthetic template and catalyzes the release of the biosynthesized peptide in linear, cyclic or branched cyclic form (Trauger *et al.*, 2000).

This domain bears a signature sequence TE (Gly-X-Ser-X-Gly) that is similar to the active site motif of acyltransferases and thioesterases. It is proposed that the full-length peptide bound to the TE domain is transferred to the hydroxyl group of the highly conserved serine residue within the TE domain to generate a transient acyl-O-enzyme intermediate. These peptides are then cleaved, resulting in a linear peptide, or a cyclic or branched cyclic product (Cane *et al.*, 1999).

1.1.5. Auxiliary Domains

In addition to the domains described above, several other modifying domains have been found in NRPS modules, which enlarge the structural diversity of the synthesized peptides. One example is the epimerization (E) – domain, about 400 residues in length, which converts the thioester-bound amino acid of an amino acyl-S-PCP or peptidyl-S-PCP from the L- into the D- configuration (Stachelhaus and Walsh, 2000). E domains are usually located downstream of the C-terminus of the corresponding PCP (Shwarzer and Marahiel, 2001).

In some NRPS modules, a N-methylation (M) domain, about 450 residues in length, is inserted into the C-terminal end of an A domain, catalyzing the transfer of a methyl group from co-factor S-adenosylmethionine (SAM) to the amino group of a PCP bound amino acid (Shwarzer and Marahiel, 2001).

An alternative mode of elongation has recently been observed in some NRPS systems. Here, a cyclization (Cy) domain substitutes for the unusual C domain in modules incorporating cysteine, serine or threonine residues. Instead of a simple peptide bond formation, the module mediates the formation of heterocyclic rings such as oxazolines or thiazolines (Du *et al.*, 2000).

The number of new catalytic domains is increasing. Newly discovered domains include a putative oxidation (Ox) domain, about 250 amino acids, that were found strictly associated with Cy domains. The Ox domain is believed to oxidize the thiazoline ring formed into the aromatic thiazol by using FMN as co-factor (Du *et al.*, 2000). Astonishingly, Ox domains can be found in two different locations within

the NRPS: downstream of the PCP or inserted into the C-terminal part of an A domain (Shwarzer and Marahiel, 2001).

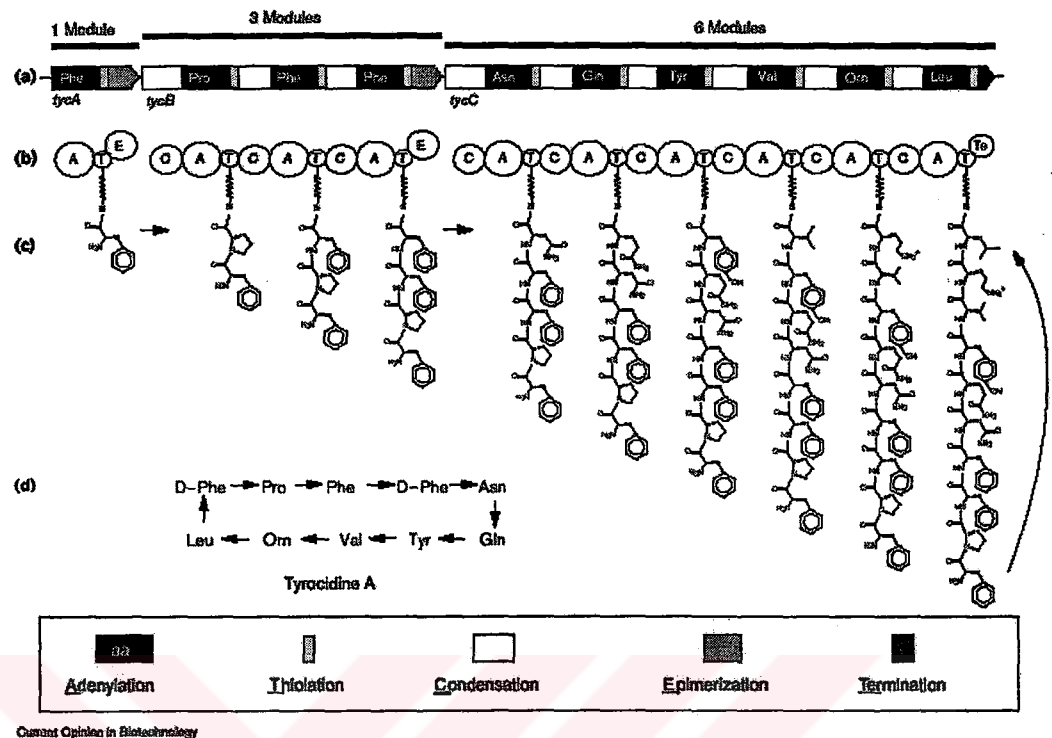


Figure 1.2: The multiple carrier thiotemplate mechanism illustrated with the example of tyrocidine A synthesis (Mootz and Marahiel, 1997). Three peptide synthetases; (a) encoded by the genes *tycA*, *tycB*, and *tycC*, act in concert for the stepwise assembly of the cyclic decapeptide. (b) The substrates 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).

1.2. *Bacillus subtilis*

Members of genus *Bacillus* represent aerobic, endospore-forming, rod-shaped Gram-positive bacteria, which also have an industrial importance coming from their capability of producing antibiotics, proteases and insecticides (Harwood *et al.*, 1990). *Bacillus subtilis* has become not only one of the most intensively studied bacteria but also one of the most clearly understood organisms found in nature (Sonenshein *et al.*, 2002 & Harwood *et al.*, 1990).

The complete genome of *Bacillus subtilis* that is placed in a 4215 kb long, single chromosome, includes 4106 putative protein-coding sequences, for about 2 300 genes with determined possible functions (Kobayashi and Ogasawara, 2002). Among 4106 protein-coding genes, 86 tRNA genes, 30 rRNA genes and three small stable RNA genes are annotated. When the sequence was published, possible functions was assigned to about 58% (2379 genes) of the protein-coding genes, however the number of the genes with assigned function has increased to 63% (2562 genes) in the current database (Kobayashi and Ogasawara, 2002). Beside these, only 4% of essential genes display unknown functions (Kobayashi *et. al.*, 2003).

Among members of this genus, *Bacillus subtilis* is a chemoorganotroph that has the ability to survive when exposed to very simple growth conditions such as simple salt medium containing glucose or other sugars as carbon and energy source, inorganic nitrogen and adequate supply of oxygen (Nicholson and Setlow, 1990). Furthermore, characteristic feature of the physiology of this organism is the tendency to a “fast and feast” existence, which is controlled by a bunch of strategies that can be observed as a complex network in post-exponential growth. Post-exponential growth corresponds to a range of responses including carbon and nitrogen repression, stringency, chemotaxis associated motility, and the production of antibiotics (Nicholson and Setlow, 1990).

1.3. Global Regulation of Gene Expression by Quorum- Sensing

Beside eukaryotes, bacteria are also capable of creating complex communication circuits and this trait makes them socially behaving microorganisms (Ruzheinikov *et al.*, 2001). Therefore these social organisms sense the “going on”s around them in the means of presence of other bacteria via the production and eventually responding to signal molecules (Taga *et al.*, 2003). The mechanism, during which bacteria communicate with the help of these chemical signal molecules in order to build up an adaptational behavior, is called quorum sensing (Miller *et al.*, 2001).

There are many examples to be given for different quorum sensing systems used by bacteria. The bacteria secrete and sense small, signalling molecules that accumulate in the growth medium as cells grow to high density. These signalling molecules include N-acyl-homoserine lactones (AHLs) (Fuqua and Greenberg, 1998) used by Gram-negative bacteria, and small peptides used by Gram-positive bacteria (Dunny

and Leonard, 1997). For instance, Gram-negative bacteria, *Pseudomonas aeruginosa* use quorum sensing for biofilm formation and virulence, *Vibrio fischeri* and *Vibrio harveyi* for bioluminescence, *Erwinia carotovora* for antibiotic production, *Agrobacterium 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 *Bacillus subtilis* show competence and additionally *Bacillus subtilis* got induced for sporulation (Sturme *et al.*, 2002; Taga *et al.*, 2003).

In *Bacillus subtilis*, sporulation and the development of genetic competence are stimulated as cells grow to high density (Solomon and Grossman, 1996; Lazazzera and Grossman, 1998). The cell density control in *B. subtilis* is mediated by extracellular peptides. The mechanisms that control two important post-exponential phase phenomena in *B. subtilis*, sporulation and competence development, illustrate how peptide signals can function in complex regulatory networks.

The dependence of competence and sporulation on both an extracellular signal and an oligopeptide permease operon, suggest that a peptide signal might accumulate in cell cultures in which they could be internalized through the oligopeptide permease and act intracellularly in the target cell. This type of signal could be a true quorum sensor, in which individual members of a *Bacillus* population gain information about how many other cells are present that must share the limited nutrients, or that could supply DNA to be taken up during competence (Solomon *et al.*, 1996; Magnuson *et al.*, 1994).

In this system of *B. subtilis*, two extracellular signaling peptides, ComX pheromone and CSF (for competence and sporulation factor), regulate the activity of the transcription factor ComA (Figure 1.3) (Solomon *et al.*, 1995 and 1996). ComA activates the expression of several genes, including one for the development of genetic competence, constituting the quorum response (Lazazzera *et al.*, 1999). ComX pheromone is the major extracellular signaling peptide, stimulating ComA activity. ComX pheromone is secreted into the medium, and as cells become crowded, ComX pheromone reaches concentrations that bind and activate the protein kinase, ComP. ComP then donates phosphate to ComA, generating ComA~P, which

expression of ComA-controlled genes. 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 (Figure 1.3) (Solomon *et al.*, 1996; Lazazzera, Solomon and Grossman, 1997; Perego, 1997; Lazazzera and Grossman, 1998).

The initiation of sporulation in *Bacillus subtilis* is regulated by a phosphorylation-mediated signal transduction pathway, known as phosphorelay (Burbulys *et al.*, 1991). The activity of master response regulator protein Spo0A depends on phosphorylation that is a typical 'response-regulator' component of the 'two-component' signaling systems, controlling entry into the developmental pathway for endospore formation (Figure 1.4) (Mirel *et al.*, 2002; Stephens, 1998).

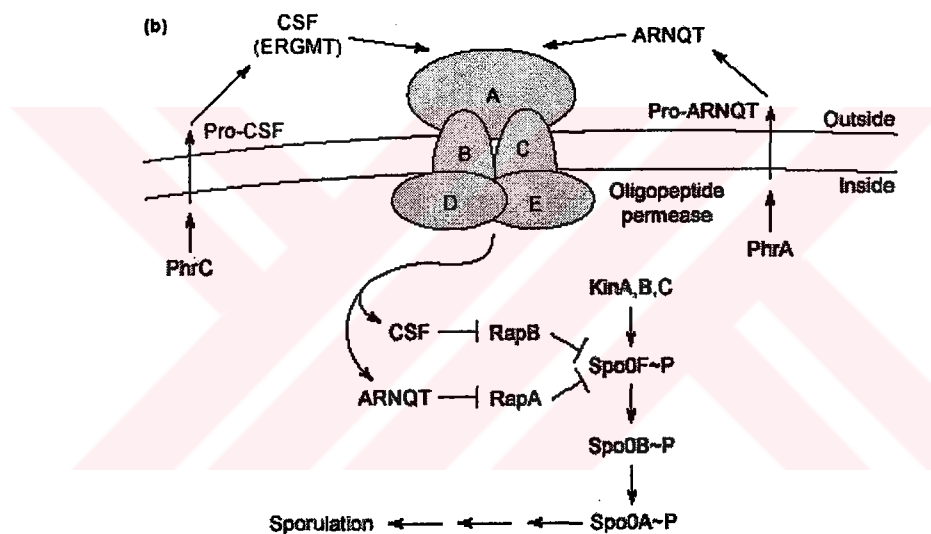


Figure 1.4: Sporulation initiation in *B.subtilis*

During the quorum sensing mediated sporulation mechanism in *Bacillus subtilis*, CSF and a peptide, ARNQT, encoded by *phrA*, are transported into the cell by Opp (figure 1.4). Each peptide inhibits the activity of a phosphatase RapB and RapA respectively. These phosphatases dephosphorylate Spo0F~P. At least three histidine protein kinases, KinA, KinB, and KinC, but mainly KinA, contribute to the production of Spo0A-P. However, unlike most response regulators, Spo0A does not normally obtain phosphate directly from histidine protein kinases. Rather, phosphate is first transferred from the kinases to Spo0F, a single-domain response regulator,

then to Spo0B, an intermediary receiving its phosphate from the Spo0F, protein, and finally to Spo0A (figure 1.5).

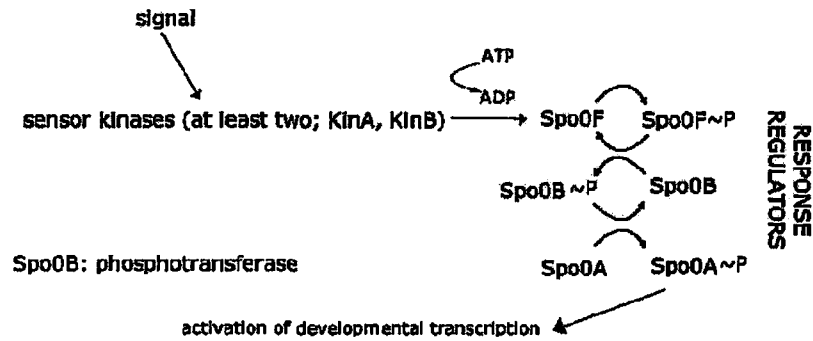


Figure 1.5: Phosphorelay signal transduction system

This phosphotransfer pathway or phosphorelay functions on the integration of multiple signals that control the initiation of sporulation. Spo0A is the key transcription factor required for the initiation of sporulation. 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 (Mirel *et al.*, 2002; Stephens, 1998).

1.4. Microbially Synthesized Bioactive Peptides

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

Table 1.1: Bioactive peptides synthesized by *Bacillus* species

Peptide	Organism	Structure
Bacilysin	<i>Bacillus subtilis</i>	Linear
Edeine	<i>Bacillus brevis Vm4</i>	Linear
Gramicidin	<i>Bacillus brevis</i>	Linear
Iturin	<i>Bacillus subtilis</i>	Cyclopeptide
Gramicidin S	<i>Bacillus brevis ATCC 9999</i>	Cyclopeptide
Tyrocidine	<i>Bacillus brevis ATCC 8185</i>	Cyclopeptide
Mycobacillin	<i>Bacillus subtilis</i>	Cyclopeptide
Surfactin	<i>Bacillus subtilis</i>	Lacton
Polymyxin	<i>Bacillus polymyxa</i>	Polypeptide
Bacitracin	<i>Bacillus licheniformis</i>	Polypeptide
Baciloscin	<i>Bacillus polymyxa</i>	Phospholipid

The potential of *B. Subtilis* to produce antibiotics has been known for 50 years. The endospore-forming rhizobacterium *B.subtilis* is known to produce more than two dozens antibiotics with an amazing variety of structures. These produced anti-microbial active compounds include predominantly peptides that are either ribosomally synthesized and post-translationally modified (lantibiotics and lantibiotic-like peptides) or non-ribosomally generated. Furthermore, non-ribosomally synthesized peptide antibiotics represent the predominant class (Stein, 2005).

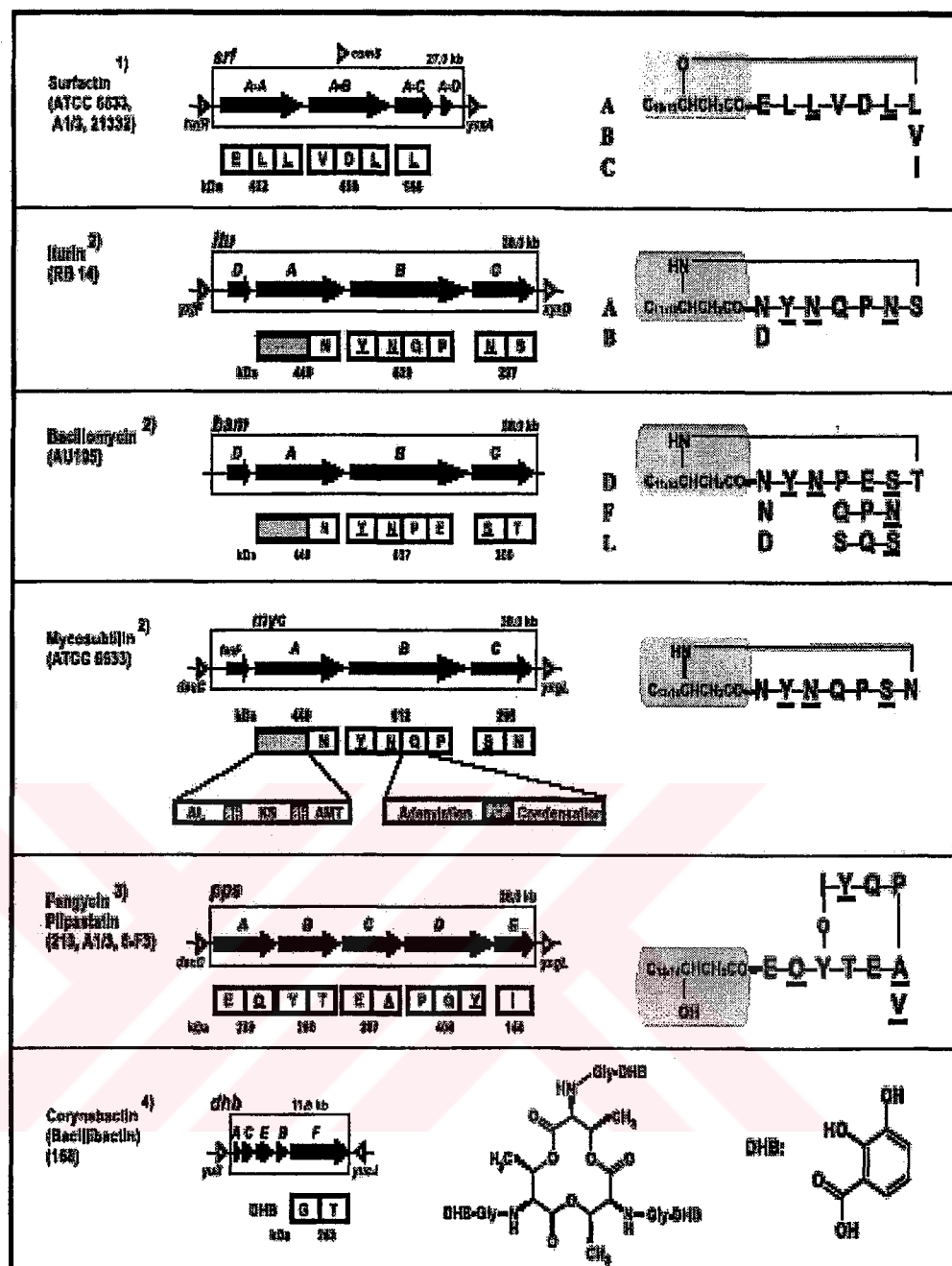


Figure 1.6: Non-ribosomally synthesized peptide antibiotics. In each line the producing *B. subtilis* strains, the genetic organization of the NRPSs (boxed), and schematic representations of produced peptide antibiotics and their possible isoforms are given.

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, catalysed by an amino acid ligase (bacilysin synthetase)(Sakajoh *et al.*, 1987).

Bacilysin production from *B. subtilis* is active when the cells are grown in a synthetic medium and becomes inhibited by certain growth conditions, like supplements and temperatures above 30°C (Özcengiz *et al.* 1990; Özcengiz and Alaeddinoglu 1991; Basalp *et al.* 1992). Its synthesis seems to be under transcription regulation via the stringent response (Inaoka *et al.* 2003) as well as under feedback regulation, likely by a component of the global quorum-sensing control system (Özcengiz and Alaeddinoglu 1991; Yazgan *et al.*, 2001; Karatas *et al.*, 2003).

In *B. subtilis*, the production of antibiotics and resistance to them are under the control of the transition state regulator AbrB. As a typical example, *tycA* operon which encodes the enzyme tyrocidine synthetase, catalyzing the synthesis of a cyclic decapeptide, is directly repressed by AbrB which interacts with sequences upstream and downstream of the promoter and controls stationary-phase expression of *tycA* (Guespin-Michel, 1971; Furbah *et al.*, 1991).

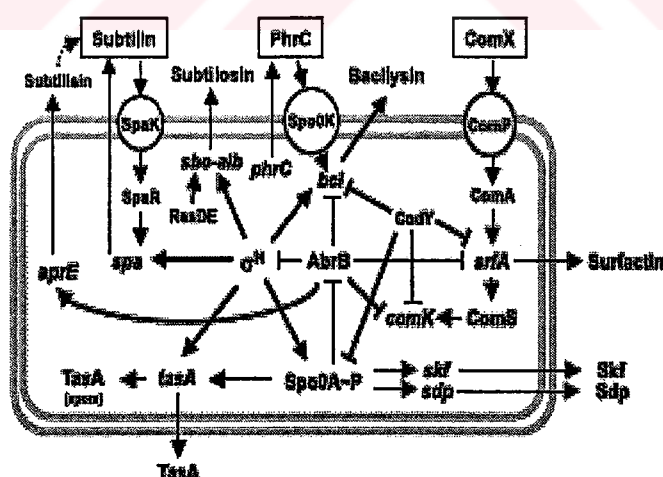


Figure 1.9: Regulatory pathways of antibiotic biosynthesis in *B. subtilis*. Survey of the regulatory pathways for the biosynthesis of the *B. subtilis* antibiotics subtilin, subtilosin, bacilysin, surfactin, the killing factor Skf and the spore-associated antimicrobial polypeptide TasA.

The biosynthesis of bacilysin depends on the *ywfBCDEFGH* cluster (Inaoka *et al.*, 2003). The unusual epoxy-modified amino acid anticapsin is generated through the action of a prephenate dehydratase and an aminotransferase encoded by *ywfBG*, respectively, as a branching off from prephenate of the aromatic amino acid pathway (Hilton *et al.*, 1988). Genes *bacDE* (*ywfEF*) have been shown to encode the functions of amino acid ligation and bacilysin immunity respectively (Steinborn *et al.*, 2004).

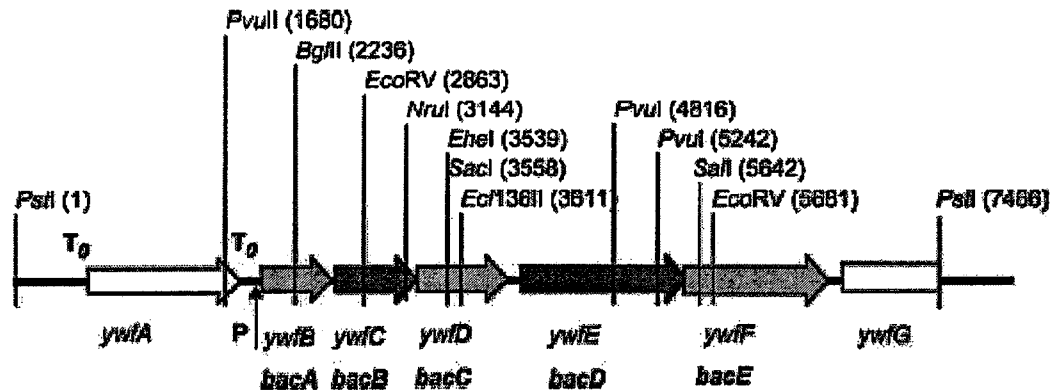


Figure 1.10: Organization of the bacilysin gene cluster *bacABCDE* relative to open reading frames *ywfABCDEFGH* of *Bacillus subtilis* 168. DNA comprises the sequence from 3875148–3867678 bp of the SubtiList database R16.1 (Kunst *et al.*, 1997). Proposed terminator (T_0) elements are indicated according 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.

Bacilysin production is regulated on different levels of positive and negative regulations. Positive regulation is conducted by guanosine 5'-diphosphate 3'-diphosphate (ppGpp) (Inaoka *et al.*, 2003) and by a quorum-sensing mechanism through the peptide pheromone PhrC (Yazgan *et al.*, 2003). Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) plays a crucial role in transcription of the *ywfBCDEFGH* 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).

The involvement of the CSF pheromone/phosphatase couple (Phr/Rap system) and the response regulator ComA in bacilysin formation which was subsequently proved by constructing *phrC* and *comA*⁻ disrupted mutants. The molecular control on bacilysin formation mainly proceeds via the inhibition of RapC phosphatase by PhrC

while the other Opp-imported pheromone PhrA is not involved. On the other hand, the other peptide factor ComX, which activates the signal transduction system composed of two component regulatory proteins ComA and ComP, is also a component of the control of bacilysin production (Lazazzera *et al.*, 1999; Lazazzera, Solomon and Grossman, 1997; Magnuson, Lazazzera and Grossman, 1994).

Bacilysin production is negatively regulated by GTP via the transcriptional regulator CodY (Inaoka *et al.*, 2003) and AbrB (Yazgan *et al.*, 2003). In wild-type (*rel*⁺) cells, a forced reduction of intracellular GTP enhances the expression of these genes and the disruption of *codY* which regulates stationary phase genes by detecting intracellular level of GTP results in an increase in their transcription. The *codY* disruption results in an increase of bacilysin production only in *rel*⁺ cells. It is known that CodY suppresses *srfA* operon and as indicated by the preliminary results, CodY also contributes to the regulation of *spo0A*; thus, increase in bacilysin production in *rel*⁺, *codY* deleted cells is due to a release from CodY repression of *oppA*, *srfA* and *spo0A*.

Bacilysin biosynthesis is under the control of quorum sensing and signal transduction phosphorelay as in the case of surfactin biosynthesis. Since the biosynthesis of surfactin and bacilysin are under the same global control, one might extend the hypothesis of Nakano *et al.* by imagining bacilysin as such an intermediate produced by *srfA* or any other peptide biosynthetic operon harbored by *B. subtilis* 168.

1.5. The Aim of the Present Study

Bacilysin is one of the simplest peptide antibiotics (125 kDa). It is produced by strain *B. subtilis* Marburg 168 and is a dipeptide containing L-alanine and the unusual amino acid L-anticapsine (Walker and Abraham, 1970). Bacilysin formation and sporulation processes are closely related (Michel and Millet, 1970; Mukherjee and Paulus, 1977; Nakano *et al.*, 1991). Moreover, its synthesis seems to be under transcription regulation via the stringent response (Inaoka *et al.* 2003) as well as under feedback regulation, likely by a component of the global quorum-sensing control system (Özcengiz and Alaeddinoglu 1991; Yazgan *et al.*, 2001; Karatas *et al.*, 2003). This biosynthesis mechanism of the simplest peptide antibiotic, thus could be expected to harbour unique features and should also be exploited with the aim of

synthesizing other specific dipeptides of commercial importance, coming from their antimicrobial, antitumor, immunosuppressive, cytostatic activities.

Under the light of this knowledge, the present study is focused on cloning and identification of the gene(s) related with bacilysin biosynthesis in *Bacillus subtilis* PY79 (a prototrophic derivative of the standard strain 168) because of its functional and applicational importance. For this aim, mini-*Tn10* transposon mutagenesis was employed to disrupt the genes necessary for the bacilysin biosynthesis.



2. MATERIALS and METHODS

2.1. Materials

2.1.1. Bacterial Strains and Plasmids

Bacillus subtilis PY79, which is a prototrophic derivative of standart strain *Bacillus subtilis* 168 was used as the wild type strain. Strains and their genotypes that are used in this project are listed in Table 2.1. *S.aureus* ATCC 9144 was used as the assay organism in bacilysin determinations. *E.coli* Top 10F' [*lacIq* Tn10 (Tetr)], *mcrA* Δ (*mrr-hsdRMS-mcrBC*), *f80lacZ* Δ M15 Δ *lacX74*, *deoR*, *recA1*, *araD139* Δ (*ara-leu*)7697, *galU*, *galK*, *rpsL* (*strr*), *endA1*, *nupG*) was used for cloning *B.subtilis* DNA. *E.coli* HB101 was used for plasmid amplification of pIC333. The mini-*Tn10* (*ori-spc*) delivery vector pIC333 was supplied from Tarek Msadek (Institut Pasteur, Department of Fundemental and Medical Microbiology Unite deBiochimie Microbienne, Paris-France). pDrive vector for cloning of PCR products was obtained from QIAGEN.

Table 2.1: Bacterial strains and their genotypes used in this project

Strain	Genotype	Source
<i>Bacillus subtilis</i> PY79	Wild type, BSP cured prototrophic derivative of <i>B.subtilis</i> 168	P.Youngman
<i>Bacillus subtilis</i> TEK1, TEK2, TEK3	<i>yvfI::Tn10::spc</i>	This project
<i>Bacillus subtilis</i> TEK4	Δ <i>yvfI::spc</i>	This project
<i>E.coli</i> Top 10F'	<i>lacIq</i> Tn10(<i>Tet</i> ^r), <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>), <i>f80lacZ</i> Δ M15 Δ <i>lacX74</i> , <i>deoR</i> , <i>recA1</i> , <i>araD139</i> Δ (<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (<i>Strr</i>), <i>endA1</i> , <i>nupG</i>	M.A.Marahiel
<i>E.coli</i> HB101	<i>F'</i> <i>traD36 lacF</i> Δ (<i>lacZ</i>) M15 <i>proA</i> ⁺ <i>B</i> ⁺ / <i>supE</i> Δ (<i>hsdM-mcrB</i>)5 (<i>r_k</i> - <i>m_k</i> - <i>McrB</i>) <i>thi</i> Δ (<i>lac-proAB</i>)	

2.1.2. Bacterial Culture Media

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

2.1.3. Buffers and Solutions

The compositions and preparation of buffers and solutions are given in Appendix B.

2.1.4. Chemicals and Enzymes

The chemicals and enzymes used and their suppliers are given in Appendix C together with their suppliers.

2.1.5. Laboratory Equipment

The laboratory equipment used during the project is listed in Appendix F.

2.1.6. Maintenance of Bacterial Strains

B.subtilis PY79, *B.subtilis* NG79 and *B.subtilis* TEK1 (*yvfI::Tn10::spc*), TEK2 (*yvfI::Tn10::spc*) and TEK3 (*yvfI::Tn10::spc*) and TEK4 (Δ *yvfI::spc*) strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates. Difco's Sporulation Medium (DSM) agar was used for the maintenance of *B.subtilis* PY79 and NG79 strains. *E.coli* and *S.aureus* strains were kept on Luria-Bertani (LB) agar plates. All cultures were stored at 4°C and subcultured monthly. 10 % glycerol stock was prepared for each strain and kept at -80°C. 2xYT medium was used for the transformation of the *E. coli* strains and HS and LS mediums were used for the transformation of *B.subtilis* strains. Perry and Abraham (PA) (Özcengiz, 1991) medium was used for the bacilysin production by *Bacillus* strains. Tryptophan containing Spizizen's Minimal Medium (SMM) (Spizizen, 1958) agar was used to determine the auxotrophic mutants. Bioassay medium (Mah et al, 1967) was used to determine the bacilysin activity against to *S.Aureus* ATCC 9144. Erythromycin (*Erm*) (1µg/ml) and Spectinomycin (*Spc*) (100µg/ml) were used as the selective antibiotics for *B. subtilis* strains, Ampicillin (*Amp*) (100 µg/ml) was used as the selective antibiotic for *E.coli* Top 10F' strain.

2.1.7. pIC333 Mini-Tn10 (*ori-spc*) Delivery Vector

Transposons were identified as mobile genetic elements over 50 years ago and subsequently became powerful tools for molecular genetics. Recently, transposon mutagenesis strategies have been developed to identify essential genes and facilitate genome sequencing analysis. In this project, to construct the transposon library, the temperature sensitive mini-*Tn10* (*ori-spc*) containing vector pIC333 (Steinmetz and

Richter, 1994) was used. This vector also contains temperature sensitive gram-positive replication origin, erythromycin resistance gene (*erm*), spectinomycin resistance gene (*Spc*) that is carried on transposon, pUC replication origin for *E. coli*. Therefore after mini-*Tn10* entegration to the chromosomal DNA, its flanking DNA segments can be cloned in *E. coli* directly.

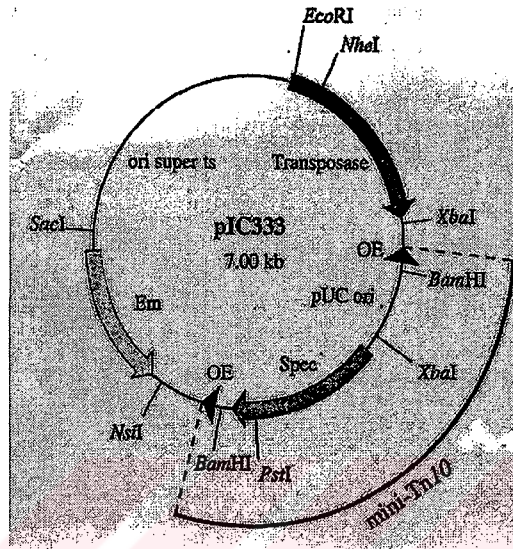


Figure 2.1: Schematic presentation of the 7 kb pIC333 vector.

2.1.8. pDrive Cloning Vector

pDrive Cloning Vector is supplied in a linear form with a U overhang at each end and was used to direct-cloning of PCR products that were generated by non-proofreading DNA polymerases just like *Taq* Polymerase. Since, proofreading DNA polymerases generate predominantly blunt-ended PCR products, they won't be efficiently ligated into the pDrive Cloning Vector. Additionally, this vector has *amp* and *kan* resistance genes, beside these, blue/white colony screening is available while using this vector for cloning purposes. pDrive also contains several unique restriction endonuclease recognition sites around the cloning site that allows easy restriction analysis of recombinant plasmids. In order to carry out transcription of cloned PCR products, as well as sequence analysis, it contains a T7 and SP6 promoter on either sides of the cloning site. Moreover, the pDrive Cloning Vector has a phage f1 origin to allow preparation of single-stranded DNA (<http://www1.qiagen.com/HB/PCRCloning>).

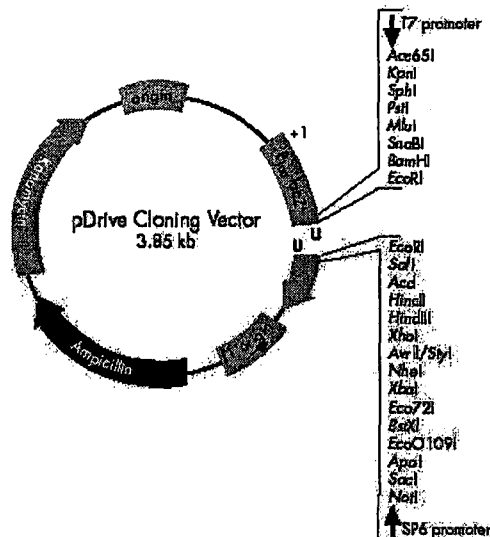


Figure 2.2: Genomic map of pDrive cloning vector including the functional genes in the structure as well as the restriction map (<http://www1.qiagen.com/HB/PCRCloning>).

2.2. Methods

2.2.1. DNA techniques and Manipulations

2.2.1.1. Plasmid DNA isolation

Plasmid DNA isolation of E.coli strains was applied through using the buffers and solutions of the “QIAquick Plasmid DNA Isolation Kit” (QIAGEN Inc.) but with a different procedure of isolation that is given below.

The bacterial cells were harvested by centrifugation at 13000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 300 μ l P1 buffer (Appendix B). 300 μ l P2 buffer (Appendix B) was added and the solution was then incubated at room temperature for 5 minutes. 300 μ l P3 (Appendix B) buffer was added and mixed through inverting until the lysate is no longer viscous. The sample was incubated for 15 minutes on ice and then centrifuged at 13000 rpm for 15 minutes. Supernatant was transferred to a new 1.5 ml eppendorf tubes and plasmid DNA was precipitated by addition 0.7 volume isopropanol and collected by centrifugation at 13000 rpm for 30 minutes. The pellet was washed with 1ml of 70% ethanol by centrifugation at 13000 rpm for 5 minutes. After removing the supernatant, ethanol was dried out at 37°C for 15. The pellet was then dissolved in 15 μ l elution buffer (EB) (Appendix B) and stored at -20°C. The isolated DNA was run on 0.8 % agarose gel.

2.2.1.2. Chromosomal DNA isolation

Chromosomal DNA of *B. subtilis* strains was isolated and purified by using “Qiagen-tips 20” (QIAGEN Inc.) following manufacturer’s protocol and alternatively by using a standard procedure devised for *Bacillus* species.

1.5 ml of overnight culture was centrifuged at 13000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 567 μ l of TE by repeated vortexing. 10 μ l of proteinase K (20mg/ml), 6 μ l of RNase (10 mg/ml), 24 μ l of lysozyme (100mg/ml) and 30 μ l of 10 % SDS were added and the mixture was incubated for 1 hour at 37°C water bath. 100 μ l of 5M NaCl solution was mixed within without vortexing until the pellet formation was observed again. 80 μ l of CTAB / NaCl (65°C) solution was added and the mixture was incubated for 10 minutes at 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. The upper phase was transferred to a new 1.5 ml microfuge tube and 0.7 volume isopropanol was mixed within and then it was centrifuged at 13000 rpm for 15 minutes. The supernatant was removed and the pellet was washed with 1ml 70% ethanol centrifuged at 13000 rpm for 5 minutes. The pellet was dried at 37°C for half an hour and dissolved in 10 μ l of TE buffer by incubation at 37°C for 3 hours at thermoshaker and stored at 4°C. The isolated DNA was run on 0.6% agarose gel and the absorbance values at 260nm and 280nm were read to determine the concentration and purity of the DNA.

2.2.1.3. Agarose Gel Electrophoresis

Depending on the purpose of the electrophoresis, different concentrations of agarose gel were used, which were given in Table 2.2. Electrophoresis of the sample DNAs were carried out in a neutral agarose gel system, composed of 1% agarose gel containing 1xTAE buffer (Appendix B) and ethidium bromide of a 0.5 μ g/ml final concentration. The gel was run in 1x TAE buffer at 4V/cm until bromophenol blue indicated that the samples have run for a sufficient distance. Following electrophoresis, the gels were visualized under UV transillumination.

Table 2.2 : Agarose gel concentrations for different samples

Sample	Concentration
Chromosomal DNA	0.6 %
Plasmid DNA	0.8 %
Digestion products of plasmid	1 %
PCR products with	1.5 % - 2%

DNA Molecular Weight Markers used in this project are Marker 1; Φ x174 DNA / *Bsu*RI (*Hae*III) and Marker 3; Lambda DNA / *Eco*RI + *Hind*III.

2.2.1.4. Gel Extraction (QIAquick Gel Extraction Kit)

The gel extraction was carried out according to “QIAquick Gel Extraction Kit” (QIAGEN Inc.) protocol. The fragment was excised from the gel and 3 volumes of buffer QG was added depending on the weight of the fragment. 10 μ l of 3M sodium acetate (pH 5.0) were added if the color of the solution was not yellow. The solution was incubated for 10 minutes at 50°C by vortexing every 2-3 minutes until the gel was dissolved completely. 1 volume of isopropanol was added and the sample was applied to the QIAquick column and centrifuged at 13000 rpm for 1 minute. The flow through was discarded and the QIAquick column was placed back into the same collection tube. 0.5 ml of buffer QG was added to the column and centrifuged at 13000 rpm for 1 minute. The flow through was discarded and 0.75 ml of buffer PE was added to wash. The column was stood for 2-5 minutes and then centrifuged at 13000 rpm for 1 minute which was followed with an additional 1 minute at 13000 rpm. Finally, the column was placed into a clean 1.5 ml microfuge tube and 30 μ l's from EB buffer was dropped to the center for the QIAquick membrane within the column and it was let stand for 1 minute and then centrifuged for 1 minute. The plasmid DNA was run on 0.8 % agarose gel and stored at -20°C.

2.2.1.5. Enzymatic Digestion

Digestion reactions were carried out as the constructor's manufacture, in a way that the amount of 10X digestion buffer was 1/10 of the total reaction mix. The reaction mix was incubated for 1-4 hours at 37°C, then enzyme was denaturated at 65°C for 10 minutes.

2.2.1.6. Polymerase Chain Reaction – PCR

PCR was performed using Taq polymerase and 10X reaction buffer from Fermentas (MBI). All cycles lasted for 1 minute. The denaturation temperature was 94°C and the extension 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. The oligonucleotide primers were purchased from IONTEK. Primers were used at 1-10 pM (equimolar) and deoxyribonucleoside 5'triphosphates (dNTPs) were used at a final concentration of 2 mM.

SacI- *yvfI* insert R : 5'-CGGGAGCTCAATATCCCGAAAGCACAT- 3'

XbaI- *yvfI* insert F : 5'-CTCTAGAACGTGTCATCATTGCTGC- 3'

*Bam*HI-*yvfI* insert R : 5' -CGGGGATCCGTGCATCCTGAATTCTTT- 3'

PaeI- *yvfI* insert F : 5'-GCCGCATGCCGTATCTGAAGGAATTTG- 3'

A master mix composed of the materials listed below was prepared according to the number of samples. Then, the master mix was divided into separate PCR tubes and 1 μl of chromosomal DNA of *Bacillus subtilis* PY79 was added into each tube as template DNA. Finally, 0.5 μl of Taq polymerase was added into each tube separately.

Reverse Primer	4 μl
Forward Primer	4 μl
10xBuffer (-MgCl ₂)	3 μl
MgCl ₂	3 μl
dNTP Mix (2 mM)	3 μl
dH ₂ O	3 μl

Total	30 μl
-------	-------

PCR Conditions:

94 °C $\Rightarrow$ 3 min(Hot Start)

94 °C 1'	} 5 cycles +	94 °C 1'	} 25 cycles	+ 72 °C 10'	Final
55 °C 1'		60 °C 1'			
Extension					
72 °C 1'		72 °C 1'			

2.2.1.7. Ligation of the PCR Products into pDrive (Qiagen) Cloning vector

Purified PCR fragments were inserted into pDrive Cloning vector (Qiagen) by using the components of Qiagen Cloning Kit, the ligation reactions were set up as follows:

pDrive Cloning Vector (50 ng/μl)	1 μl
PCR product	2 μl (1-4 μl)
Distilled water	2 μl (variable)
Ligation Master Mix	5 μl
Total Volume	10 μl

Reaction mixture was incubated at 16°C for 1.5 hours then ligase was denatured at 70°C for 10 minutes. After ligation was completed, the mixture was used for transformation into *E.coli* Top10F'.

2.2.1.8. Self-Ligation of Digested Chromosomal DNA

Totally digested chromosomal DNA was self ligated as follows:

DNA	15 μg/ml
10xBuffer	30 μl
Ligase	3 μl
Distilled Water	variable
Total	300 μl

Reaction mixture was incubated at 16°C for 16 hours then ligase was denatured at 65°C for 10 minutes. After ligation was completed, the mixture was precipitated with ethanol and used for transformation into *B.subtilis*.

2.2.2. Transformation

2.2.2.1. Preparation of *E.coli* Electrocompetent Cells and Transformation of electrocomponent *E.coli* Top 10F' Cells

E.coli Top10F' overnight inoculum was diluted 1:100 fold into 400 ml 2XYT medium containing Tetracyclin (20 μg/ml) and incubated at 37°C with 250 rpm shaking until OD₆₀₀ (Optical Density at 600 nm) reached to 0.6. Then cells were stayed on ice for 30 minutes and after incubation period cells were harvested by centrifugation at 5000 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended in 40 ml of cold distilled water and centrifuged at 5000 rpm for 15

minutes. Supernatant was removed and pellet was resuspended in 20 ml cold distilled water and centrifuged at 5000 rpm for 15 minutes. Then supernatant was discarded and pellet was resuspended in 1 ml of cold sterile 10 % glycerol and dispensed into aliquots of 40 µl into 1.5 ml eppendorf tubes. Aliquots were frozen immediately by immersing within liquid nitrogen and stored at -80°C.

For transformation, 10 µl of ligation mix was transferred into *E.coli* Top10F' by electroporation as follows:

Following a short incubation on ice, competent *E.coli* cells (Each microfuge tube contains about 40µl of *E.coli* Top10F' electrocompetent cells) were mixed with 10 µl of ligation mixture and transferred into electroporation tube. The sample was placed onto electroporation machine (Eppendorf Electroporator 2510) and the process was carried out at 1800V. After addition of 1 ml from LB broth the mixture was transferred to a 1.5 ml tube. The mixture was incubated for 1 hour at 37°C with 250 rpm shaking and then centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 100 µl of 0,85% NaCl. 100 µl of culture was spread out for each Amp/IPTG/X-gal/LB plate and incubated overnight at 37°C.

2.2.2.2. Preparation of *E.coli* CaCl₂ Competent Cells and Transformation of CaCl₂ competent *E.coli* HB101 Cells

E.coli overnight inoculum was diluted 1:100 fold into 100 ml LB broth and incubated at 37°C with 250 rpm shaking until OD₅₅₀ (Optical Density at 550 nm) reached to 0,4. Then cells were harvested by centrifugation at 3000 rpm for 10 minutes. Supernatant was discarded and pellet was resuspended in 40 ml of cold Tfb I (Appendix B) then cells were stayed on ice for 10 minutes. Following the cells were centrifuged at 3000 rpm for 8 minutes. Supernatant was discarded and the cells were gently resuspended in 4 ml of cold Tfb II (Appendix B). The cells were dispensed into aliquots of 200 µl into 1.5 ml eppendorf tubes. Aliquots were frozen immediately by immersing within liquid nitrogen and stored at -80°C.

For transformation, 10 µl of ligation mix was transferred into *E.coli* HB101 by heat-shock method as follows:

Following a short incubation on ice, CaCl₂ competent *E.coli* cells (Each microfuge tube contains about 200 µl of HB101 competent cells) were mixed with 10 µl of

ligation mixture and incubated on ice for 30 minutes. After heat pulse for 90 seconds in 42°C in water bath and cells were put on ice for 5 minutes. 0.9 ml of LB broth was added and cells were incubated at 37°C for 1 hour with 250 rpm shaking and then centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 100 µl of 0,85% NaCl. 100 µl of culture was spread out for each LB plate containing antibiotics and incubated overnight at 37°C.

2.2.2.3. Preparation of *B. subtilis* Competent Cells and Transformation

Preparation of *B. subtilis* competent cells and transformation were performed as described previously (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 OD₆₀₀ 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 hours 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 hours.

2.2.3. Southern Blot Analysis

For probe [mini-*Tn10(ori-spc)* element] labeling and Southern blotting, the ECL Random Prime Labeling and Detection System (Amersham/Buchler, Braunschweig, Germany) was used according to the protocol described by the manufacturer.

2.2.3.1. Probe Labeling

The DNA fragment [mini-*Tn10(ori-spc)* element] was labelled fluorescently by using the “ECL Random Probe Labeling System” (Amersham). 1 µl of probe DNA was taken and total volume completed to 34 µl with distilled water. It was denatured by heating for 5 minutes in a boiling bath, and then chilled on ice for 2 minutes. 10 µl of nucleotide mix, 5 µl of primers and 1 µl of Klenow polymerase enzyme were added and mixed gently by pipetting. It was spinned briefly to collect the contents at the bottom of the tube and incubated at 37°C for 16 hours. Labeled probe was stored at -20°C.

2.2.3.2. Southern Blotting

Electrophoresis of sample DNAs were carried out in a neutral agarose gel system. Following electrophoresis, the gel was visualized under UV transillumination. Then the gel was denatured in denaturation solution (1.5 M NaCl, 0.5 M NaOH), twice for 15 minutes per wash. After washing with sterile distilled water, the gel was neutralized in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) twice for 15 minutes per wash. After incubating in 2xSSC for 2 minutes, the gel was placed onto capillary blot apparatus which was assembled as described by Sambrook *et al* (1989). 20xSSC was used as transfer buffer and the transfer was performed by placing the gel overnight on to Hybond-N⁺ membrane and the DNA was fixed to the membrane by baking at 80°C for 2 hours.

2.2.3.3. Hybridization

20 ml of hybridization buffer (Appendix B) was preheated to 50°C. The blot was then replaced into this buffer and prehybridized for at least 30 minutes at 50°C with constant shaking. The probe was denatured by boiling it for 5 minutes and then cooled on ice. The denatured probe was centrifuged and added to the prehybridization buffer and mixed gently. The blot was hybridized overnight at 50°C with gentle agitation in the hybridization oven.

A stringency wash solution (1xSSC, 0.1 % SDS) was prepared and preheated to 40°C. The blot was carefully transferred to this solution and washed for 15 minutes with gentle agitation. A further wash was carried out in 0.5xSSC, 0.1 % SDS at 40°C for 15 minutes.

2.2.3.4. Blocking, Antibody Incubation and Washes

The blot was placed in a clear container and rinsed with buffer A (Appendix B). It was then transferred into liquid block 20 fold diluted in buffer A and incubated for 30 minutes. The anti-fluorescein-HRP conjugate was 1000-fold diluted in freshly-prepared 0.5 % BSA fraction in buffer A. The blot was incubated in dilute conjugate solution for 30 minutes. The blot was then washed 3 times, each for 10 minutes with 0.1 % Tween 20 in buffer A to remove unbound conjugate.

2.2.3.5. Signal Generation and Detection

Equal volumes of detection solution 1 and detection solution 2 were mixed to give a final volume sufficient to cover the blot. The blot was placed on a sheet of Saran Wrap and detection reagent was added directly to the blot on the side carrying the DNA. After 1 minute incubation, excess detection reagent was drained off and the blot was wrapped in Saran Wrap. The blot was placed in a film cassette. A sheet of autoradiography film was placed on the blot at dark and the cassette was closed. The film was exposed at least for 1 hour, and then developed.

2.2.4. Transposon Tn10 Mutagenesis

2.2.4.1. Transposon *Tn10* Mutagenesis and Selection of Bacilysin-Negative Mutants

For transposon mutagenesis, the temperature-sensitive mini-*Tn10* (*ori-spc*) containing vector pIC333 (Steinmetz and Richter, 1994) was used. This vector contains temperature sensitive gram positive replication origin, erythromycin resistance gene, spectinomycin resistance gene which is carried on transposon, and pUC replication origin for *E.coli*. Therefore after mini-*Tn10* (*ori-spc*) integration to the chromosomal DNA, flanking regions of *Tn10* was cloned in *E.coli* directly.

For this aim pIC333 was introduced into competent PY79 cells and transformants were selected on erythromycin (1 µg/ml) and Spectinomycin (100 µg/ml) LB agar plates at 28°C. The resultant *Erm*^R and *Spc*^R colonies were grown overnight in *Spc* containing LB broth medium at 28°C, diluted 100 fold in the same medium and grown for 3 hours. After that temperature was shifted to 42°C for 4 hours. At the end of this time cells were harvested by centrifugation at 5000 rpm for 15 minutes. Then pellet was resuspended in 1 ml of sterile saline solution and spread on Spectinomycin (100 µg/ml) containing LB agar plates dispensing 200 µl volume and incubated overnight at 42°C. All of the resultant colonies were collected by washing the plates with saline solution, next their chromosomal DNA's were isolated. Then chromosomal DNA was introduced into competent PY79 by transformation and transformants were selected on *Spc* containing LB agar plates at 37°C. Auxotrophic mutants and the mutants resistant to both *Spc* and *Erm* were next eliminated.

The remaining prototroph colonies resistant to *Spc* but sensitive to *Erm* were further

screened for the loss of activity against *S. aureus* ATCC 9144. These strains were grown in PA medium for 16 hours under standard conditions.

2.2.4.2. The Cloning of Mini-Tn10 (*ori-spc*) Insertion and Its Flanking DNA

This mini-*Tn10* (*ori-spc*) insertion and its flanking DNA segments were rescued in a single step, taking advantage of pUC origin of replication present on transposon. The chromosomal DNAs of mutant strains were totally digested with one of the suitable restriction enzymes (such as *EcoRV*, *PvuII*, *ClaI*, *EcoRI*, *HindIII*) and resultant fragments were self-ligated at 15 µg/ml DNA concentration as given above. The ligation mixture was precipitated and transformed into *E.coli* Top 10F' and selected for spectinomycin resistance.

2.2.4.3. Sequencing Reactions

The cloned fragments adjacent to mini-*Tn10* (*ori-spc*) were sequenced by using the DNA primers 5'-GCCGCGTTGGCCGATTC-3', 113 bp to 98 bp and 5'-GATATTACGGTTTAC-3', 2235 bp to 2249 bp hybridized to the ends of the mini-Tn10 (Steinmetz and Richter, 1994). Sequencing reactions were carried out by the chain termination method (Sanger *et al*, 1997) with dye-labeled dideoxy terminators by ABI Prism 3100 Avant automated sequencer. Deduced nucleotide sequence data were compared with National Center for Biotechnology Information (NCBI) database using the BLAST search at the web site <http://www1.ncbi.nlm.nih.gov/BLAST>.

3. RESULTS AND DISCUSSION

3.1. Transposon Mutagenesis

3.1.1. Transposon (Tn10) Mutagenesis and Detection of Mutants Defective in Bacilysin Production

In order to isolate the mutants defective in bacilysin biosynthesis, transposon mutagenesis was performed in strain PY79 using the mini-*Tn10* insertion delivery vector pIC333. Of ca. 4000 Spc^R and Erm^S mini-*Tn10* insertion mutants from the transposon library screened for the loss of activity against *S. aureus* ATCC 9144, three mutants remained zone-free on assay plates. These bacilysin-negative mutants were selected for further analysis and designated as TEK1, TEK2 and TEK3, respectively (Figure 3.1).

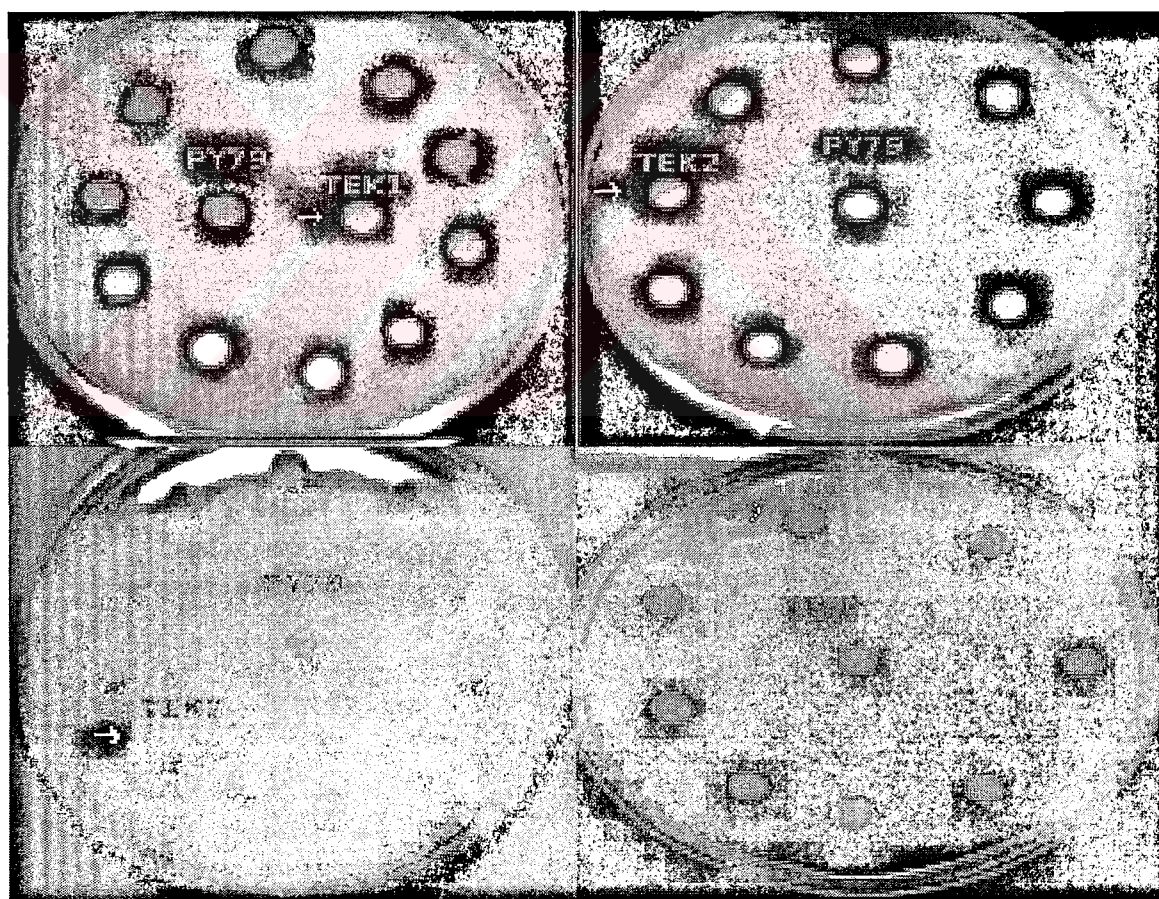


Figure 3.1: Bioassay plates of Bacilysin-defective mutants. *B.subtilis* PY79 used as the positive control.

3.1.2. Southern Blot Hybridization Analysis of Bacilysin–Negative Mutants

The chromosomal DNA's from TEK1, TEK2 and TEK3 were digested with restriction enzymes *Cla*I and *Hind*III. Resulting DNA segments were separated on 1% agarose gels, blotted and hybridized to a mini-*Tn10* (*ori-spc*) probe for the purpose of determining location of the insertion of mini-*Tn10* in the chromosome of these mutants. As shown in the figure 3.2, single mini-*Tn10* insertions were detected in the chromosomes of these mutants.

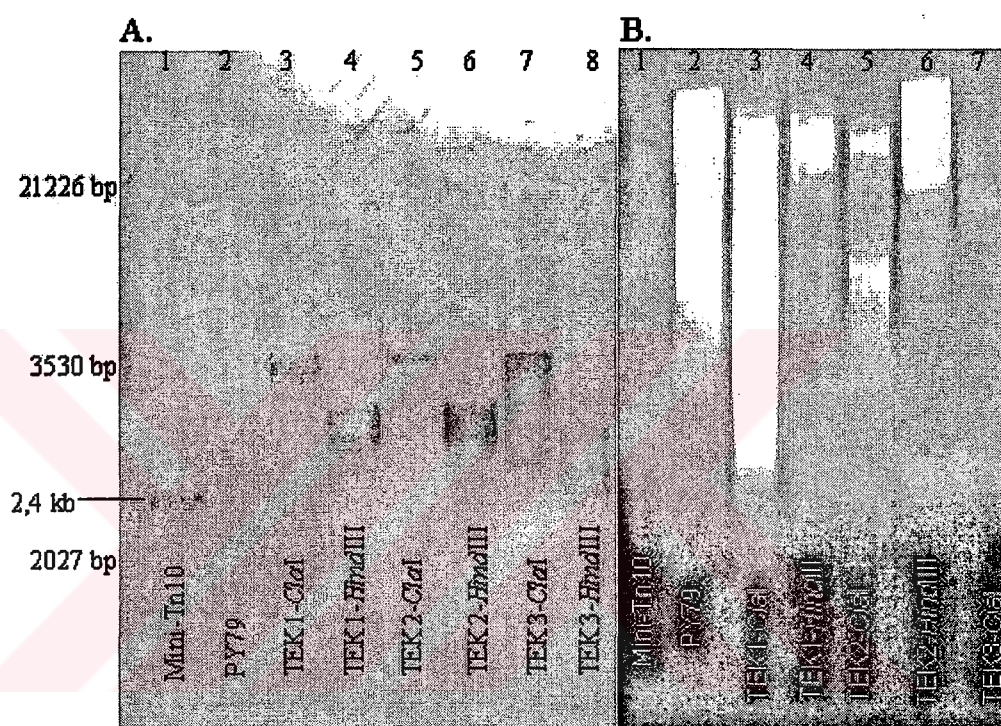


Figure 3.2: Southern Blot Hybridization Analysis of the mini-*Tn10* (*ori-spc*) insertional mutants TEK1, TEK2 and TEK3 [*yvfI::Tn10(ori-spc)*]. **A.** hybridized with fluorescently labelled app.2.4 kb *Bam*HI fragment of plasmid pIC333 containing mini-*Tn10* (*ori-spc*) sequence as probe. Unlabelled probe was run as positive control at the positions of lane 1. **B.** Chromosomal DNA from *B.subtilis* PY79 (lane 2), TEK1 (lane 3), TEK2 (lane 5), TEK3 (lane 7) were digested with *Cla*I and chromosomal DNA from TEK1 (lane 4), TEK2 (lane 6), were digested with *Hind*III.

3.1.3. Cloning of Tn10 Insertions and Their flanking Regions

Following the aim of identifying the gene(s) of the mutant loci affecting bacilysin biosynthesis in PY79, inserted mini-*Tn10* (*spc*) transposons and their flanking DNA segments were cloned and sequenced. 3,5 kb plasmids were rescued from the chromosome of TEK1, TEK2 and TEK3 after *Eco*RV total digestion and self ligation (Figure 3.3).

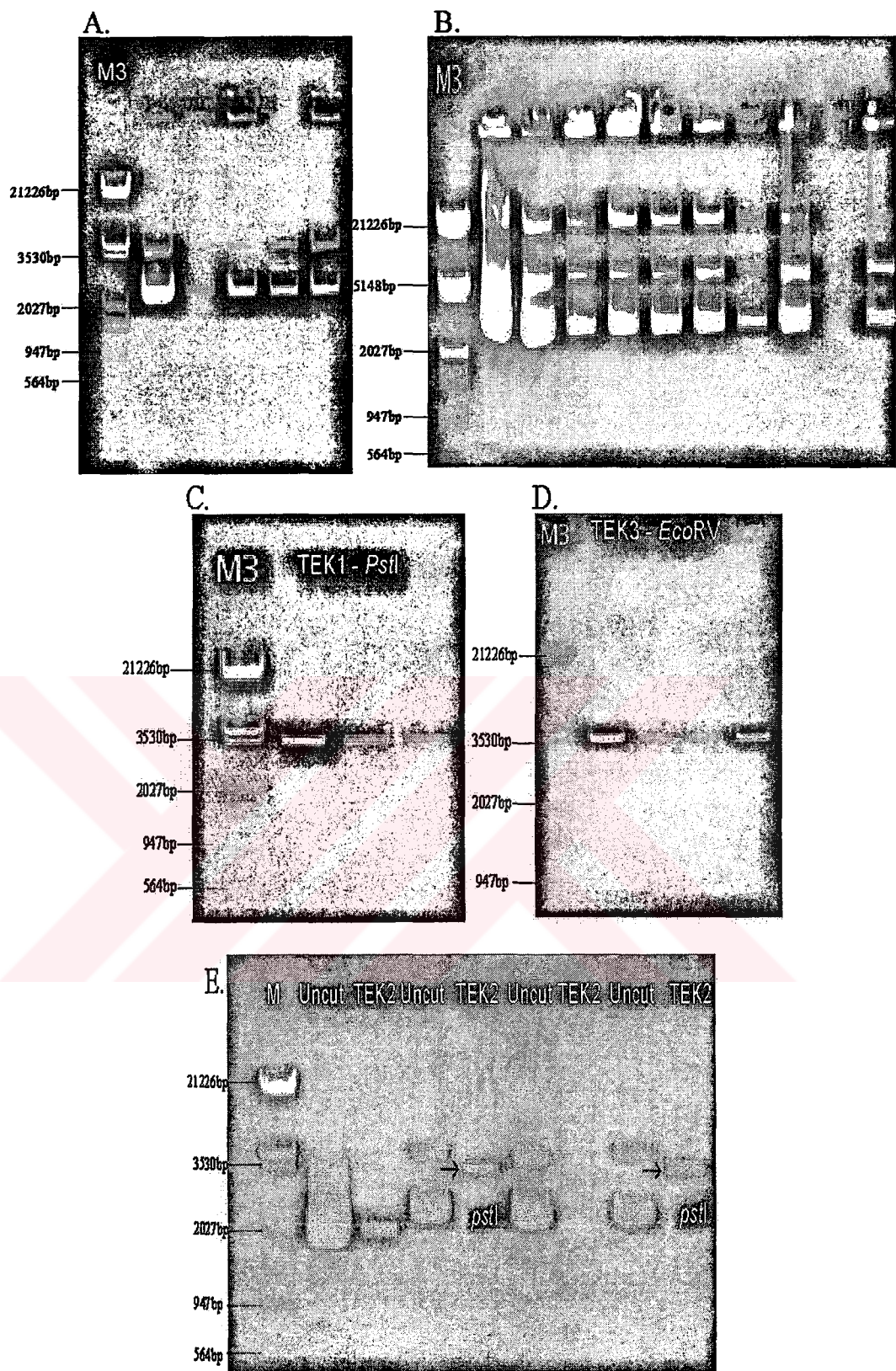


Figure 3.3: A. Undigested plasmids rescued from *Tn10* insertional mutant TEK1, B. Undigested plasmids rescued from *Tn10* insertional mutant TEK3, C. 3,5 kb rescued plasmid from *Tn10* insertional mutant TEK1 : digested with *Pst*I, D. 3,5 kb rescued plasmid from *Tn10* insertional mutant TEK3 : digested with *Eco*RV, E. 3,5 kb rescued plasmid from *Tn10* insertional mutant TEK2 : digested with *Pst*I. M3: Lambda / *Eco*RI+*Hind*III Marker DNA fragments

Nucleotide sequence analysis revealed that the insertions took place within the *yvfI* (unknown; similar to transcriptional regulator factor) gene as based on the *B. subtilis* genome consortium (Kunst et al.; 1997).

Chromosomal DNAs isolated from these mutants were used to backtransform the wild-type strain PY79 against spectinomycin resistance in order to confirm that the observed loss of bacilysin biosynthetic ability was due to the inserted transposon. For each individual mutation, 50 transformants were tested for their ability to synthesize bacilysin. Introduction of *yvfI::Tn10* mutations to the parental strain conferred the same phenotype in the wild-type background since 92-100 % of backtransformants were nonproducers (Figure 3.4).

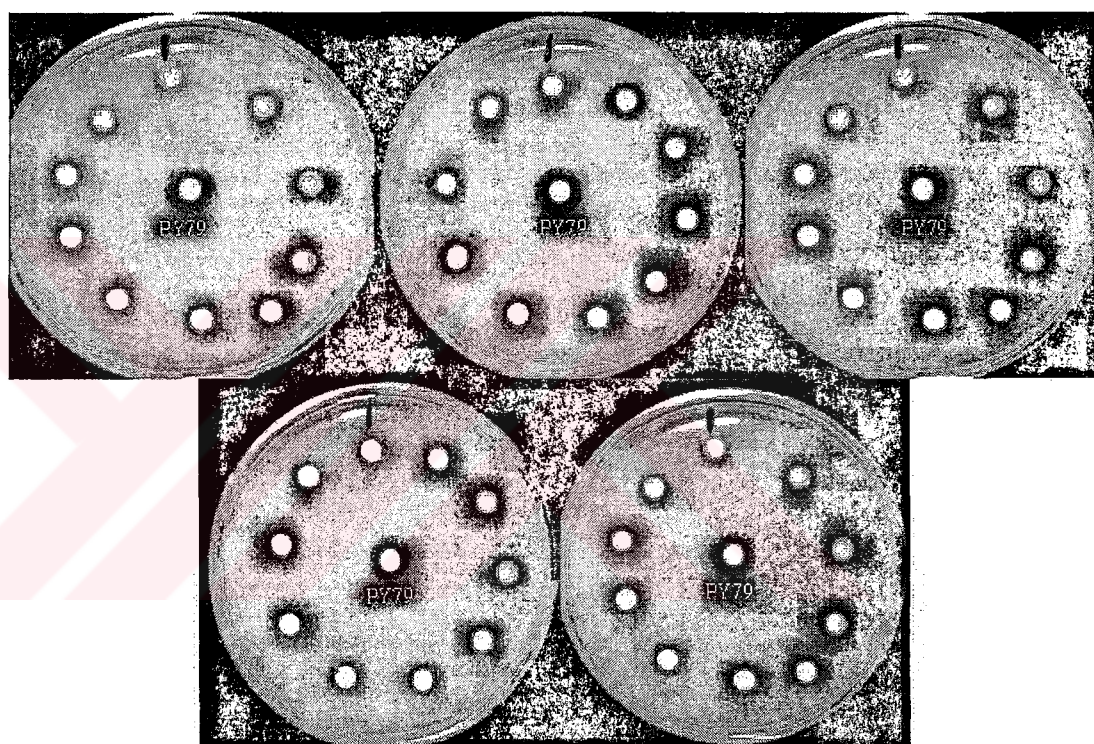


Figure 3.4: Bioassay plates of backtransformants

3.2. Construction of *yvfI* deletion vector

3.2.1. Obtaining *spc* cassette

7.0 kb long pIC333 vector was digested with *Bam*HI for the interest of obtaining 2.4 kb long mini-*Tn10* fragment (Figure 3.5). Afterwards, mini-*Tn10* fragment was double digested with *Xba*I and *Bam*HI and 900 bp long *spc* cassette was obtained which had been isolated from the agarose gel.

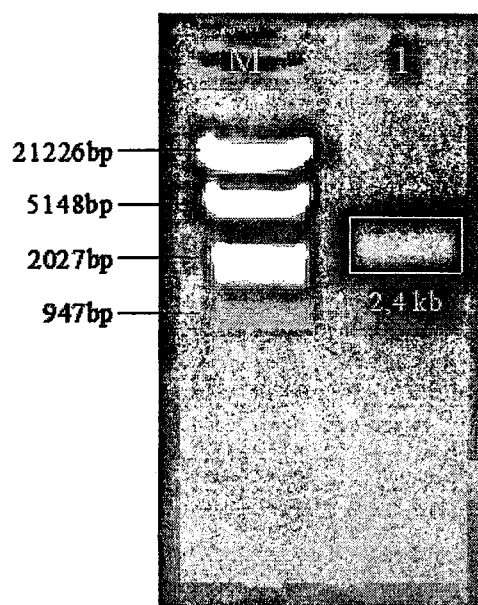


Figure 3.5: 2.4 kb long mini-*Tn10* fragment obtained from pIC333 vector (lane 1). M: Marker 3: Lambda DNA / *EcoRI* + *HindIII*

Under the aim of cloning it into pDrive Cloning Vector (Qiagen), *spc* cassette was digested with *XbaI* and *BamHI* restriction enzymes and ligated into similarly digested pDrive Cloning Vector (Qiagen). This ligation mixture was used to transform electrocompetent cells of *E.coli* Top10 and transformants were selected on LB agar plates containing 100µg Ampicillin ml⁻¹.

Resulting transformants were picked up and 10 of them were used for plasmid DNA isolation for the verification of the cloning of 900 bp *spc* cassette fragment (Figure 3.6).

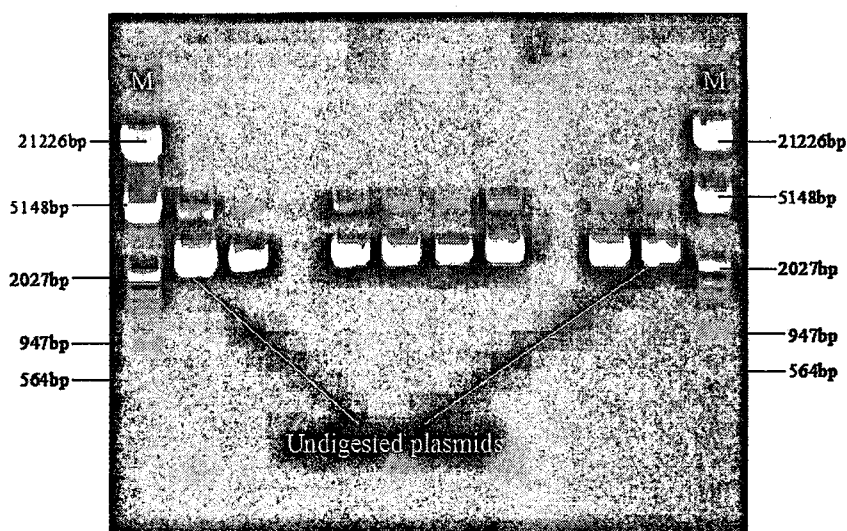


Figure 3.6: Plasmid DNA' s isolated from *E.coli* Top10 Amp^R transformants and M: Lambda / *EcoRI*+*HindIII* Marker DNA fragments.

Plasmid DNA's isolated from transformants were double digested with *Bam*HI and *Xba*I restriction enzymes for further confirmation (Figure 3.7). The vector pDrive itself was 3850bp long while the insert was 900 bp long. Thus, molecular weight of the resulting recombinant plasmid was expected to be about 4750 bp long.

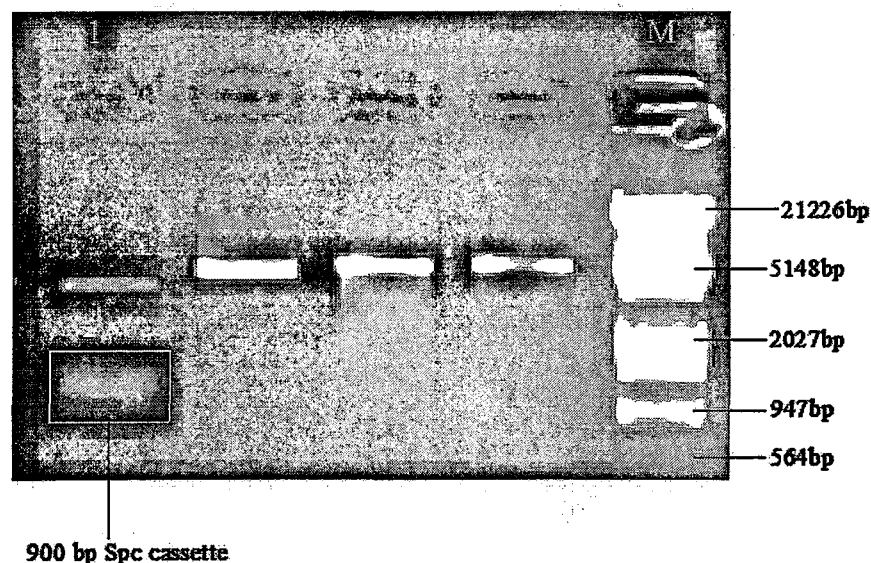


Figure 3.7: pDrive cloning vector and *spc* cassette, after *Xba*I and *Bam*HI double digestion (1). M : Marker 3: Lambda DNA / *Eco*RI+*Hind*III

As a consequence of plasmid double digestion (Figure 3.7 lane 1), a 900 bp DNA fragment, which is equal to the cloned *spc* cassette from pIC333 (Figure 3.7 lane 1) and a 3850 bp DNA fragment which represents linear pDrive Cloning Vector DNA (Figure 3.7 lane 1) were observed on the agarose gel. Therefore, plasmid DNA's isolated from this transformant was selected as the desired construct.

3.2.2. Insertion of the PCR Fragments into pDrive Cloning Vector Containing *spc* cassette

An internal fragment of the *B. subtilis* PY79 528 bp *yvfI* gene, which stands between 314 to 490 bp downstream of translational codon, was amplified by PCR using the chromosomal DNA of wild type strain *Bacillus subtilis* PY79 as template. The reverse sequence was representing recognition site for *Sac*I and the forward sequence was representing recognition site for the *Xba*I restriction endonucleases.

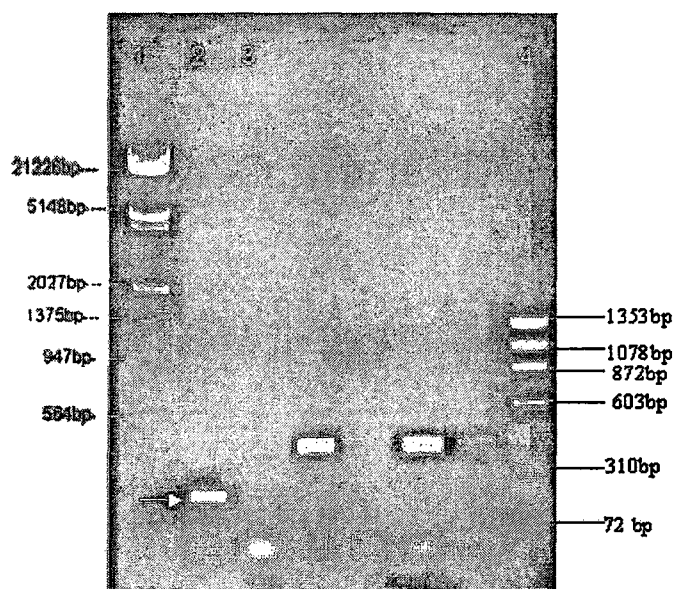


Figure 3.8: 176 bp *yvfI* fragment amplified with PCR (lane 2). Control PCR (lane 3). Marker 3: Lambda DNA / *EcoRI*+*HindIII* (lane 1). Marker 1: PhiX174 DNA / *BsuRI* (*HaeIII*) (lane 4).

In order to clone into the constructed plasmid, the resulting PCR fragment was digested with *SacI* and *XbaI* restriction enzymes and ligated into similarly digested pDrive containing *Spc* cassette vector. The ligation mixture was used to transform electrocompetent cells of *E.coli* Top10 and transformants were selected on LB agar plates containing 100µg Ampicillin / ml.

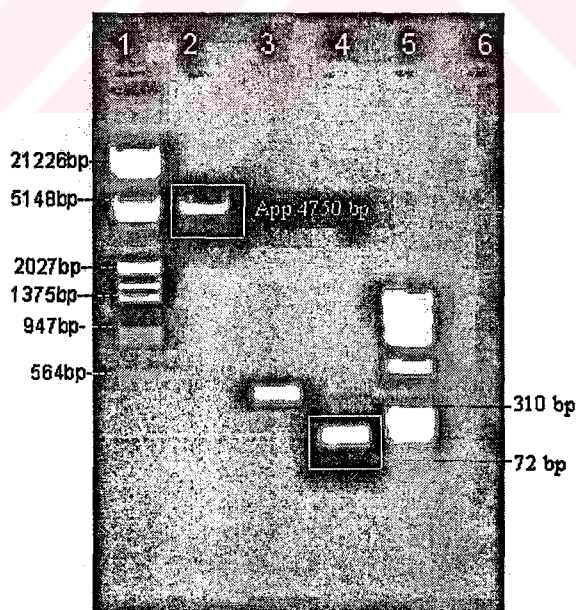


Figure 3.9: Gel extraction result of *spc* cassette containing pDrive cloning vector (lane 2) and 176 bp long PCR fragment double digestions with *XbaI* and *BamHI* after gel extraction (lane 4). Marker 1: PhiX174 DNA / *BsuRI* (*HaeIII*) (lane 5). Marker 3: Lambda DNA / *EcoRI*+*HindIII* (lane 1).

Resulting transformants were picked up and all of them were used for plasmid DNA isolation for the purpose of verifying both the cloning of 900 bp *spc* cassette fragment and PCR product. Following this aim, isolated plasmids in different conformational structure were chosen and double digested with *Xba*I-*Bam*HI and also with *Xba*I-*Sac*I in order to obtain the *spc* cassette and the cloned PCR fragment, respectively (Figure 3.10).

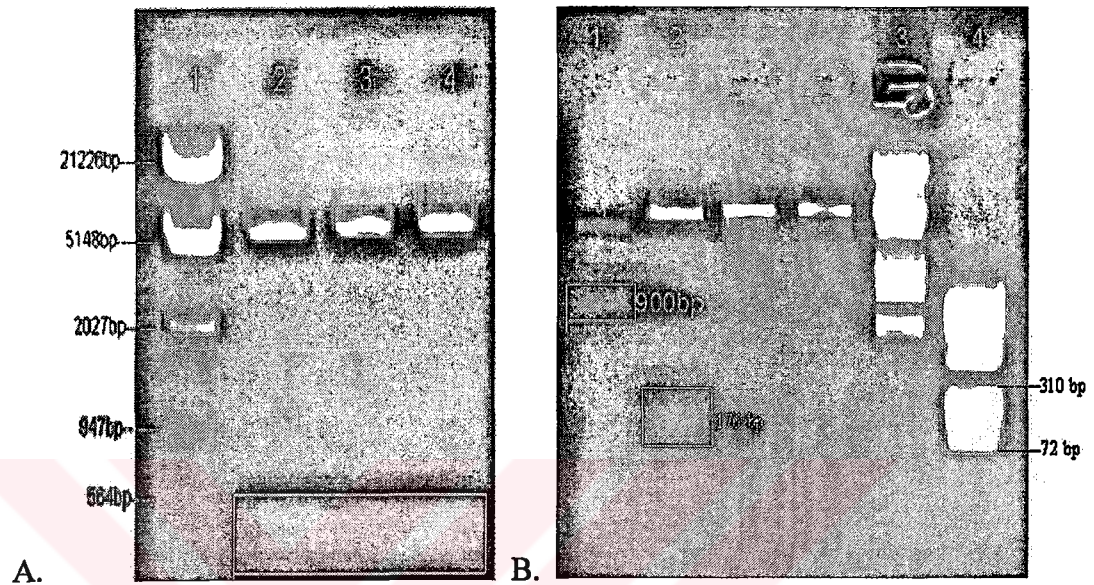


Figure 3.10: A. App.176 bp long *Sac*I-*Xba*I PCR product (lane 2, 3, 4). Marker 3: Lambda DNA / *Eco*RI+*Hind*III (lane 1). B. 900 bp long *spc* cassette (lane 1). 176 bp PCR product (lane 2). Marker 3: Lambda DNA / *Eco*RI+*Hind*III (lane 4). Marker 1 : PhiX174 DNA / *Bsu*RI (*Hae*III) (lane 3).

As a result of these double digestions (Figure 3.10,(A. lane 2,3,4 and B. lane 2)), an app. 176 bp DNA fragment, which is equal to cloned *yvfI* PCR fragment (Figure 3.10 lane 5) and a 900 bp DNA fragment which represents *Spc* cassette DNA (Figure 3.10 (B.lane 1) were observed on the agarose gel.

Following that, the second internal fragment of the *B. subtilis* PY79 *yvfI* gene, a fragment which stands between 20 to 196 bp downstream of translational start codon was amplified by PCR using the chromosomal DNA of wild type strain *B.subtilis* PY79 as template(Figure 3.11). The reverse sequence were representing recognition site for *Bam*HI and the forward sequence were representing recognition site for the *Pae*I (*Sph*I) restriction endonucleases.

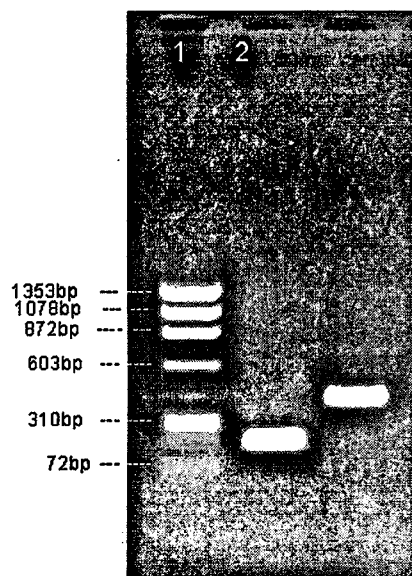


Figure 3.11: 176 bp *Bam*HI-*Pae*I PCR fragment after gel extraction (lane 2). Marker 1: PhiX174 DNA / *Bsu*RI (*Hae*III) (lane1).

To clone this second PCR product into the constructed vector containing both *Spc cassette* and *Xba*I-*Sac*I fragment, and second PCR product was double digested with *Bam*HI-*Pae*I and ligated into similarly digested constructed vector. The ligation mixture was used to transform electrocompetent cells of *E.coli* Top10 and transformants were selected on LB agar plates containing 100µg Ampicillin / ml.

The resulting transformants were picked up and all of them were used for plasmid DNA isolation (Figure 3.12.A) to verify the cloning of the inserted fragments. Following this, restriction digestion analysis was carried out and the rescued plasmids were linearized with *Sac*I (Figure 3.12.B) and double digested with *Sac*I-*Pae*I (Figure 3.12.B). As a consequence of these digestions, only one plasmid was identified as having the expected molecular weight and containing the cloned fragment (Figure 3.12.B). For further confirmation, obtained plasmid and the construct which contains *Spc cassette* and *Sac*I-*Xba*I PCR product were double digested with *Bam*HI-*Pae*I and *Bam*HI-*Xba*I, respectively (Figure 3.12.C) in order to observe the molecular weight difference between these two constructs.

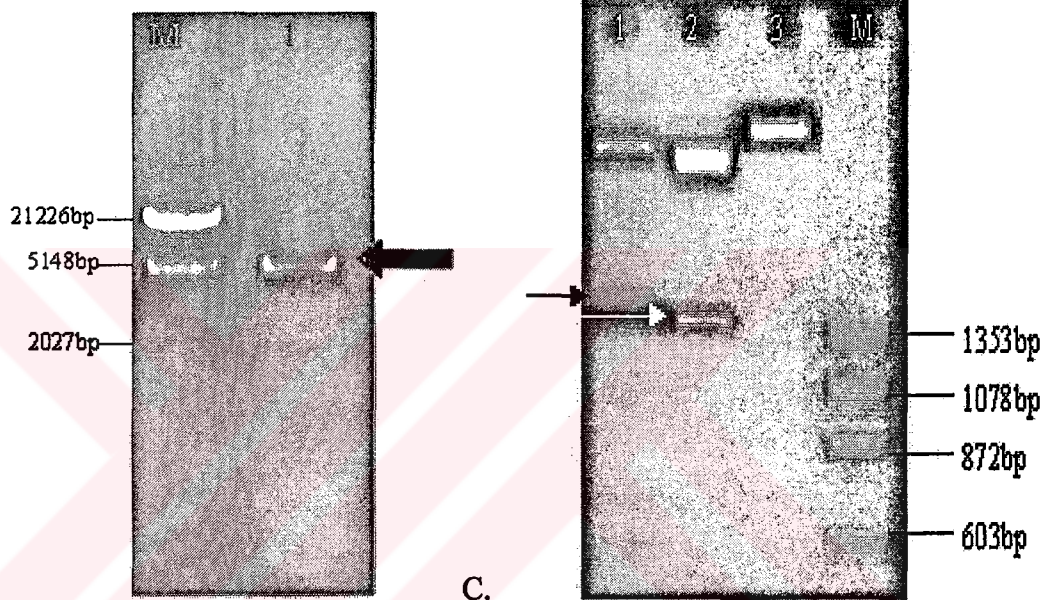
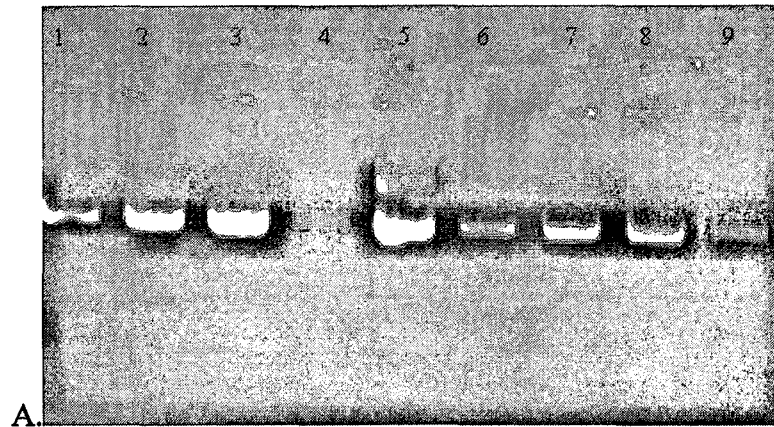


Figure 3.12: A. Undigested plasmids, B. Linearization of the obtained plasmid with *SacI* (lane 1) M: Marker 3: Lambda DNA / *EcoRI*+*HindIII*, C. Linearized plasmid with *SacI* (lane 3), double digestion with *Bam*HI-*Xba*I (lane 1), double digestion with *Bam*HI-*Pae*I (lane 2), M : Marker 1 : PhiX174 DNA / *Bsu*RI (*Hae*III).

The vector pDrive itself was 3,85 kb long while the inserts were totally 1,252 bp long. The molecular weight of the resulting recombinant plasmid was expected to be about 5,1 kb long. Therefore, a band app. 5,1 kb in size was obtained from the plasmid digestion with *SacI* (Figure 3.12.B). There had to be app.176 bp difference in size between the bands given by *Bam*HI-*Pae*I and *Bam*HI-*Xba*I double digestions of the construct coming from the fact that when the construct is double digested with *Bam*HI-*Pae*I, the restricted site contains not only the *spc* cassette and the first PCR fragment but also the second cloned fragment. Thus, in Figure 3.12.C, it was also shown that these two construct differ in size as expected.

With the objective of size determination, this chosen plasmid was amplified in *E. coli* Top10, and the recombinant plasmid was further screened with the sequence analysis, using the specific primers to the ends of *yvfI* gene; *PaeI* forward and *SacI* reverse and M13 forward(-40) specific primer, respectively. It was shown that this constructed vector contains both the ends of the *yvfI* gene and also the *spc* cassette (Figure 3.13).

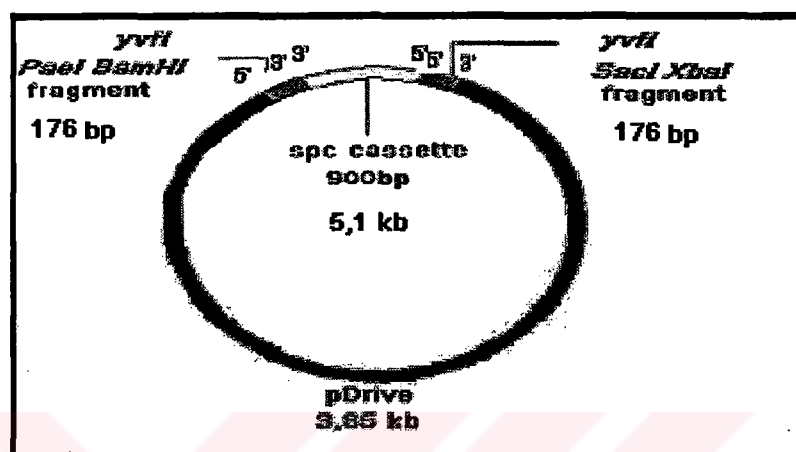


Figure 3.13: Constructed Deletion Vector

3.3. Construction of *yvfI* deletion in *B. Subtilis*

In order to provide construction of *yvfI* deletion mutant, constructed deletion vector DNA was used to transform competent cells of *B. subtilis* PY79 to spectinomycin resistance. Recombinant plasmid was driven into the chromosomal DNA of *B. subtilis* PY79 by a double cross over event. Resultant transformants were screened for the loss of activity against *S. aureus* ATCC 9144. Six $\Delta yvfI::spc$ mutants were remained zone-free on assay plates (Figure 3.14).

Chromosomal DNAs of these bacilysin-negative Spc^R transformants were isolated and cloned into pDrive Cloning Vector. For this aim, chromosomal DNAs were double digested with *SacI* and *PaeI* and ligated into similarly digested pDrive Cloning Vector. The ligation mixtures were used to transform electrocompetent cells of *E. coli* Top10 and transformants were selected on LB agar plates containing 100 μ g Ampicillin / ml.

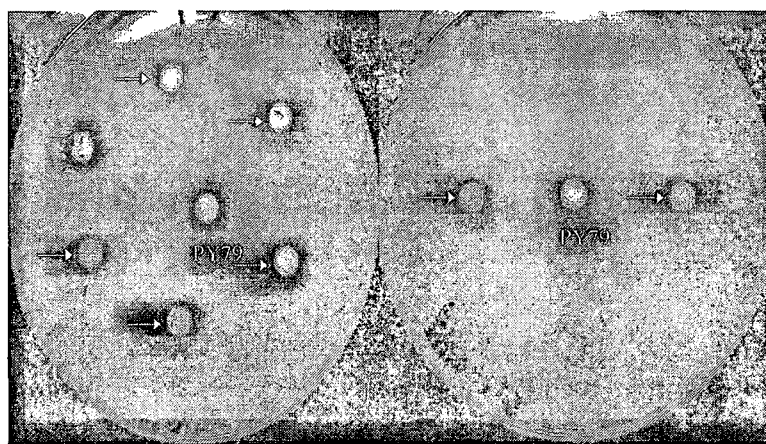


Figure 3.14: Bioassay plates of bacilysin – negative $\Delta yvfl::spc$ mutants

The resulting transformants were picked up and all of them were used for plasmid DNA isolation and obtained plasmids were double digested with *SacI*-*PaeI* in order to obtain the cloned DNA. After the verification of insertion, the recombinant plasmid was further screened with the sequence analysis, using the specific primers to the ends of *yvfl* gene; *PaeI* forward and *SacI* reverse and M13 forward (-40) specific primer.

Under the light of the information driven from the results of this project, this newly identified gene, *yvfl*, encoding an unknown protein similar to GntR transcriptional regulator, was found to be necessary in the biosynthesis of bacilysin. Moreover, it can be speculated that this synthesis can also be a part of transcriptional regulation mechanisms as well as quorum sensing global regulation system. In order to identify the exact regulation route for bacilysin production, all genes involved in these kinds of regulation cascades should be tested for their effects on bacilysin synthesis, as recommended for further studies.

Regulation of transcription through the action of small molecules that directly bind to transcription factors is widespread in all life forms. In prokaryotes, classical studies on gene expression have shown that operons or regulons, encoding proteins involved in a particular metabolic pathway, are often regulated by transcription factors that bind metabolites, which are a part of that pathway. The GntR family of transcription factors is one of the most prevalent superfamilies of transcription factors in bacteria. These proteins are regulators for a very diverse set of operons and regulons, which respond to a range of stimuli, in the form of different small molecules (Aravind and Anantharaman, 2003).

The family contains a repressor of *Bacillus subtilis* gluconate operon (GntR), regulators for histidine utilization in *Pseudomonas putida* (HutC_{PP}) and *Klebsiella aerogenes* (HutC_{KA}), a repressor (FadR) of fatty acid degradation in *Escherichia coli*, a regulator involved in the conjugal transfer of the broad host range plasmid pIJ101 (KorA), and three proteins of unidentified function in *E. coli* (GenA, P30 and PhnF). The proteins share amino acid sequence similarities in a 69-residue N-terminal region. A helix-turn-helix motif is predicted in the most highly-conserved segment of each protein suggesting that they are members of a new family of helix-turn-helix DNA-binding proteins (Haydon and Guest, 1991).

It is widely believed that thousands of genes and their products (i.e. RNA and proteins), in a given living organism, function in a complicated and orchestrated way that creates the mystery of life. One should keep in mind that it is important to identify the regulation mechanism in the synthesis and gene expression pattern and timing as well as to identify new genes. To obtain such a data, more sophisticated investigations should be carried out which will bring the researchers from one point to the whole picture. However, traditional methods in molecular biology generally work on a "one gene in one experiment" basis, which means that the throughput is very limited and the "whole picture" of gene function is hard to obtain. In the past several years, a new technology, called DNA microarray, has attracted tremendous interests among biologists. This technology promises to monitor the whole genome on a single chip so that researchers can have a better picture of the interactions among thousands of genes simultaneously. Base-pairing (i.e., A-T and G-C for DNA; A-U and G-C for RNA) or hybridization is the underlining principle of DNA microarray. An array is an orderly arrangement of samples. It provides a medium for matching known and unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns. There are two major application forms for the DNA microarray technology: Identification of sequence (gene / gene mutation); and determination of expression level (abundance) of genes.

Under the fact of this knowledge, the further studies should be focused on the identification of new genes related to bacilysin biosynthesis as well as obtaining new data which will let us understand the roles of the genes in the synthesis and in the whole cell. Understanding the synthesis of this simple peptide with its unique feature, will let us apply this knowledge into the biotechnological applications.

4. CONCLUSIONS

To identify new gene(s) involved in the bacilysin biosynthesis, transposon mutagenesis was employed. Following this aim, mini-*Tn10* insertional mutants were selected phenotypically from the transposon library using bacilysin bioassay. Three mutants were isolated and designated as TEK1(*Bac*::*Tn10*), TEK2(*Bac*::*Tn10*) and TEK3(*Bac*::*Tn10*). The mini-*Tn10* insertion and its flanking DNA's in these mutants were cloned in *E. coli* TOP10F' cells. Sequence analysis revealed that mini-*Tn10* insertion took place in *yvfI* gene (*yvfI*::*Tn10*) related with the loss of bacilysin activity. Gene *yvfI* is an unknown gene and it encode a protein similar to transcriptional regulator factor belonging GntR family of transcriptional regulators.

In order to verify that the loss of activity in bacilysin biosynthesis depends on the disruption of *yvfI* gene or the mini-*Tn10* insertional mutation itself, a *yvfI* deletion vector was constructed and introduced into *Bacillus subtilis* PY79 strain. Resulting mutant, TEK4 ($\Delta yvfI::spc$) was further investigated for the loss of bacilysin activity, and this mutation resulted in the elimination of bacilysin biosynthesis. Thus, it was confirmed that *yvfI* gene is involved in the biosynthesis of dipeptide antibiotic bacilysin, as well as *thyA*, *ybgG* and *oppA*.

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APPENDIX A

Compositions and Preparation of Culture Media

Perry and Abraham (PA) Medium (pH 7.4)

KH ₂ PO ₄	1 g/L
KCl	0.2 g/L
MgSO ₄ .7H ₂ O*	0.5 g/L
Glutamate.Na.H ₂ O	4 g/L
Sucrose*	10 g/L
Ferric citrate**	0.15 g/L
Trace elements**	1 ml
CoCl ₂ .6H ₂ O	0.0001 g/L
Ammonium molybdate	0.0001 g/L
MnCl ₂ .4H ₂ O	0.001 g/L
ZnSO ₄ .7H ₂ O	0.0001 g/L
CuSO ₄ .5H ₂ O	0.00001 g/L

*Autoclave separately

**Filter sterilization

DSM (Schaeffer's sporulation medium / agar) (1000 ml)

Nutrient Broth	8 g
KCl (10% w/v)	10 ml
MgSO ₄ .7H ₂ O (1.2%)	10 ml

NaOH (1M) 0.5 ml

Autoclave for 30 minutes and cool down to 50°C.

Ca (NO₃)₄ (1M) 1 ml

MnCl₂ (0.01M) 1 ml

FeSO₄ (1mM) 1 ml (resuspend before use)

% 1.5 Agar can be added if necessary before autoclaving.

Luria Bertani (LB) Medium (1000ml)

Tryptone 10 g/L

Yeast Extract 5 g/L

NaCl 5 g/L

Distilled H₂O was added up to 1000ml and then autoclaved for 15 minutes.

Bioassay Medium (pH 7.1)

Na₂HPO₄.2H₂O 3.3 g/L

KH₂PO₄ 1 g/L

NaCl 1 g/L

Glucose* 10 g/L

MgSO₄.7H₂O* 0.7 g/L

Na₃.citrate.2H₂O 0.5 g/L

Glutamic acid.Na.H₂O** 2.4 g/L

12 amino acid*** 0.025 g/L (each)

FeSO₄.7H₂O** 0.01 g/L

Agar****

*Autoclave glucose and MgSO₄.7H₂O together, separately

**Filter Sterilization

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

****Autoclave separately

SMS/MM (Spipizen's Minimal Salts/Medium)

$(\text{NH}_4)_2\text{SO}_4$	2 g/L
K_2HPO_4	14 g/L
KH_2PO_4	6 g/L
$\text{Na}_3\text{.citrate.}2\text{H}_2\text{O}$	1 g/L
$\text{MgSO}_4\text{.}7\text{H}_2\text{O}$	0.2 g/L

Autoclave, cool down to 50°C, add the following sterile solutions to one liter of SMS:

50% (w/v) D-Glucose*	10 ml
L-tryptohan (5 mg/ml)*	10 ml

*Filter sterilization

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

10X-S-base

$(\text{NH}_4)_2\text{SO}_4$	20 g/L
$\text{K}_2\text{HPO}_4\text{.}3\text{H}_2\text{O}$	140 g/L
KH_2PO_4	60 g/L
$\text{Na}_3\text{.citrate.}2\text{H}_2\text{O}$	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 H₂O 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 sterilized)	50 µl

Freshly prepare and complete up to 20 ml with sterile distilled H₂O.

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

Plasmid Isolation Solutions

P1 (pH 8)

Tris	10 mM
EDTA	1 mM
RNase	100 µg/ml

P2

NaOH	1 N
SDS	1%

P3 (pH 4.55)

Potassium acetate	5 M
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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 ₂ O 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.

Low Melting Agarose Gel (2%)

Agarose	1 g
TAE buffer (1X)	50 ml

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

Solutions for CaCl₂ Competent Cell Preparation

TfbI Solution

Stock	From stock to preparation	500 ml
30 mM KOAc	1 M	15 ml
50 mM MnCl ₂	1 M	25 ml
100 mM KCl	1 M	50 ml
10 mM CaCl ₂	1 M	5 ml
15% (w/v) glycerol	75%	100 ml

Add 305 ml distilled water and keep at 4°C.

TfbII Solution

Stock preparation of 100 ml solution

10 mM Na-MOPS, pH:7	1 M	1 ml
75 ml CaCl ₂	1 M	7.5 ml
10 mM KCL	1 M	1 ml
15% glycerol	75%	20 ml

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)

Solutions Required for Southern Blotting and Hybridization

Depurination Solution

HCl 250 mM

Denaturation Solution

NaCl 1.5 M

NaOH **0.5 M**

Neutralization Solution (pH 7.5)

NaCl 1.5 M

Tris-HCl 0.5 M

20XSSC

Na₃citrate 0.3 M

NaCl **3 M**

Buffer A (pH 7.5)

Tris-HCl 100 mM

NaCl 600 mM

Hybridization Buffer

5XSSC

0.1 % (w/v) SDS

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

100 mg/ml denatured heterologous DNA (optional)

20 fold dilution of liquid block

Combine all the components and heat the solution gently with continuous stirring to dissolve the dextrane sulphate.

APPENDIX C

ENZYMES AND CHEMICALS

Enzyme	Supplier
<i>Cla</i> I	Fermentas
<i>Xba</i> I	Fermentas
<i>Eco</i> RV	Fermentas
<i>Eco</i> RI	Fermentas
<i>Pst</i> I	Fermentas
<i>Sac</i> I	Fermentas
<i>Bam</i> HI	Fermentas
<i>Hind</i> III	Fermentas
Taq polymerase	Fermentas
T4 DNA Ligase	Fermentas

Chemical	Supplier
Agar	Sigma
Agarose	Sigma
Calcium chlorid (CaCl_2)	Merck
D(+)-Glucose monohydrate	Merck
Iron(III) sulfate – 7 – hydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	Riedel-de Haën
L- Amino acids	Merck
Lysozyme	Sigma
Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	Riedel-de Haën
Natruim hydroxid (NaOH)	Riedel-de Haën

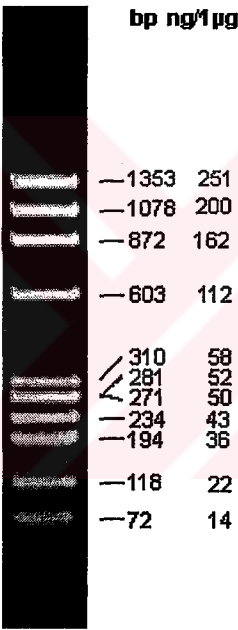
Natrium sulfate (Na_2SO_4)	Riedel-de Haën
Polyethyleneglycol ($\text{HO}(\text{C}_2\text{H}_4\text{O})_n\text{H}$)	Merck
Potassium chloride (KCl)	Riedel-de Haën
Potassium di hydrogen phosphate (KH_2PO_4)	Riedel-de Haën
di Potassium hydrogen phosphate (K_2HPO_4)	Riedel-de Haën
Sodium chloride (NaCl)	Riedel-de Haën
Sodium hydrogen phosphate($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$)	Merck
Tris (hydrocymethyl) aminomethane	Merck
Triton-X100	Sigma
Tryptone	Sigma
Yeast Extract	Sigma



APPENDIX D

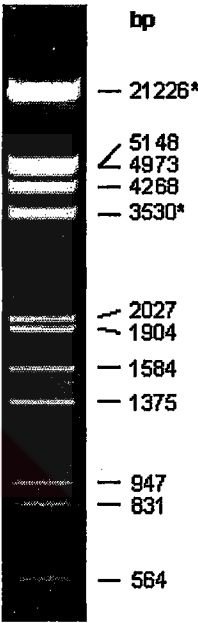
MARKERS.....Fermentas

**Marker 1 : PhiX174
DNA / *Bsu*RI (*Hae*III)
Marker, 9**



1.7% agarose
0.5μg/lane,
8cm length gel,
1X TBE, 12V/cm

**Marker 3: Lambda DNA /
*Eco*RI+*Hind*III Marker, 3**



1.0% agarose
0.5μg/lane,
8cm length gel,
1X TAE, 17V/cm

APPENDIX E

yvfI DNA Sequence

```
1 atgaaacagg gagaaggcac gtatctgaag gaatttgagc tcaatcaaat ttctcagccg
61 ctctcagccg cccttctgat gaaaaaagag gacgtcaagc agctgctcga ggtcagaaaa
121 ctgcttgaaa tcggcgtggc ttcactagcg gctgaaaaaa ggacagaagc agatctcgaa
181 agaattcagg atgcactaaa ggaaatgggc agcattgaag cggacgggga gctgggagag
241 aaagcagact ttgcatttca tcttgcgctt gcggacgctt ctcaaaatga acttcttaaa
301 cacttgatga atcacgtgtc atcattgctg ctggaaacaa tgagggaaac gaggaaaatc
361 tggctgtttt ccaagaagac ctccgttcag cggctgtatg aggagcacga acggatttac
421 aatgctgtgg ctgccgggaa cgggtgcacag gcggaagccg ccatgctggc gcatttgacg
481 aatgtggaag atgtgctttc gggatatttc gaggaaaatg tgcaataa
```


APPENDIX F

LABORATORY EQUIPMENT

Autoclave: Tuttnauer Systec Autoclave (2540 ml)

Balances: Precisa 620C SCS

Precisa 125 A SCS

Centrifuge: Beckman Coulter, Microfuge 18

Centrifuge rotor: F241.5P

Deep freezes and refrigerators: -80°C Heto Ultrafreeze 4410

-20°C Arçelik 209lt

+4°C Arçelik

Electrophoresis equipments: E – C mini cell primo EC320

Gel documentation system: UVI PHotoMW Version 99.05 for Windows

Incubators: Nüve EN400

Nüve EN500

Orbital shaker incubators: Sertomat S – 2

Thermo 430

Pipettes: Gilson pipetteman 10 µl, 20 µl, 200 µl, 1000 µl

Volumate Mettler Toledo 10 µl, 20 µl, 200 µl, 1000 µl

Eppendorf research 10 µl, 20 µl, 200 µl, 1000 µl

pH meter: Mettler Toledo MP220

Spectrophotometer: PerkinElmer Lambda25 UV/VIS Spectrometer

Thermocycler: Techne FTGENE 5D

Thermomixer: Eppendorf thermomixer comfort (1.5ml)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

RESUME

Türkan Ebru (Aydoğdu) Köroğlu was born in KADIKÖY-İSTANBUL in 1981. She is married. After getting her high school diploma from Kadıköy Fenerbahçe high school, in 1999, she started to study in Istanbul University, Cerrahpaşa Medicine Faculty, Department of Medical Biology in 1999. She graduated in 2003 and at the same year, she was accepted to Advanced Technologies in Molecular Biology, Genetics and Biotechnology's program in Department of Molecular Biology and Genetics. She is still pursuing her studies in the same department. Microbial biotechnology, molecular microbiology, molecular genetics are among her professional interest topics. The titles of her publications are as follows:

ABSTRACTS

Köroğlu, T.E., İrigül Ö., Karataş, A. 2005. Identification of New Genes Related with Bacilysin Biosynthesis

Köroğlu, T.E., İrigül Ö., Özcengiz G., Karataş, A.2005. Identification of new genes releated with Bacilysin Biosynthesis by Tn10 Transposon Mutagenesis