Molecular mechanisms controlling bacilysin biosynthesis in plant growth promoting rhizobacterium- *Bacillus amyloliquefaciens FZB42*

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M.Sc Aruljothi Mariappan

Präsident der Humboldt-Universität zu Berlin

Prof. Dr. Jan-Hendrik Olbertz

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I

Prof. Dr. Andreas Herrmann

Gutachter/in: 1. Prof. Dr. Rainer Borriss
2. PD. Dr. Joachim Vater
3. Prof. Dr. Thomas Eitinger

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Summary

Bacillus amyloliquefaciens FZB42 is a Gram-positive, pathogen-suppressing and plant-growth promoting rhizobacterium. It has recently been shown that GFP-labelled FZB42 was able to colonize the roots of three different plant genera. Apart from this ability, it produces a vast array of secondary metabolites, which includes both ribosomal and non-ribosomal peptides. Amylocyclicin A and plantazolicin are ribosomally synthesized antimicrobials by FZB42 which were recently identified.

In case of non-ribosomally synthesized peptides, five gene clusters (*srf*, *bmy*, *fen*, *nrs*, and *dhb*) direct the biosynthesis of lipopeptides, and three other gene clusters (*mln*, *bae*, and *dfn*) are involved in the synthesis of antibacterial polyketides. In addition to this, a dipeptide antibiotic bacilysin is encoded by a single operon (*bacABCDE*) along with the adjacent, *ywfGH* genes. It is a simple peptide antibiotic consisting of L-alanine and L-anticapsin as its molecular backbone.

In this work, the transcriptional activation and regulation of bacilysin biosynthesis were studied at the promoters of *bac* and *ywfH* genes. The promoter of bacilysin was identified using 5'-deletion analysis. Sigma factor A (σ^A) was found to start transcription via conserved promoter elements (-10 and -35) of *bac* and *ywfH* genes. *lacZ* reporter fusion studies were performed in wild type and regulatory mutants. The results show the involvement of transcriptional regulators to activate the expression of bacilysin genes. Several global regulators such as DegU, ComA, Hpr and AbrB were identified and found to influence gene expression. In particular, I confirmed DegU binding in *bac* and *ywfH* promoters using radioactive DNase I footprinting.

Furthermore, Hpr, a transition state regulator was found negatively to control bacilysin biosynthesis. Hpr binding to *bac* promoter was demonstrated using radioactive DNase I footprinting. Remarkably, Hpr does not influence the promoter of the monocistronic gene, *ywfH*. The other transcriptional regulators, such as ComA and AbrB, were correlated indirectly to affect the gene expression of bacilysin via DegQ and Hpr, respectively. The gene regulation of *hpr* was studied in this work. It was demonstrated that AbrB, a global regulator, directly controls the promoter of the *hpr* gene. However, the consensus sequence for AbrB binding was not identified, since it covers the entire promoter region in the DNA-protein interaction study.

To conclude, this study provides new information regarding the genetic regulation of bacilysin biosynthesis in *B. amyloliquefaciens* FZB42.

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Dedication

I would like to dedicate this work to my beloved parents who supported me all my life with great care, affection and love

Abbreviations

aa	Amino acid
aa-tRNA	Amino acyl tRNA
ACN	Acetonitrile
ABC transporter	ATP-binding cassette transporter
Ap	Ampicillin
APS	Ammoniumpersulfate
ATP	Adenosine-5'-triphosphate
BCIP	5-Bromo-4-chloro-3-indolylphosphate
BSA	Bovine serum albumin
Cpm	Counts per minute
CIAP	Calf intestine alkaline phosphatase
Cm	Chloramphenicol
DIG	Digoxin
EDTA	Ethylendiaminetetraacetate
EtOH	Ethanol
EMSA	Electrophoretic mobility shift assay
FAS	Fatty acid synthase
Fig.	Figure
h	Hours
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography - Mass spectrometery
H_2HPP	Dihydro hydroxy phenyl pyruvate
Km	Kanamycin
LB	Luria Bertani broth
MALDI-TOF MS	Matrix assisted laser desorption/ionization time of flight mass spectrometry
MS	Mass spectrometry
MU	Miller unit

NRPS	Nonribosomal peptide synthetase
NRP	Non-ribosomal peptide
NBT	Nitro-blue-tetrazolium chloride
N-MT	N-methyltransferase
OD	Optical density
ONPG	Ortho-nitro phenyl-beta-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
PNK	T4-polynucleotidekinase
Poly dI-dC	Poly(deoxyinosinic - deoxycytidylic)
PKs	Polyketides
RNAP	RNA polymerase
RPM	Revolution per minute
RT	Room temperature
SOE	Splicing over extension
SDS	Sodiumdodecylsulfate
TCS	Two component regulatory system
TEMED	N,N,N,N-Tetramethylethylendiamine
Tris	Tris-(hydroxymethyl)-aminomethane
X-Gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1 Introduction

Bacilli are ubiquitous and successful among bacteria. They are members of division firmicutes. The survival success of the many species of this genus is mainly due to the physiological abilities that allow living in a wide range of environments. Today, there are 65 valid species of *Bacillus* clustered in at least five different groups based on 16S rRNA analysis [1-2]. Since *Bacillus* can thrive in a wide range of environments, it is difficult to characterize them into particular ecological niche [3]. Each cell forms a single endospore under nutritional deprivation or adverse climatic conditions. The spores are resistant to heat, cold, radiation, desiccation and disinfectants and they are carried to long distance. Upon suitable conditions, the spores germinate into viable cells.

During starvation, *Bacillus* species not only activate the sporulation process, but also other regulons, such as competence development, the production of extracellular degradative enzymes and antibiotics are induced. These phenomena reflect the ability of *Bacillus* to adjust rapidly to a changing environment. The production of antibiotics is considered to be a major step in enhancing the competitiveness of producing organism under an environment with limited resource [4].

Bacillus produces a wide variety of antibiotics and extracellular degradative enzymes. The synthesis of these antibiotics and enzymes are temporally controlled and subject to regulation by a large number of global regulators such as DegU, ComA, ScoC, SinR and AbrB [5-8]. The production of antibiotics by *Bacillus* includes NRPs, PKs, dipeptide antibiotics, and several other secondary metabolites [9]. The regulations of these secondary metabolites are carried along with other regulons such as competence development and sporulation. It has been reported that the production of these secondary metabolites promotes plant growth in the vicinity of root [10-12].

1.1 *Bacillus amyloliquefaciens FZB42*

The *Bacillus* strains of plant growth promoting activity were isolated from plant-pathogen-infested soil [13]. *B. subtilis* can be differentiated from *B. amyloliquefaciens* by its ability to produce lipase from lactose [14]. *B. amyloliquefaciens* is a naturally occurring rod shaped, spore forming, and Gram-positive bacterium. The bacterial ecological niche is rhizosphere,

where they colonize and mutually benefit plant-microbe interaction. Initially, it was isolated and used industrially for the production of α-amylase to liquefy starch [15]. The commercially available strain FZB24 is the closest relative of *B. amyloliquefaciens* [16]. The genome sequencing of *B. amyloliquefaciens strain* FZB42 was completed in 2007 by Chen *et al*. The circular chromosome of this genome is 3,918,589 bp and is smaller than those of *B. subtilis* 168 and *B. licheniformis.*

The smaller size of the genome is due to the absence of prophage islands which are abundant in other *Bacillus* species. There are 214 unique genes present in this strain clustered in 17 genomic DNA islands. Apart from the unique genes, *B. amyloliquefaciens* FZB42 harbour many genes for unusual sugar metabolic pathway involved in degrading the plant sugars which are available around plant roots. *B. amyloliquefaciens* FZB42 has gene for a novel sigma factor and its corresponding gene for anti-sigma factor, whereas the genes for other common sigma factors are similar to the genes of *B. subtilis* 168 [17]. A successful root colonizing involves the formation of sessile, multicellular colonies called biofilm. *B. amyloliquefaciens* FZB42 forms robust pellicles in the liquid-air interface, whereas, *B. subtilis*168 forms weak pellicles. There are three unique genes present in *B. amyloliquefaciens* FZB42 which are involved in surface adhesion or biofilm formation. The biofilm formation at the root surface has also been demonstrated by using SEM and TEM (Fan Ben pers.communication).

B. amyloliquefaciens FZB42 has nine giant gene clusters involved in biosynthesis of secondary metabolites. Nearly 8.5% of the genome is devoted to the production of antibiotics and siderophores not involving ribosomes. Nine clusters direct the synthesis of antimicrobial peptides and polyketides by modularly organized mega-enzyme complexes of non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). Four clusters *bmyD*, *pks2*, *pks3* and *nrs* are not found in *B. subtilis* 168 [17], but the genes (*bac*) for bacilysin biosynthesis are conserved in both species of *Bacillus*. For the production of other NRP and polyketides, Sfp is required. Sfp is an enzyme that transfers 4'-phosphopantetheine from coenzyme A to carrier proteins of nascent peptide or polyketide chains. All the gene clusters have been assigned with biological functions [18].

The synthesis of non-ribosomally produced cyclic lipopeptides in *B. amyloliquefaciens*

FZB42 makes this organism competent in the rhizosphere and helps it to act against phytopathogenic viruses, bacteria, fungi, and nematodes in the rhizosphere. Most of these peptides are produced at sophisticated modular multienzyme complexes and contain a β-amino or β-hydroxy fatty acid component that is integrated into the peptide moiety. Gene clusters involved in surfactin, bacillomycinD, and fengycin biosynthesis were identified in the genome of FZB42. Bacillomycin D and fengycin are shown to act in a synergistic manner, enabling the bacteria to cope with competing organisms within plant rhizosphere [18]. The functional characterization of the gene clusters involved in lipopeptide synthesis was performed by using MALDI-TOF- MS [19]. Due to the enormous capacity of FZB42 to produce a wide range of antimicrobial peptides and antibiotics, it has been used as biocontrol agent to improve plant growth promotion (RhizoVital®42-AbiTEP GmbH).

1.2 Antibiotics

Antibiotics are produced by a large number of bacterial species as secondary metabolites. They are useful to the producing organism even at very low concentrations [20]. Antibiotics have a wide range of applications in the fields of chemotherapy, plant pathology, food preservation, veterinary medicine and as research tools in biochemistry and molecular biology. The production of antibiotics by bacteria also benefits plant-microbe interaction by warding off plant pathogenic fungi and bacteria and thereby promotes the health of plants [21]. At present, approx.7000 antimicrobial compounds are known and hundreds of them are produced commercially by microbial fermentation processes [22]. A list of antibiotics and their producing microorganism are given in the Table 1.

Table 1: Antibiotics and its Producers

Penicillium	Penicillin
B. licheniformis	Bacitracin
Cephalosporium acremonium	Cephalosporin
Nocardia uniformis	Norcardins
Streptomyces caespitosus	Actinomycins

S. antibioticus	Mitomycin
S. erythreus	Erythromycin
S. griseus	Streptomycin, cycloheximide
S. virginae	Virginiamycin
B. subtilis 168	Ericin, bacilysin, subtilin, sublancin, mersacidin, subtilosin A
B. amyloliquefaciens FZB42	Bacillaene, bacillomycinD, bacilysin, macrolactin, fengycin, difficidin

Peptide antibiotics are produced either by gene encoded ribosomal synthesis or via multimodular enzyme templates as non-ribosomally synthesized peptides. The path of these peptides is based on their evolutionary acceptance of genes over the period.

In general, syntheses of proteins are carried out by ribosomes. The message carried by mRNA is translated into protein using ribosomes. The translation machinery is one of the big complexes in the cell containing a large variety of proteins and RNAs [23-24]. Transfer RNA (tRNA) functions as an adaptor molecule between the codon on mRNA and the respective amino acid specified by the codon. The tRNAs are aminoacylated by aminoacyl-tRNA synthetases [25]. The decoding of mRNA and peptide synthesis takes place on ribosomes and carries out protein synthesis in the cytosol [26-27]. All phases of protein synthesis, i.e., initiation, elongation, and termination require the action of translation factors that interact with the ribosome at defined stages of translation [28].

The endospore-forming rhizobacterium *Bacillus* species produces two dozens of antibiotics with an amazing variety of structures [9]. Their antagonistic activity extends to a wide range of potential phytopathogens, including bacteria, fungi and oomycetes [29]. Peptide antibiotics represent one of the major classes of antibiotics. The predominant bioactive molecules include either ribosomally synthesized and post-translationally modified (lantibiotics and lantibiotic-like peptides) or non-ribosomally generated antibiotics. Non-peptidic compounds such as polyketides, aminosugars, and phospholipids are also synthesized in *Bacillus*. The genome of

B. subtilis has devoted 350kb of genes for the antibiotic production which accounts for 3-5% of its genome capacity [30].

The structures of lantibiotics are rigid due to their unusual D-amino acids. The thioether linkages make them resistant to proteolysis and oxidation. Peptide antibiotics with thioether linkages are named as 'Lantibiotics' (lanthionine containing antibiotics) [31]. The mechanism behind lanthionine formation includes dehydration of serine and threonine residues, respectively, and subsequent addition of neighbouring cysteine thiol groups during the post-translational modification. Based on their structural properties, lantibiotics have been grouped into type A lantibiotics (21–38 amino acid residues), killing Gram-positive bacteria by forming voltage dependent pores in cytoplasmic membranes, [32], whereas, type B lantibiotics exhibit more globular structures and inhibit cell wall biosynthesis by forming complex with lipid II.

Lantibiotics	Mol wt.kDa	No.of aa	Unusal amino acids	Charges	Properties
				postivie/ negative/ total	
Group "A" lantibiotics					
Pep 5	3,48	34	6	8 / 1/ 7+	elongated, helical
Nisin	3,35	34	8	4 / 1 / 3+	cationic, amphiphilic
Subtilin	3,31	32	8	4 / 2 / 2+	tensioactive
Epidermin	2,16	32	4	3 / 0 / 3+	membrane active
Mersacidin	1,89	20	5	3 / 1 / 2+	amphiphilic
Group "B" lantibiotics					
Cinnamycin	2,04	19	3	3 /2 / 1+	compact,almost
Duramycin A	2,01	19	3	3 / 2 / 1+	neutral,amphiphilic
Duramycin B	2,01	19	3	3 / 2 / 1+	enzyme inhibitor
Duramycin C	2,01	19	3	3 / 2 / 1+	immunologically active
Ancavenin	1,95	19	4	2 / 2 / 0+	

Figure 1: Classification of lantibiotics in groups "A" and "B" according to the charges, conformation, and biological activity [32].

The lantibiotic producers are always self-protected. They carry immunity genes within its operon. The ATP binding cassette (ABC) transporter exports the lantibiotic from the cytoplasmic membrane into the extracellular space [9, 32]. Subtilin, a 32 amino acid pentacyclic lantibiotic is produced by a gene cluster containing ten genes encoding for prepeptide, transportation and immunity [33-35]. Its biosynthesis is positively feedback regulated. The growth phase sigma factors SigH and the global regulator AbrB regulate the subtilin production [36-37].

Lantibiotics are flexible as it could be seen in the production of ericin. Ericin is similar to subtilin, except for four amino acids differing in former. Its biological activity is comparable to that of subtilin [38]. It is produced by two structural genes, *eriA* and *eriS.* Ericin S and ericin A are produced by common synthetases, EriBC. Ericin A is a variant of ericin S except for the 16 amino acid substitutions and a different ring organization. Mersacidin belongs to type B lantibiotics and exhibits globular structure. It is produced by the structural gene *mrsA*. The operon includes genes for post-translational modification, immunity and transport. Mersacidin is controlled by the regulators MrsR2/K2. [39]. Expression of mRNA occurs in early stationary phase, however, the link between mersacidin and growth regulation is not yet clear. MrsD, a member of the homo-oligomeric flavin-containing cysteine decarboxylases (HFCD) family, modifies the C-terminus of cysteine of the mersacidin prepeptide. The dodecameric MrsD and its close relative EpiD are involved in epidermin biosynthesis, representing the only example of lantibiotic modifying enzymes with known three-dimensional structure [40].

Bacillus produces unusual lantibiotic such as sublancin 168. It has a β-methyllanthionine and two disulfide bridges which are unusual for lantibiotics [41-42]. The structural gene for sublancin was acquired from a temperate bacteriophage SPβ. Even though the sublancin gene cluster is not essential for the survival of *B. subtilis*, one attractive hypothesis is that sublancin might be contributing to the survival of the bacteriophage [41]. For example, sublancin kills only non-lysogenized cells, thus enriching the percent of a lysogenized *B. subtilis* population [43]. Subtilosin A, an unusual antibiotic, is produced by several *Bacillus* strains. It contains a macrocyclic ring structure with three inter-residual linkages, and thioether bonds between cysteine sulphurs and amino acid alpha-carbons [44-46]. The unusual lantibiotics can act against a variety of Gram positive bacteria including *Listeria* [47-49].

1.3 Non- ribosomal peptides

Non-ribosomal synthesis of peptides is widespread among bacteria and fungi [50-51]. Many useful antibiotics are produced via the non-ribosomal path. NRPSs are composed of large multi-enzymatic, modularly arranged catalytic domains which perform all necessary steps of selection and condensation of amino acids [52-53]. Each elongation cycle in non-ribosomal peptide synthesis requires the cooperation of three domains which includes i) an adenylation domain ii) a thiolation or peptidyl carrier domain and iii) a condensation domain located between each pair of adenylation and peptidyl carrier domains [52, 54]. In an overview, it can be

concluded that both the machineries of ribosomal and non-ribosomal peptide biosynthesis are similar, but are unique in its own respective features. The need for primary metabolism and several proofreading mechanisms in ribosomal synthesis of peptides are not needed in case of non-ribosomal peptide synthesis [54].

Only twenty standard amino acids make proteins, whereas the non-ribosomal peptide synthesis uses a large range of substrates to produce wide varieties of peptides. It is possible because of the massive assembly line synthetases that produce these non-ribosomal peptides [55]. In NRPSs the arrangement and sequence of modules defines the fate of resulting peptides. The synthesized peptides are usually 3-15 amino acids long, while the maximum length is being imposed by the enormous size of NRPSs. They produce not only bigger molecules, but also structurally diverse NRP that exceed ribosomal production of peptides. This is mainly achieved by incorporating proteogenic, non-proteogenic amino acids, as well as β-hydroxy and carboxylic acids for their synthesis [56].

The enzymatic conversion of building blocks into their stereoisomer, heterocyclic rings, acylation, glycosylation, *N*-*C*-, and *O*-methylation are factors responsible for structural variability of NRP. NRP had been classified into three groups, linear NRP (type A), iterative NRP (type B), and nonlinear NRP (type C), according to their biosynthetic ability [55]. NRP often contain high levels of hydrophobic residues. The incorporation of long chain fatty acids render the peptides more hydrophobic allowing them to diffuse easily out of the cell wall and other biological membranes. In general, most NRP has modified or unusual amino acids at C or N termini [54], making them resistant to cleavage by proteases and other destroying enzymes. Furthermore, the enormous structural diversity and peptide structures allow them to fit into a wide variety of targets. As a result, NRP is biologically active and structurally stable. Producers of nonribosomal peptides are mostly members of the Gram positive such as *Actinomycetes* and *Bacilli* genera. The filamentous fungi and marine microorganisms are also included in this list [18].

1.3.1 Modules and Domains

NRPSs consist of an arrangement of modules. A module can be defined as a unit responsible for incorporating a building block into a growing polypeptide chain [50]. Modules are dissected into domains, which are enzymatic centres to catalyze peptide synthesis. Thus, NRPSs

act as a template as well as a biosynthetic machinery for polypeptide synthesis. The catalytic sites residing in domains can perform various functions ranging from covalent binding, substrate activation, and peptide bond formation of nonribosomal peptide synthesis [52]. Domains with common functions share similar sequence, the so-called 'signature sequences' that can be used to predict cognate substrates of the newly identified NRPS. The change of selectivity for substrates can be obtained using site-directed mutagenesis, which might serve as a powerful tool to obtain new products [55].

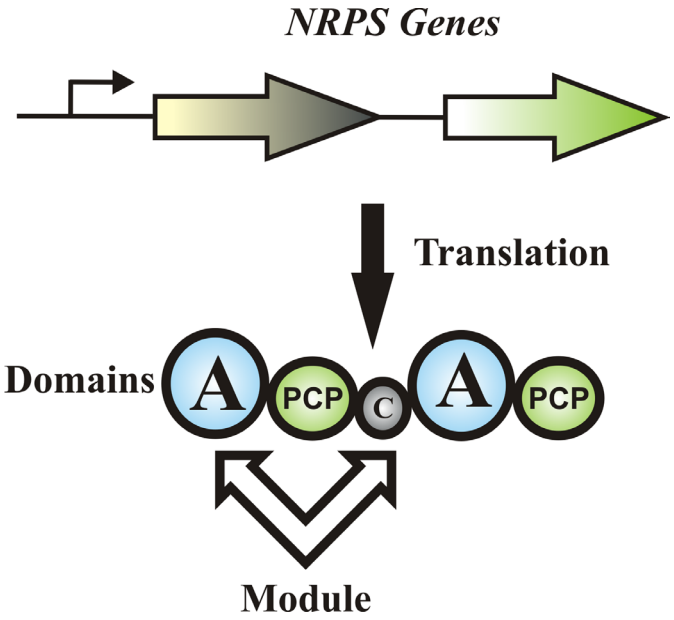


Figure 2: Genes involved in the synthesis of each module and domains. The module recruits a single amino acid into its pockets. Modules are dissected into domains that harbour catalytic activities for substrate activation (A-domain), covalent loading and transport (CP-domain), and peptide bond formation (C-domain).

1.3.2 Additional domains

The NPRS contain enzymatic machineries and a big repository of enzymes which are involved in peptide building up and processing of products [57]. The most common tailoring domains are Cy-domains and E-domains. Tailoring domains carry out their function as an integral part of NRPS acting in *cis* or *trans* to mature products [58].

The formation of a peptide bond takes place in a three step mechanism along with heterocyclization of cysteine, serine and threonine residues [59]. In addition, a Cy-domain adds thi-

ozoline or oxazoline rings to the peptides which help them to intercalate or chelate with metals and thus adding important functional elements. [60].

The occurrence of D-amino acid is one of the striking features of nonribosomal peptide synthesis. The incorporation of D-amino acids occurs via E-domains which are located at the C-terminal end of modules. The enzyme catalyses the epimerization of the PCP-bound L-amino acid or C-terminal amino acid of the growing polypeptide chain [61]. The E-domain is a peptidyl epimerase which determines the enantiomer of NRP [60].

A number of NRPS contains methyltransferase responsible for the methylation of N- and C-terminal amino acids, making the protein less susceptible to preoteolytic cleavage. N-MT is usually located between the corresponding A- and T-domains, and catalyzes the transfer of the methyl group from *S*-adenosyl methionine (SAM) to the α-amino group of the thiosterified amino acid. Glycosylation is also another important post processing modification for the functionality of the peptide [62].

Posttranslational modifications of domains are essential for the functionality of NRPS [63-64]. On this account, one of the main players of NRPS, the PCP domain has to be posttranslationally modified in order to carry out its function as an active enzyme. Intermediate building blocks of NRP are tethered to this domain posttranslationally into the flexible arm. For this purpose, the 4'PP moiety of CoA is transferred to a conserved serine residue of the *apo*-CP which is converted into an active *holo*-CP [62]. The transfer of 4'PP is catalyzed by a PPTase in an Mg2+-dependent reaction. PCPs are sometimes erroneously acylated by PPTases using acyl-CoA instead of CoA as the 4'PP donor. The latter process of misacylation requires a deacylation step that is executed by type II thioesterases (TEIIs) [65].

1.4 Regulation in antibiotic genes

The endospore forming bacterium *B. subtilis* is able to produce more than a dozen of antibiotics. The antibiotics are secondary metabolites, produced under starving condition of nutritional stress. The regulatory proteins that are involved in the regulation of antibiotic genes are produced during the early transition period to late stationary phase. The control mechanisms are primarily at the level of transcription initiation [36]. The transcriptional regulators which

are regulating antibiotic production are discussed below. Most of these global regulators control antibiotic production either by direct or indirect regulation.

1.4.1 Bacillomycin D regulation in *Bacillus amyloliquefaciens* FZB42

B. amyloliquefaciens FZB42 is a potential synthesizer of several antimicrobial compounds which helps in warding away pathogens at rhizosphere [19]. Bacillomyin D is a lipopeptide of the iturin family synthesized nonribosomally by *B. amyloliquefaciens* FZB42 according to the multicarrier thiotemplate mechanism. It is synthesized during stationary phase. At the molecular level, the *bmyD* gene is transcribed through *bmy* operon. It consists of four genes (*bmyD*, *bmyA*, *bmyB*, *bmyC)* without orthologues in *B. subtilis* 168.

The transcriptional regulatory mechanism of bacillomycin D biosynthesis has been elucidated by Koumoutsi *et al*. [66]. It was shown that *bmy* was dependent on a single σ^A dependent promoter favoured by a small regulatory protein DegQ. Furthermore, it has been shown that the global regulator DegU, YczE and ComA are essential for the full activation of the *bmy* promoter.

1.4.2 Regulation of the *srfA* operon

Surfactin is a cyclic lipopeptide produced by *B. subtilis* and other *Bacillus* species. Due to the amenability of *Bacillus* for genetic manipulation, it has been a subject of several studies. Surfactin-producing colonies are phenotypically identified by a zone of lysis on the erythrocyte agar medium. The *Tn917* insertion mutations that are defective in surfactin were isolated and found to contain two loci originally called *srfA* and *srfB* (Nakano *et al.* 1988; 1992).

The *srfA* is an operon consisting of 4 different genes. The *orfi (srfAA)* encodes the peptide synthetase subunit that functions in the incorporation of the constituent amino acids Glu, Leu, and D-Leu. *orf2 (srfAB)* encodes the subunit that catalyses the incorporation of Val, Asp, and D-Leu. *orf3 (srfAC)* functions in the incorporation of Leu, and *orf4 (srfAD)* encodes a protein with significant sequence similarity to that of the thioesterase-like product of *grsT gene* which is a member of the gramicidin S synthetase operon, *grs.* The transcription of *srfA* is dependent on the early competence genes (*com* genes) [67-68]. SrfB was found to be essential for devel-

opment of competence. The production of surfactin is limited to *Bacillus* which has the ability to produce Sfp protein [69].

1.5 Bacilysin - a dipeptide

Bacilysin is a dipeptide antibiotic. It consists of L-anticapsin and L-alanine. The name bacilysin was first given to an antibiotic produced by the strain of *B. subtilis* N.C.T.C. 7197, which was isolated from the soil at Oxford [70]. Despite its simple structure, it has an impressive antimicrobial activity against a wide range of bacteria and some fungi [71]. The mode of its antimicrobial activity is mainly due to inhibition of the glucosamine synthesis, leading to defects in microbial cell wall [72-73]. It has been well established that prephenate, an intermediate of the aromatic amino acid biosynthetic pathway, is the primary metabolic precursor of the anticapsin moiety of bacilysin [74-75]. Anticapsin is responsible for the antimicrobial activity of bacilysin. It is released from bacilysin on hydrolysis and transported into the cell [76].

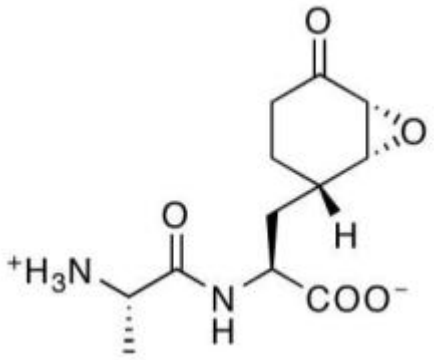


Figure 3: Structure of bacilysin indicating N-terminal alanine and C-terminal anticapsin.

The *bacABCDE* operon was shown to encompass the core biosynthetic genes of bacilysin production [77]. Besides these five genes, a monocistronic gene *ywfH*, is also essential for the complete cycle of bacilysin production [78]. The function of these genes has been proposed by C.T. Walsh *et al*. [75]. It was demonstrated that BacA and BacB are involved in converting prephenate into dihydro-4-hydroxyphenylpyruvate (H_2HPP 4) and (H_2HPP 5), respectively. The YwfH is involved in converting H_2HPP 5 to H_4HPP 6, making the precursor for anticapsin, whereas the function of BacC is unclear. The BacD and BacE were shown direct amino acids ligation and impose self-protection to the host bacterium, respectively. Concerning the

production of bacilysin under physiological conditions, it was demonstrated that a temperature of 25°C and pH 6.8 are suitable conditions for bacilysin production [79].

Regulation of bacilysin production has been the subject of study for a long time. In *B. subtilis* 168, bacilysin production is controlled by a dual regulation system composed of the guanine nucleotides ppGpp and GTP sensed by CodY to mediate repression [78]. In additon, the competence regulating gene products such as ComP-ComA, ThyA, YbgG, OppA, Spo0A and AbrB are shown to affect bacilysin production [80-81].

1.6 Organization of *bac* genes

Most of the structural genes for bacilysin biosynthesis are organized in a single operon. The *ywfABCDEFG* was renamed as *ywfA-bacABCDE-ywfG-ywfH* [77]. The putative functions of each gene have been assigned. The function of *ywfA* , a monocistronic gene upstream of *bacABCDE* , is still unknown. Its putative function has been assigned to the major facilitator super family (MFS) class of proteins. This family also includes a wide range of uniporters, antiporters and symporters, helping in the transportation of secondary metabolites across cytoplasmic membrane. The first three genes determine the fate of anticapsin production, while the fourth gene, *bacD*, was shown to ligate amino acid, catalyzing peptide bond formation between L-Ala and anticapsin. The fifth gene, *bacE*, is involved in host resistance

The putative functions of the first three genes have been proposed by Mahlstedt *et al*. (2010) based on bioinformatic analysis. BacA is homologous to prephenate dehydratases which are involved in decarboxylation of prephenate. BacB is a member of bicupin iron enzyme family. Finally, BacC is proposed to have nicotinamide-dependent reductase or dehydrogenase activity. Whereas, the function of *ywfG*, the sixth gene of bacilysin production is predicted to code for an aminotransferase, the gene next is the *ywfH* which has a putative nicotinamide-dependent reductase activity (see Fig.4). However, Mahlstedt *et al*. have reported BacA, BacB, YwfH and YwfG to be involved in the synthesis of the nonproteinogenic amino acid, tetrahydrotyrosine (H_4Tyr) which is a precursor for anticapsin.

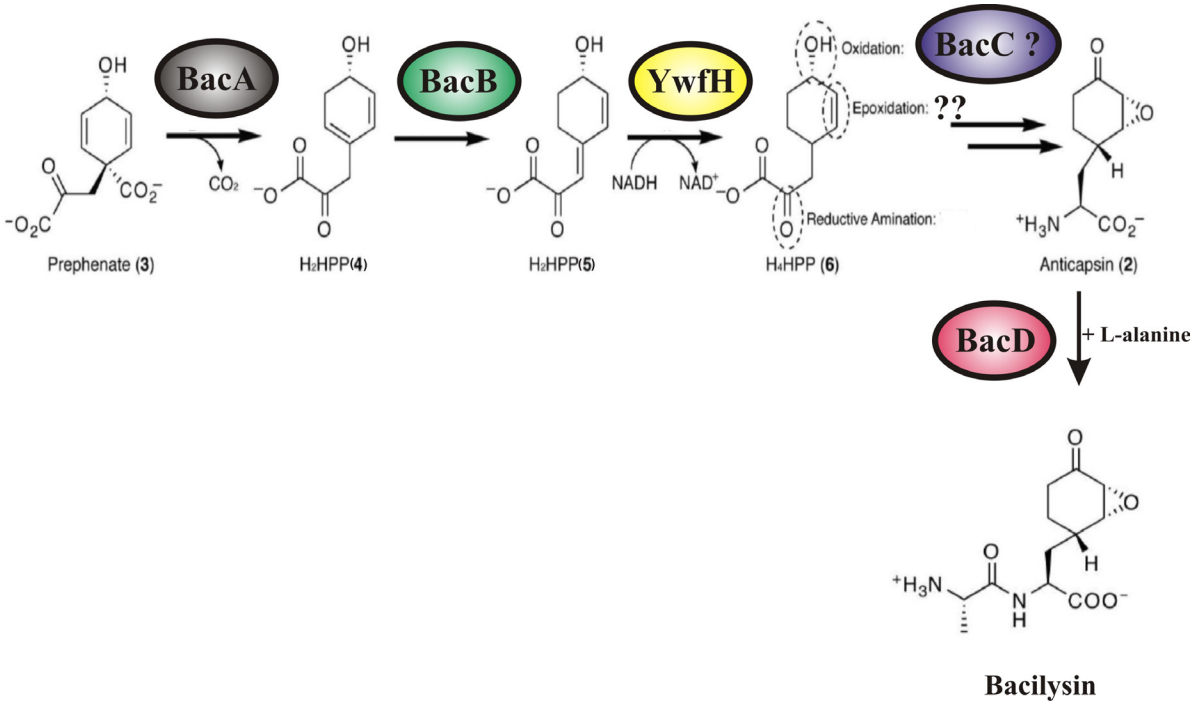


Figure 4: The pathway of bacilysin biosynthesis from prephenate was depicted as proposed by Mahlstedt *et al.* (2010). The function of each protein was described in the text.

1.7 Research objectives

B. amyloliquefaciens FZB42 is a strain harboring an enormous potential to synthesize a wide range of antimicrobial, antifungal and nematocidal compounds. Bacilysin is a dipeptide antibiotic having antimicrobial activity and it is synthesized by FZB42 during the exponential and transition phase. The main objectives of this research have been to establish the promoter of bacilysin, deciphering the transcriptional start point and identifying the major transcriptional regulators involved in the molecular mechanism of bacilysin production.

2 Materials and Methods

The chemicals and instrumental materials used in this study are listed below.

2.1 Chemicals and Materials

Table 2: Chemicals used in this work.

Manufacturer	Products
Fermentas	Restriction enzymes, DNA markers, dNTPs, prestained protein ladder, RevertAid M-MuLV reverse transcriptase (200 U/µl), RiboLock ribonuclease inhibitor (40 U/µl), T4 DNA ligase, T4 kinase, and T4 polynucleotide kinase.
USB	Thermo Sequenase cycle Sequencing kit
MP Biomedicals	Urea pure
Carl Roth	Agarose NEEO (ultra-quality), chloramphenicol, citric acid, $CuSO_4$, DEPC, $FeCl_2$, $FeCl_3$, $Fe_2(SO_4)_3$, formaldehyde, L-glutamic acid, glycerol, HEPES, IPTG, KCl, K_2HPO_4, H_2KPO_4, maleic acid, $MgSO_4$, $MnCl_2$, $MnSO_4$, Na-acetate, Na-citrate, Na_2CO_3, NaCl, NaOH, $(NH_4)_2SO_4$, peptone, SDS, proteinase K, Rotiphorese Gel 40 (19:1), Rotiphorese Gel 40 (29:1), TEMED, Tris, Triton-X 100, Tween 20, XGal, yeast extract, $ZnCl_2$
Roche	Anti-DIG AP, Ampicillin, blocking reagent, DIG-dUTP, kanamycin
Macherey-Nagel	Nitrocellulose membrane porablot NCL, Nucleo Spin ® Extract II, Nucleo Spin RNA L, Porablot NY plus, Protino® Ni-1000 kit
Promega	BCIP (50 mg/ml), NBT (50 mg/ml), pGEM-T® Vector systems
Qiagen	QIAEX II gel extraction kit, QIAprep Spin mini prep kit, Qiaquick PCR purification kit
Bio-Rad	Blotting grade blotter non-fat dry milk
Hartmann Analytic	[γ- ^{32}P]ATP
Fluka	$CaCl_2$, EDTA
Santa Cruz Biot.	His-probe H15 sc-803 rabbit polyclonal IgG (200 mg/ml)
Sigma	Oligonucleotides, Anti-rabbit IgG AP

2.2 Bacterial strains, Plasmids and Primers

All the bacterial strains, plasmids and primers used in this study are listed below as tables.

2.2.1 Bacterial strains

Table 3: Bacterial strains used in this study.

Bacterial Strains	Genotype	Reference
B.amyloliquefaciens FZB42	Wild type	FZB
RSO6	FZB42 Δ*bacA*::Cm^r, Δ*sfp*::Em^r	[82]
AA1	AK13 Δ *amyE*::P_{bac}0-*lacZ* (Spec^r)	This study
AA2	AK13 Δ *amyE*::$P_{bac892bp}$-*lacZ (*Cm^r)	This study
AA3	AK13 Δ *amyE*::$P_{bacbp335bp}$-*lacZ* (Cm^r)	This study
AA4	AK13 Δ *amyE*::$P_{bac305bp}$-*lacZ* (Cm^r)	This study
AA5	AK13 Δ *amyE*::$P_{bac257bp}$-*lacZ* (Cm^r)	This study
AA6	AK13 Δ *amyE*::$P_{ywfH540bp}$-*lacZ* (Cm^r)	This study
AA7	AK13 Δ *amyE*::$P_{ywfH600bp}$-*lacZ* (Cm^r)	This study
AM01	FZB42 Δ*alsD*::Spec^r	This study
AM02	FZB42 Δ*alsS*::Cm^r	This study
AM03	FZB42 Δ*thyB*::Cm^r	This study
AM04	FZB42 Δ*thyA*::Spec^r	This study
AM05	FZB42 Δ*sigD*::Spec^r	This study
AM06	FZB42 Δ*sigM*::Km^r	This study
AM07	FZB42 Δ*abrB*::Km^r	This study
AM08	FZB42 Δ*ydjL*::Spec^r	This study
AM10	FZB42 Δ*hpr*::Spec^r	This study
AM11	AA2 Δ*comA* ::Em^r	This study
AM12	AA2 Δ*degU* ::Em^r	This study
AM13	AA2 Δ*hpr* ::Em^r	This study
AM14	AA6 Δ*degU* ::Em^r	This study
AM15	AA7 Δ*abrB* ::Em^r	This study
AK38	*E. coli* DH5α pREP4 pAK54	[66]
CH23	FZB42 Δ*comA*::Em^r	X.-H. Chen, un-published
TF1	FZB42 Δ*degU*::Em^r	T.-F. Huang, un-

		published
AK57	Derivat FZB42 Δ*sigB*::Emr Δ*rapX*::Cmr	Laboratory stock
AK48	Derivate FZB42 Δ*sigX*::km^r	Laboratory stock
CH30	FZB42 *sigV*::*Em* ^{r}	X.-H.Chen

2.2.2 Plasmids

Table 4: Plasmids used in this study.

Plasmid/Reference	**Description**	**Reference**
pGEM-T/Promega	Cloning vector, Ap^r	
pET22b/New England Biolabs	Expression vector, IPTG-inducible promoter, His_6-Taq, Ap^r	This study
pAFN0	pAK9 carrying a fragment of *bacA* from -400 to +126 bp	This study
pAFN1	pAK9 carrying a fragment of *bacA* from -400 to +126 bp	This study
pAFN2	pAK9 carrying a fragment of *bacA* from -400 to +126 bp	This study
pAFN3	pAK9 carrying a fragment of *bacA* from -400 to +126 bp	This study
pAFN4	pAK9 carrying a fragment of *bacA* from -400 to +126 bp	This study
pAFN5	pAK9 carrying a fragment of *bacA* from -400 to +126 bp	This study
pAYN1	pAK9 carrying a fragment of *ywfH* from -400 to +126 bp	This study
pAHN1	pAK9 carrying a fragment of *hpr* from -400 to +126 bp	This study
pAM01	pGEM-T carrying *alsD*::*spec* ^{r}	This study
pAM02	pGEM-T carrying *alsS*::Cm ^{r}	This study
pAM03	pGEM-T carrying	This study

	thyB::Cm ^{r}	
pAM04	pGEM-T carrying *thyA*::Spec^{r}	This study
pAM05	pGEM-T carrying *sigD*::Spec ^{r}	This study
pAM06	pGEM-T carrying *sigM*::Km^{r}	This study
pAM07	pGEM-T carrying *abrB*::Km^{r}	This study
pAM08	pGEM-T carrying *ydjL*::Spec^{r}	This study
pAM10	pGEM-T carrying *hpr*::Spec^{r}	This study

2.2.3 Primers

Table 5: Oligonucleotides used in this study.

Primer name (restriction site)	**Sequence (5' to 3' end)/ Restriction sites***	**Use**
alsSfp	TTATTCCGGGCTTCCTTCG	AM02
alsSrp	CGGCAACGGCAATAAAGTATT	AM02
alsDfp	TGACTCTTATCTCGTTTCCGC	AM01
alsDrp	ATGAGCCGCTGAAACTGTTGAT	AM01
SigD fw	TTAGCAGGTTCCTATTTAACGG	AM05
SigD rw	TTGGTTGACATAGTACATGCC	AM05
sigM Lfw-sacI	AATTATAGAGCTCAACCGAATTGCAGATTCGTATCAAC	AM06
SigM LRw-speI	ATATTAACTAGTCAGCGGCTGAAAGCACTTTATAATAG	AM06
SigM Rfw-sacII	AATATACCGCGGTTTTGTCATGGAGAGCAGGAAC	AM06
Sigm Rrw-NotI	AATATAGCGGCCGCAACTCCTCGGCATCCATTTAG	AM06
ThyA fw	TTCATTCCGCCATTCGTCATGTC	AM03
ThyA rw	TCAGTCCCAGCAATCGAAAGC	AM03
AbrB Lfw-sacI	AATAAGAGCTCTATACGAAAGAGATCCGCACG	AM07
AbrB Lrw-speI	AATAAACTAGTAAGGCGCTGAGCAAATCATC	AM07
AbrB Rfw-sacII	ATAATACCGCGGTTTCATTAACAGTCTCCTCCCGAGAG	AM07
AbrB Rrw-	ATAATAGGGCCCATCAAGCGCCATCAGCATAATCG	AM07

ApaI		
Hpr FW	TGAAATAACCGCATACCGAAACC	AM10
Hpr RW	TCGGATTCCTGGTCAATCAGAC	AM10
ThyB fw	AAGGTTCCGTATCATCACGC	AM04
ThyB rw	ATGTGCGGATATTACACGCTCATCCAGTCAGG	AM04
Amyback1fw	AAGAGTCCACATGGATGAGTG	AA1
Amyfront2fw	TACAGCCATTCAGACATCTCC	AA1
LacZfw	ACCAGACCAACTGGTAATGG	AA1
LacZrw	TTGTTCCCACGGAGAATCC	AA1
AK BacN1Fw-HindIII	ATATTAAAGCTTACGGCATGTATTCCTTTCTC	AA2
AKBacN1 Rw-SalI	ATATTGTCGACGCACGATTCAAATGTATCATGC	AA2
AkBacN3Fw HindIII	ATTAAAGCTTAAATGTTAATTTTAACACCC	AA3
AkBacN4Fw HindIII	TTATTAAGCTTTGTTTTCTAATATATAGG	AA4
AkBacN5Fw HindIII	TTTATAAGCTTTGACAGCTTGAACATCTATG	AA5
bacFP1a	ATGAGCATCAGGCCGACCAAAC	FP/EMSA
bacFP1b	ATCCATAGATGTTCAAGCTGTC	FP
bacFP2a	TCATAGGGTGTTTTCTAATATATAG	FP
bacFP2b	TTAAGTAAATATTATCCATAGATG	FP
bacFP3a	TTCAAATAATATTGACAGCTTG	FP
bacFP3b	TGATCGTAATCAGTTTAGATATTG	FP/EMSA
PeRW3 Pe RW2	ACTACTTGTCCCTTCAGGACCG TCCAATATAATCATGAGCACC	PE
BacFp1aRW	ATTCCTATATATTAGAAAAC	FP
Hpr FPF1	GGAGGAAATCAAACCGCACC	FP/EMSA
Hpr FPF2	CCTCTATTATGCCAATAAAATAAAG	FP/EMSA
Hpr FPF3	TATTTTATTGGCATAATAGAGG	FP/EMSA
HprFPF4	TTGGAAAATTTCAGATATCCC	FP/EMSA
Hpr2Fw NdeI-	TTAATACATATGAATCGTGTTGAACCGCC	Protein expression
Hpr2Rw BamHI-	ATTAATGGATCCTTTATTGAGATTATGAAGCAC	Protein expression
YwfH PE 2	ATAATAAACGCGGTTCGTTTTGAC	PE
Hpr PE 2	AAGGCGGTTCAACACGATTC	PE
ywfH-hindIII	ATATATAAGCTTAGCGATGATGTGCTTCAGTTC	AA6
ywfH-SalI	ATATTGTCGACTTCTTTCCATAGGTTTCCGACG	AA6
ywfH FW FP	TCGACACGCTTCCGAAGTTTTTG	FP
ywfH RW FP	ACGCGGTTCGTTTTGACAACTG	FP
Spec Fw	CTCAGTGGAACGAAAACTCACG	AM10
Spec Rw	TAAGGTGGATACACATCTTGTC	AM10

FP-primers used for footprint, EMSA. PE-Primer extension, restriction sites used are underlined.

2.2.4 Media and supplements

Table 6: All media were prepared and sterilized according to the standard procedure.

Media	**Ingredients**
GA medium	7 g/l K_2HPO_4, 2 g/l KH_2PO_4, 0.1 g/l $MgSO_4$, 1 g/l $(NH_4)_2SO_4$, 0.05 g/l Yeast extract, 0.15 mg/l $Fe_2(SO_4)_3$ x $6H_2O$, 5 mg/l $MnSO_4.H_20$, 0.16 mg/l $CuSO_4.x$ $,5H_2O$, 13 g/l sucrose.
LB Medium	1 % w/v Peptone, 0.5 % w/v yeast extract, 0.5% w/v NaCl
Antibiotics	
Amplicillin	100 µg/ml
Chloramphenicol	20 µg/ml (for *E. coli*), 5 µg/ml (for *Bacilli*)
Erythromycin	1 µg/ml (for *Bacilli*)
IPTG	1 mM
Kanamycin	20 µg/ml (for *E. coli*), 5 µg/ml (for *Bacilli*)
Lincomycin	25 µg/ml (for *Bacilli*)
Xgal	40 µg/ml

2.2.5 Buffers

Table 7: All the buffers used in this study.

Buffers	**Ingredient**
Transformation buffer	
MDCH	1X PC buffer, 0.1 M Glucose, 0.005 % tryptophan, 0.04 M $FeCl_3$/Na-citrate, 3 mM $MgSO_4$, 0.5 % w/v Na-glutamate, 0.1 % casein hydrolysate.
MD	1X PC buffer, 0.1 M glucose, 0.005 % tryptophan, 0.04 M $FeCl_3$/Na-citrate, 3

	mM $MgSO_4$
10X PC	0.8 M K_2HPO_4, 0.45 M K_2HPO_4, 0.028 M Na-citrate
Transformation buffer	1X SMM, 1 mM EGTA, 0.025 M glucose, 0.02 M $MgCl_2$
Southern Blot	
Denaturation buffer	1.5 M NaCl. 0.5 M NaOH
Neutralization buffer	1.5 M NaCl, 1 M TrisHCl pH 8.0
Hybridisation buffer	5X SSC, 1 % blocking reagent, 0.1 % N-laurylsarcosin
P1-Dig buffer	1 M Maleic acid (pH 7.0), 5 M NaCl, 1 % w/v blocking reagent
Wash buffer	0.1 M Maleic acid, 0.1 % NaCl, 0.3 % v/v Tween20
Ap buffer	0.1 M TrisHCl, 0.05 M $MgCl_2$, 0.1 M NaCl
ß-Galactosidase	
Z-buffer	100 mM Na-P-buffer pH 7.0, 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 1 mM DTT, 0.3 mg/ml lysozyme, 100 µg/ml chloramphenicol, 0.005 % Triton X100, 0.5 U/ml benzonuclease
ONPG	4 mg/ml in Z-buffer
Stop solution	1M $NaCO_3$
SDS-PAGE	
Storage buffer	50 mM TrisHCl pH 8.0, 0.5 mM EDTA, 100 mM NaCl , 0.5 mM DTT, 50 % glycerol

1X SDS-loading buffer	100 ml 10X Laufpuffer, 10 ml 10 % SDS make up to 1L with dH_20
10X TBS	100 mM TrisHCl (pH 8), 1.5 M NaCl
10X loading buffer	0.25 M TrisHCl (pH 8.3) , 1.92 M glycin
Fixing solution	1 Vol.acetic acid (100 %), 1.Vol.EtOH (96 %), 5.vol.dH_2O
Protein staining solution	0.2 % Coomassie R 250, 0.05 % Coomassie G250, 42.5 ethanol, 10 % acetic acid, 5 % methanol
RNA Preparation	
Killing buffer	20 mM TrisHCl pH7.5, 5 mM $MgCl_2$, 20 mM NaN_3
10X MEN	200 mM MOPS, 50 mM Na-acetate, 10 mM EDTA pH 7.0
EMSA buffers	
5X Binding buffer	100 mM TrisHCl pH8.0, 500 mM KCl 25 mM $MgCl_2$ 2.5 mM DTT, 50 % glycerol, 0.25 Nonidet P40, 0.025 w/v polydI-dC, 0.025 w/v BSA
6 % 7 M urea polyacrylamide gel	25 g urea, 6 ml 10X TBE, 9.2 ml AA/BAA (19:1) to 60 ml with dH_2O
10X TBE	121.1 g/l Tris Base, 51.3 g/l boric acid, 3.72 g/l EDTA
Foot print buffers	
DNase I – Stop solution	0.4 M Na-Acetate, 50 µg/ml Calf Thymus DNA
Stop solution	95 % deionised formamide, 20 mM EDTA pH 8.0, 0.05 % bromophenol

	blue, 0.05 % xylencyanol
Other buffers	
Sodium phosphate buffer	1 M Na_2HPO_4/NaH_2PO_4
Phosphate buffer	1 M KH_2PO_4/ K_2HPO_4
10X TE	100 mM TrisHCl (pH 7.5), 10 mM EDTA (pH 8)

2.3 Methods

2.3.1 Bacterial transformation

Competent cells of *B. amyloliquefaciens* FZB42 were obtained as previously described [83]. Cells were grown overnight in LB medium at 28°C (170 rpm). The next day, they were diluted in glucose-casein hydrolysate-potassium phosphate (GCHE) buffer to an OD_{600} of 0.3. The cell culture was then incubated at 37°C under vigorous shaking (200 rpm) until the middle of exponential growth (OD_{600} ~1.2). Dilution with an equal volume of GC medium followed and the cells were further incubated under the same conditions for 1 hour. Further on, the culture was divided in 2 ml Eppendorf tubes and cells were harvested by centrifugation at 6000 rpm for 5 min (room temperature). The pellets were resuspended in 100 µl of the supernatant and the desired DNA (1 µg) with 2 ml transformation buffer was added to them. After incubation at 37°C under shaking at 75 rpm for 20 min, 1 ml LB medium containing sublethal concentration (0.1 µg/ml) of the appropriate antibiotic was added. The cells were grown under vigorous shaking for 90 min and platted on selective agar plates.

2.3.2 Antibiotic sensitivity test (AST) and bioautography

For the antibacterial tests, *B. amyloliquefaciens* FZB42 and its derivatives were grown and treated in the same manner. The indicator strain was grown overnight at 37°C under vigorous shaking. The indicator plates were prepared by mixing 1 ml of the culture to 3 ml soft agar and poured onto petri dishes. Supernatants obtained from the *B. amyloliquefaciens* FZB42 strains, grown in GA medium for 24 hours, were applied to the plates and incubated at 37°C.

Bioautography of bacilysin was performed essentially as previously described (Chen *et al.*, 2006). Samples were collected from the supernatant of cultures grown in GA medium. 20 µl of the samples were loaded onto thin-layer chromatography (TLC) aluminum sheets (20 by 20 cm; Merck, Darmstadt, Germany), for *B. megaterium* bioautography.TLC was performed using a mobile phase consisting of 1-butanol/acetic acid/water 4:1:1 (v/v/v). The TLC plate was cut into strips. The strips containing spots were placed onto the solid GA medium containing the indicator strain. Strips were allowed to stay on the agar for 30 - 60 min and removed. Plates were incubated at 30^{o}.

2.3.3 Southern blot

Southern blotting involves transfer of DNA molecules from an agarose gel onto a membrane. It is designed to locate a specific sequence of DNA in a complex mixture. The shorter the probe, the more specific is the detection. For Southern hybridization, an appropriate probe was PCR labelled with digoxigenin-11-dUTP (DIG-dUTP), according to the Ready-to-Go kit from Roche. The DNA region was amplified with specific primers. (DNA 50 ng, dNTP(2 mM), 1 mM Dig-11-dUTP, 0.1 units of Tag to a final volume of 50 µl). The labelled product was then stored at -20°C until use.

1-2 µg of the chromosomal DNA of FZB42 were digested overnight with a suitable restriction endonuclease. Samples were initially separated on a 0.8 % agarose gel in 1 x TAE buffer at 70 Volt. The gel was washed twice with denaturation buffer and subsequently with neutralization buffer for 20 minutes. ssDNA was transferred onto a positivated nylon membrane with the pore size of 0.45 µM. It has a binding capacity of about 500 µg/cm. Transfer on the nylon membrane was performed using the Bio-Rad vacuum blotter (model 785). The DNA was cross-linked to the membrane using UV radiation.

Hybridisation and detection The membrane was initially incubated for 1 hour at 65°C with 40 ml hybridization buffer and was hybridized overnight at 55°C with 5-10 ml hybridization buffer containing 5-25 ng/ml of denaturated DIG-labelled probes. The membrane was washed twice for 15 minutes, first with 2 x SSC/0.1 % SDS at room temperature and again with a lower amount of SSC (0.5 x SSC/0.1 % SDS at 55°C).

Detection was achieved by a colorimetric approach. The membrane was first equilibrated with P1-DIG buffer and was then incubated for 30 minutes with P1-DIG buffer containing 3.75 units of the antibody Anti-Digoxigenin-Alkaline-Phosphatase. Unbound antibody was removed after a fifteen minute washing step. The 10 ml AP buffer containing 2.25 mg nitroblue tetrazolium salt (NBT) and 1.75 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was added to the membrane in a petri dish and incubated in the dark allowing visualization of the hybridized DNA with our labelled probe.

2.3.4 HPLC and HPLC-MS assay of bacilysin

High pressure liquid chromatography was performed with the HPLC 1100 device from HP Agilent, Waldbronn, Germany, essentially as previously described (Schneider *et al.*, 2007). In brief, 10 µl sample was injected onto a HPLC column (Luna® 5 µm C18 (2) 100 Å LC column 100 x 4.6 mm). The temperature was kept at 30°C during the experiment. The run was performed with a flow rate of 1.5ml/min and a gradient of solvents A (H_20 + 0.1%, HCOOH) and B (CH_3CN + 0.1 % HCOOH), which reached 100 % B after 12 min. To equilibrate the column, it was treated with 5 % CH_3CN–HCOOH for 3 min. A diode-array detector in the range from 190 nm to 550 nm was used for detecting peaks. Bacilysin was detected at 230 nm.

HPLC- MS of bacilysin was performed on an Exactive Orbitrap system (Thermo Fisher Scientific, Bonn, Germany) coupled with an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). Aliquots of the culture filtrates of wild-type and mutant strains were fractionated by reversed-phase HPLC on a Zorbax Eclipse Plus C18, Rapid Resolution HD (2.1 x 50 mm, 1.8-Micron from Agilent) at a flow rate of 1.5 ml/min with a gradient of 5 % CH_3CN plus 0.1% formic acid to 100% CH_3CN plus 0.1% formic acid in 8 min and further to 5 % CH_3CN plus 0.1% formic acid in 10 min. Every sample was measured in the negative and positive mode, and mass spectra were acquired in an m/z range of 50 to 500 at a scan rate of 1,000 atomic mass units/s.

2.3.5 Quantification of specific β-galactosidase enzymatic activity

Specific β-galactosidase activity was determined from growing liquid cultures in GA medium, according to [84]. At different times of the growth curve the optical density of the culture at

600nm was determined and cells were harvested. Their pellets were frozen in order to be further used in the β-galactosidase assay.

Pellets were resuspended in 800 µl Z-buffer. After short vortexing, they were incubated on ice for 10min and at 30°C for 10 min. The reaction began by addition of 200 µl ortho-nitrophenyl-β-D-galactopyranoside (ONPG) 4 mg/ml at 30°C and was stopped by addition of 400 µl 1 M Na_2CO_3, when their colour changed to yellow. The samples were then centrifuged for 5 min and the supernatant's absorbance was measured at 420 and 550 nm. Specific β-galactosidase activity was calculated in Miller units (MU) [85].

Miller units (MU) = 1000 x (OD 420 - 1,75 x OD 550) / (t x V x OD 600)

According to the formula,

OD420, OD500, OD600 = Optical density at 420,550 and 600nm

t = reaction time (min)

V = volume of the sample of bacterial cells used for the reaction (ml)

2.3.6 Overexpression and purification of His-tagged DegU and Hpr

The DegU protein was prepared similar to previous work [66]. Strain AK38 was grown overnight at 37°C in LB medium containing 100 µg/ml ampicillin and 20 µg/ml kanamycin. The culture was diluted in 500 ml LB-Ap/Km to an OD_{600} of 0.03 and was further grown at 30°C under vigorous shaking. When the cells had grown to an OD_{600} of 1, ethanol was added to a final concentration of 3 % to induce chaperone synthesis and minimize formation of inclusion bodies. After 15 minutes, IPTG was added to the cultures at 1 mM final concentration. The cultures were grown for 2.5 hours and were then centrifuged at 6000 rpm for 20 minutes. The pellets were stored at -80°C. The protein was then purified with the Protino® Ni-1000 kit according to the manufacturer's instructions (Macherey Nagel) and was subsequently dialysed overnight against storage buffer at 4°C.

Hpr protein was overexpressed by amplifying a fragment using the primers Hpr2Fw NdeI/ Hpr2Rw BamHI and cloned into pET 15b (Novagen) vector which carries an N-terminal His Tag (see primers in Table 2.2.3). The overexpression was performed by growing *E. coli* BL21 (DE3) harbouring pET 15b-*hpr* overnight in LB lactose (1 % final concentration) in the medium. Subsequently, the purification was performed using the Protino Ni-1000 kit, according to the manufacturer's instructions (Macherey Nagel).

2.3.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to [86]. The proteins in the gel were separated using the "Mini-Protean II" apparatus of Bio-Rad. Gels were run at 200 Volt in 1 x SDS running buffer and were stained with protein staining solution (see buffers section).

2.3.8 Urea gel

The gel was left to prerun before loading the samples for 1 hour at 60 Watt in 1x TBE buffer, using the SequinGen Sequencing Cell of Bio-Rad. After loading the samples, DNA separation was performed for approximately 100 minutes more using the same running conditions. The gel was dried at 80°C for 1 hour using the vacuum SlaB Gel Dryer Model SE1160. An IP screen was put on the top of the dried gel and visualization was achieved using the Molecular Imager FX scanner (Bio-Rad).

2.3.9 Radiolabelling of primers

Primers were radiolabelled using the T4 polynucleotide kinase (PNK). PNK catalyses the reactions at the 5'-OH end by transferring of the γ-phosphate from ^{32}P- ATP. The reaction was carried out with 40 pmol of primer and 4 µl of [γ-^{32}P] ATP (10µCi/ml). The reaction mixture was incubated at 37 °C for 1 hour and heat inactivated at 70°C for 10 minutes.

2.3.10 Sequencing of radioactive DNA

Sequencing reactions were carried out using the Thermo sequenase cycle Sequencing kit (USB) according to the manufacturer's instructions. 100 ng of PCR fragment containing the desired fragment and 1 pmol of the radioactive primer were included in the reaction. Amplifi-

cation was performed using a 23 cycle PCR pro [T_{den}=94°C (30 sec), T_{anneal}=58°C (sec), T_{ext}=72°C (30 sec)]

2.3.11 RNA preparation

Transition-phase cells of *B. amyloliquefaciens* FZB42 were harvested for preparation of total RNA.10 ml of the culture was mixed with 5 ml "killing" buffer (stops mRNA production and nucleases) and centrifuged for 10 minutes at 4°C and 12000 rpm. The pellet was washed with 1 ml "killing" buffer and stored at -80°C.

Isolation of RNA was performed using the Nucleo Spin RNA L (Macherey Nagel). In order to remove possible DNA contaminations, the isolated RNA was additionally extracted with an acidic phenol: chloroform: isoamylalcohol (25:24:1) mixture and then chloroform - ethanol precipitation followed .The pellet was resuspended in 20 µl DEPC-H_2O. The concentration of total RNA was spectrophotometrically determined according to [1], whereas its quality was checked on a 1.5 % RNA agarose gel under denaturating conditions (1x MEN, 16 % formaldehyde) (For MEN see Table 2.2.5). The samples were mixed with 1.6 volume loading buffer and were incubated at 65°C for 5 minutes prior to loading on the gel. The gel was run in 1 x MEN buffer at 60 Volt.

2.3.12 Primer extension

Primer extension was used to map the 5' termini of mRNAs. 40 µg of total RNA was mixed with 0.15 µM radioactively (^{32}P) labelled primer at 70°C for 5 minutes to anneal. Then 4 µl 5 x reverse transcriptase buffer, 2 µl dNTPS (10 mM each) and 1 µl ribonuclease inhibitor (40 units) were added to a final volume of 19 µl. After incubation at 37°C for 5 minutes, 1 µl reverse transcriptase (200 units) was added to the mixture and further incubation was allowed for 1 hour at 42°C. The primers used for identifying the transcriptional start of the bacilsysin operon are given in the table 2.2.3.

2.3.13 Gel retardation assay

Gel retardation assay or electrophoretic mobility shift assay (EMSA) is a common technique used to characterize protein-DNA/RNA interactions. In my case, the desired DNA fragment of

the *bacA* promoter region was amplified by PCR using primers bacFP1a and bacFP3b (Table 2.2.3), one of which was previously labelled at its 5'-end with [γ-^{32}P] ATP. The radio-labelled product (264 bp) was purified with the Qiagen PCR purification kit. After dilution of the labelled DNA fragment to attain final activity of 20.000 cpm, the DNA was incubated at 37°C for 30 minutes with increasing concentrations of DegU protein in the 1x binding buffer. The reaction mixtures were separated on 8 % polyacrylamide gels under non-denaturating conditions in 1 x TBE buffer at 60 V. The gels were visualized using the Bio-Rad Molecular Imager FX scanner.

Similarly, the EMSA for p_{ywfH} and p_{Hpr} was performed with the fragment synthesized using the primers ywfH FWFP, ywfH RWFP and hpr FPF1, hpr FPF4, respectively (Table 2.2.3).

2.3.14 DNase I footprinting

DNase I footprinting experiments were performed as described previously [87]. A DNA fragment carrying the extended version of the *bacA* promoter (271 bp) was obtained by PCR amplification using primers bacFP1a and bacFP3b. It was incubated in binding buffer with different amounts of DegU protein (0, 1, 2, 5 and 10 µM) for 30 min at 37°C. Complexes were then treated with DNase I for 1 min, and the reaction was stopped by addition of DNase I stop solution containing non-specific calf-thymus-DNA (50 mg/ml). The treated complex was extracted using 95 % ethanol and dried at 50°C. Once the sample was completely dried, it was resuspended in 5 µl of stop solution. The samples were then separated on 7 M urea - 7 % polyacrylamide sequencing gels and visualized using the Molecular Imager FX Pro Plus (Bio-rad).

For deciphering DegU binding on *ywfH* promoter, a DNA fragment containing 150 bp was PCR amplified using primers ywfH FW- FP and ywfH RW- FP. DegU protein of different amounts (0, 0.75, 1, 2, and 5 µM) was incubated for 30 min at 37°C. The rest of the procedure was performed as described for the *bacA* promoter. Similarly, a DNA fragment containing all of Hpr putative sites was PCR amplified (180bp; obtained using primers bacFP2a and bacFP3b) and incubated with different concentrations of Hpr protein (0, 1, 2, 4, and 8 µM) for 30 min at 37°C. The rest of procedure was performed as described for the *bacA* promoter.

3 Results

3.1 Growth pattern of *B. amyloliquefaciens* FZB42 in GA medium

Bacilysin, a dipeptide (L-alanyl-[2, 3-epoxycyclohexanone-4]-L-alanine) is composed of non-proteogenic L-anticapsin at the C-terminus and L-alanine at the N-terminus. It is produced by *B. amyloliquefaciens* FZB42 during vegetative and transition growth stages. It is synthesized in a non-ribosomal fashion, however, it is independent of *sfp* which is required for multi-modular NRPS [82]. In order to understand the nature of growth pattern, FZB42 was grown in GA medium developed by Scholz *et al*. [82]. It is a minimal media containing glucose and ammonium sulphate as carbon and nitrogen source, respectively. The production of bacilysin was found to peak at 30°C (170 rpm) and pH 6.5. The Figure 5 indicates the growth pattern of FZB42 in GA medium.

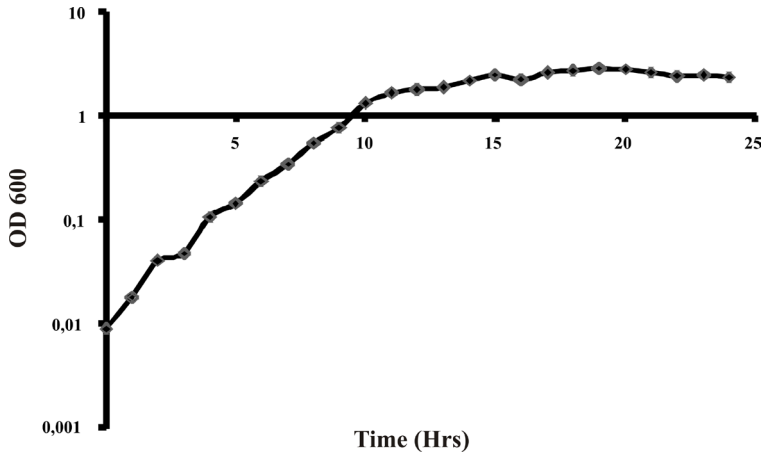


Figure 5: Growth curve of FZB42 (wild type) which was grown in GA medium at 30°C.

3.1.1 Antibiotic sensitivity test (AST)

Studying the antibiotic activity of bacilysin, the antibiotic sensitivity test or the agar diffusion test was used to get information about the anti-bacterial activity. *B. megaterium* was used as indicator strain. To understand the regulation of bacilysin production, I used the inhibition-test with different deletion mutants such as TF1 (Δ*degU*), CH23 (Δ*comA*), AM10 (Δ*hpr*), RS06 (Δ*bacA* and Δ*sfp*) and CH3 (Δ*sfp)*). Since the *sfp*-dependent nonribosomally synthesized anti-

biotics can inhibit the growth of *B. megaterium,* a *sfp*-deletion mutant was used as positive control. RS06, where Δ*sfp* and Δ*bacA* were knocked out, was used as negative control.

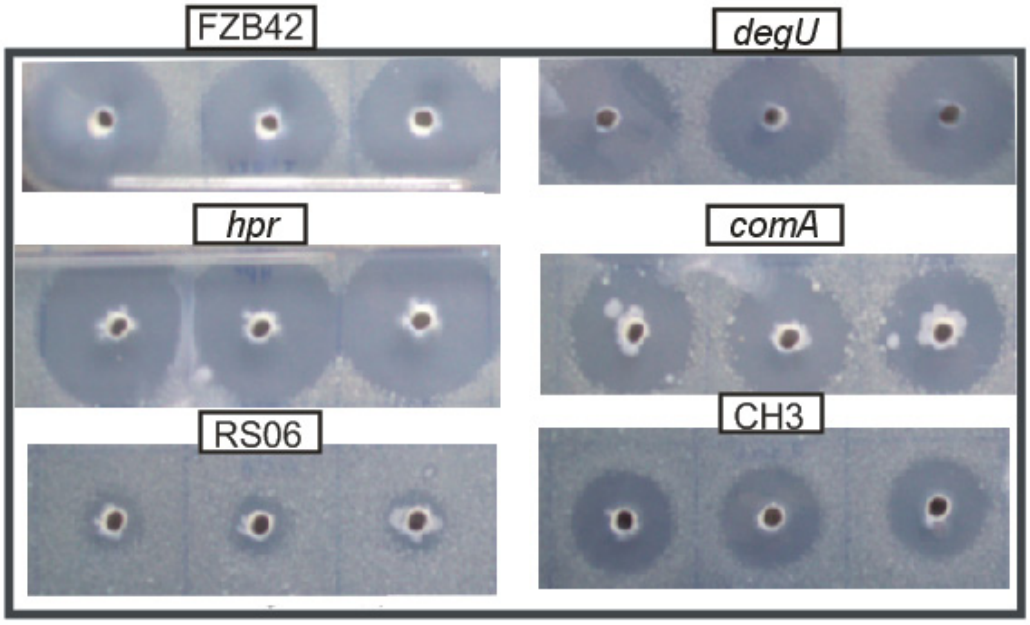


Figure 6: Agar diffusion test indicates the antibiotic activity of bacilysin on *B. megaterium.* Different mutants showed different activity. The *hpr* mutant indicated highest activity, whereas *degU* and *comA* had a similar or less effect compared to wild type. RS06 (Δ*sfp* and *bacA*) was used as negative control. CH3 (Δ*sfp*) used a positive control.

Comparing the results, CH3 (1.8 cm) had a smaller inhibition zone than wild type (2.5). Here, I have shown that *comA* and *degU* mutants had a smaller inhibition zone (2.1 and 1.9 cm respectively) compared to the wild type. On the other hand, *hpr* mutant had a larger zone of inhibition (3.0 cm). The result indicated the possible regulators which might be involved in the gene expression of bacilysin biosynthesis

3.1.2 Bioautography of bacilysin

Bioautography was performed essentially as described in Chen *et al.* [82]. Cultures were grown to OD600 nm of 0.8-1.0. Supernatant was spotted directly on precoated TLC plates. 20 µl of samples were loaded onto the plates which were placed in a chamber containing mobile phase solvents (1-butanol/ acetic acid/ water in 4:1:1 (v/v/v)). The gel was run for 2 hrs and thereafter it was dried at room temperature for an hour or more. Then the plate was stripped into small pieces containing individual samples. Meanwhile, GA agar plates containing indi-

cator strains were prepared (see Materials and Methods). The strips were incubated on the plates for 30 min. Later, they were incubated at 37°C overnight.

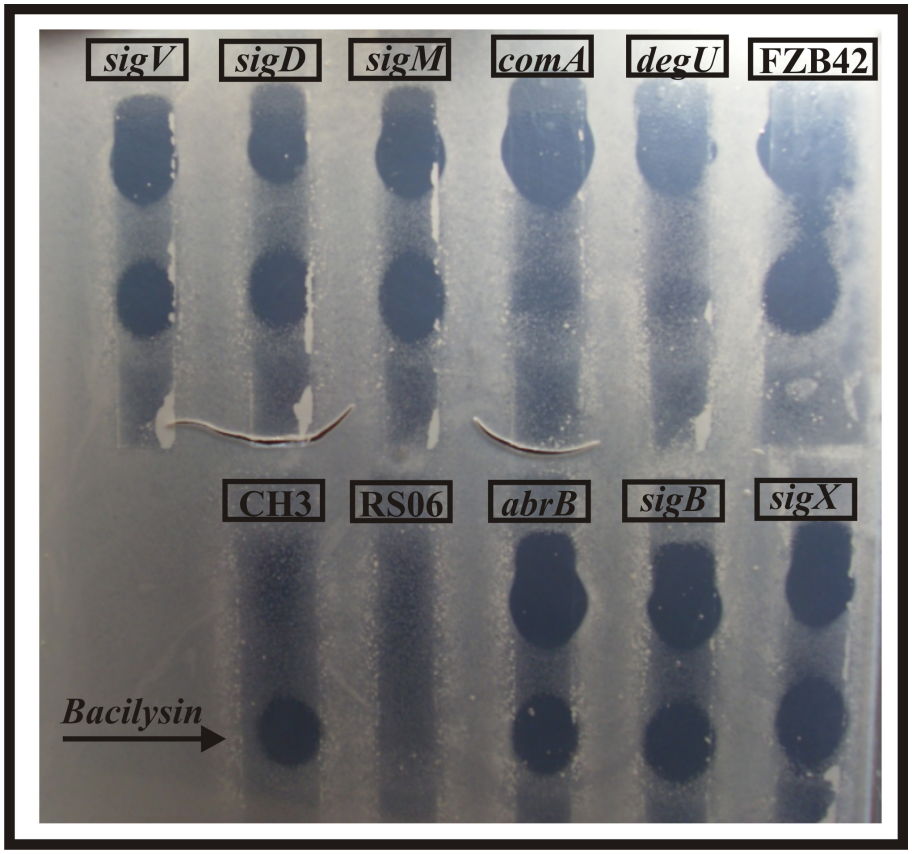


Figure 7: Bioautography of bacilysin was performed using *B. megaterium* as indicator strain. The second zone of clearance is the position of bacilysin. Both *degU* and *comA* mutants indicated lower production of bacilysin. RS06 is a double mutant of *bacA* gene and *sfp* gene, whereas CH3 is a mutant lacking *sfp*. They were used as negative and positive control, respectively.

The results of TLC indicated production of bacilysin by different mutants and by wild type. The second zone of clearance in the bioautography indicates the position of bacilysin (see Fig.7). The obtained results show that *degU* and *comA* mutants affected the production of bacilysin. Other regulatory and sigma factor mutants did not affect bacilysin biosynthesis.

3.1.3 HPLC of bacilysin production in FZB42

High performance liquid chromatography was performed with a C18 column (see Materials and Methods). Cell culture supernatant was used immediately after spinning down the cell debris. Bacilysin appeared early in the run (4.9 min) and it is highly hydrophilic. Therefore, it was not retained on the column. There are several other compounds in the chromatogram which could be metabolic remnants and medium components. Due to the unavailability of a standard, the bacilysin peak was identified by comparison with the chromatogram of non-producing mutants (see Figure 25) and with an earlier work by Scholz *et al*. [82].

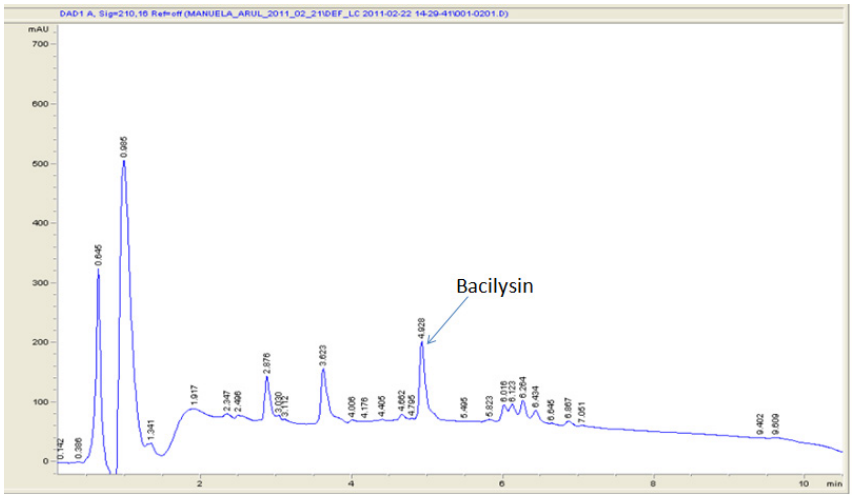


Figure 8: HPLC of bacilysin. Cell culture supernatant was used directly to measure bacilysin at 210nm. It appeared at 4.9 min which is indicated by an arrow.

3.2 Identifying the transcriptional start point of *bacA* and *ywfH* promoters

The transcriptional initiation site (TIS) of the *bac* promoter was determined. Total RNA was extracted from *B. amyloliquefaciens* FZB42 from culture growing in GA medium. The sample was collected during vegetative growth phase of FZB42 (see Materials and Methods). Primer extension was used to identify the adenine (A) as +1 which is the start point of transcription for *bac* promoter. The upstream region of the mapped transcriptional start site confirmed the presence of consensus regions -10 (TAATAT) and -35 (TTGACA) for sigma factor σ^A. The well conserved elements of sigma A indicated that the substance is produced during vegetative growth of FZB42, whereas most of secondary metabolites are produced during stationary phase. The spacer between the -35 and -10 region is 18 bp long and has a well conserved -16 region i.e. CATGC. The conserved elements coincide with reported σ^A

sequences [88]. It has well conserved promoter elements and a unique ribosomal binding site (TGGTTGGT) instead of conserved AGGAGGT. At this level, we propose that FZB42 utilizes this less conserved RBS to avoid expression of *bac* genes all the time avoiding the energy expensive process of synthesizing and transport.

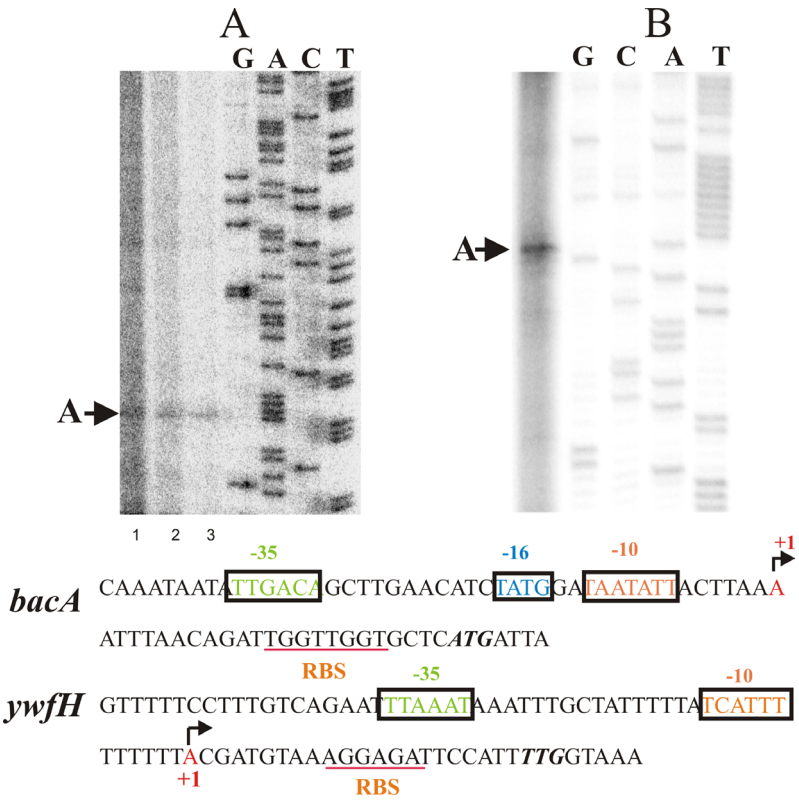


Figure 9: The primer extension of p_{bac} and p_{ywfH} is indicated above. A) The transcriptional start of p_{bac} was identified as +1 which is an adenine. 1, 2 and 3 (1, 2 and 3 µl of 2 µM primer) represent different concentrations of primer used to obtain the transcriptional start point. B) Similarly, adenine was identified as +1 of the transcriptional start of p_{ywfH} which is marked by a black arrow.

The transcriptional start of p_{ywfH} was determined by primer extension. The start point was seen as single strong band which is more pronounced than *bacA* (see Fig.9). Interestingly, the start points of both p_{ywfH} and p_{bac} are similar being an adenine base. Unlike the promoter of *bacA*, p_{ywfH} has not well conserved elements of promoter like -35 (TTAAAT) and -10 (TCATTT) region. In this promoter, the ribosome binding site (AAGGAGA) is quite conserved and has a match of nine out of 12 nucleotides compared to consensus sequence.

3.3 Transcriptional regulation of bacilysin production in *Bacillus amyloliquefaciens* FZB42

Bacilysin is produced during the exponential and transitional growth phase which was shown in an earlier part of this section [89]. Despite its small size, it is tightly regulated by transcriptional regulators. It was found in this study that bacilysin is regulated by global regulators such as DegU (degradative and protease regulator) and Hpr, while ComA, CodY and AbrB are indirectly controlling the expression [90]. The presence of these regulators is essential for positive or negative regulation of bacilysin genes [78]. In this work, the promoters of *bacA* and *ywfH* genes were studied which are responsible for the biosynthesis of bacilysin. The promoter of the Hpr was also investigated and found to be regulated by AbrB, a global regulator affecting gene expression.

3.3.1 Promoter analysis of *bacA* (p_{bac}) and *ywfH* (p_{ywfH})

The promoter of *bacA* and *ywfH* has been analysed in order to understand their role in expression of bacilysin genes. Several molecular techniques were employed to understand their mechanism of action. The *bac* operon was considered to be the major player in bacilysin production; later it was reported that *ywfH,* a monocistronic gene, is also essential in the production of bacilysin. Walsh *et al*. reported the function of YwfH to be an enzyme involved in converting intermediate substrate into pro-anticapsin moiety, which is a major part of the dipeptide [75]. The Figure 10 shows the complete operon of *bac* genes and the monocistronic gene *ywfH*.

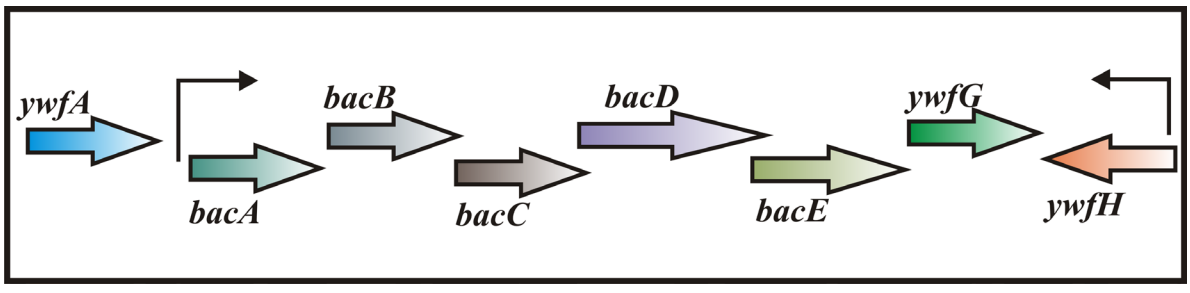


Figure 10: The *bac* operon and the monocistronic gene, *ywfH*, responsible for bacilysin biosynthesis.

The synthesis of bacilysin starts from prephenate of the aromatic amino acid pathway [91]. The function of each gene in the operon has been assigned bioinformatically, the first three genes (*bacA, bacB and bacC*) and the *ywfH* have been assigned to the formation of anticapsin

which is critical for the functionality of bacilysin [77]. The rest of the genes is involved in amino acid ligase and imposesw self-immunity to the producer [78, 92].

The sections below will reveal the results of different molecular studies used to decipher the promoter and regulators of bacilysin.

3.3.2 β-Galactosidase activity of *bacA* and *ywfH* promoters in wild type

In order to determine the *in vivo* expression of the putative *bacA* promoter (upstream region of the first gene of *bac* operon), five reporter fusions were constructed using *lacZ* with common downstream ends and variable upstream ends. A series of four nested fragments was generated using the polymerase chain reaction (PCR) with help of primers AFN1 to AFN5 (see Table 2.2.3). The generated fragments contain 5'-end deletions of *bac* promoter along with restriction sites, which can later be inserted into the vector PAK9. The vector was then integrated at the *amy*E locus of *B. amyloliquefaciens* FZB42. The correct chromosomal integration was confirmed using chromosomal PCR. The newly constructed strains containing variable lengths of promoter fused to *lacZ* were named as AA1, AA2 (-671 to +221bp), AA3 (-114 to +221bp), AA4 (-82 to +221bp), and AA5 (-36 to+221bp). The AA1 strain represents the construct without promoter fragment which is prepared to facilitate the transformation of FZB42 (see Fig.11).

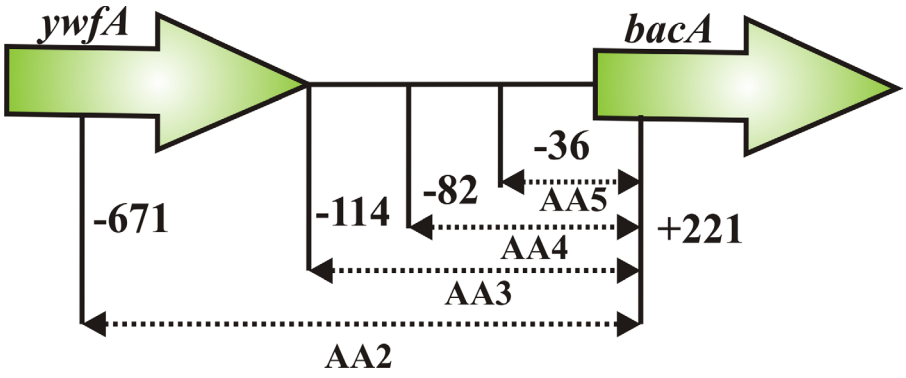


Figure 11: Schematic representation of different fragments used in *lacZ* fusion.

The expression of the p_{bac} promoter under *in vivo* conditions was assayed by the β-galactosidase activity. The expression pattern was studied for the complete growth cycle of FBZ42. The activity starts after four hours of growth indicating its expression during the exponential growth stage and it reaches the maximum after ten hours (See Fig.5).

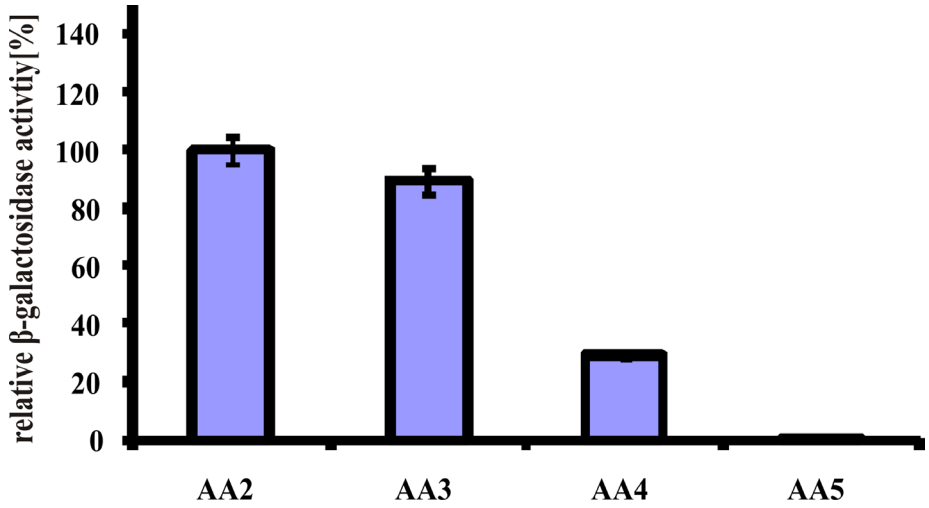


Figure 12: 5'deletion of p_{bac}. Different constructs were grown in GA medium and their β-galactosidase activity was determined. The activity decreased, while the fragment size was reduced from 5'of the promoter end. AA5 was the strain harbouring a fragment with deleted regions of essential promoter elements like -35 and -10 indicating less or no activity.

AA2 is the strain harboring the entire promoter region of $p_{bac.}$ The activity of AA2 reached the maximum after 10 hour concurrent with a published report for the production of bacilysin during transition growth stage [79]. The activity of AA3 got reduced compared to AA2. In this case, a part of the putative binding site of DegU (Identified through *in silico* analysis) was removed. AA4 exhibited much lower activity (~80 %) than AA2 (see Fig.12). AA4 contains the deleted version of two putative DegU binding sites. Furthermore, AA5 strain which has an extended region of deletions removing the -35 region had a deleterious effect on the expression of transcripts and remains silent during the entire growth cycle. In conclusion, the experiments indicate that the selected gene region harbours the bacilysin promoter.

3.3.3 β-Galactosidase activity of *bacA* and *ywfH* promoters in regulatory mutants

The expression of bacilysin was studied in regulatory mutants of *B. amyloliquefaciens* FZB42. Mainly, *degU, comA* and *hpr* mutants were transformed with the whole promoter region via double crossover using the chromosome. The *degU* and *comA* mutant was used from the strain collection of our laboratory, while the *hpr* mutant was prepared in this study. The β-galactosidase activity was studied for these regulatory mutants along with the wild type strain for comparison.

With this approach, several regulators involved in bacilysin were identified. The most important being the two-component signal system response regulators, DegU and ComA, which

were found to play a major role in the expression of bacilysin genes. DegU is the global regulator involved in the regulation of degradative enzymes and initiation of competence. ComA is the regulator of late competence genes and surfactin production. Apart from these positive regulators, Hpr and AbrB seem to play a negative role in controlling bacilysin synthesis.

The expression in the *degU* mutant (AM12) is not completely silenced, while there remained a basal activity. It was probably due to the activity of the vegetative growth phase sigma factor, sigma A. The *in silico* binding site analysis of *bac* indicated three putative sites for DegU binding, which reflected the results of β-galactosidase assay. Since DegU acts as a positive regulator, its main role played could be to bring the distant sequences together for the efficient binding of RNA polymerase.

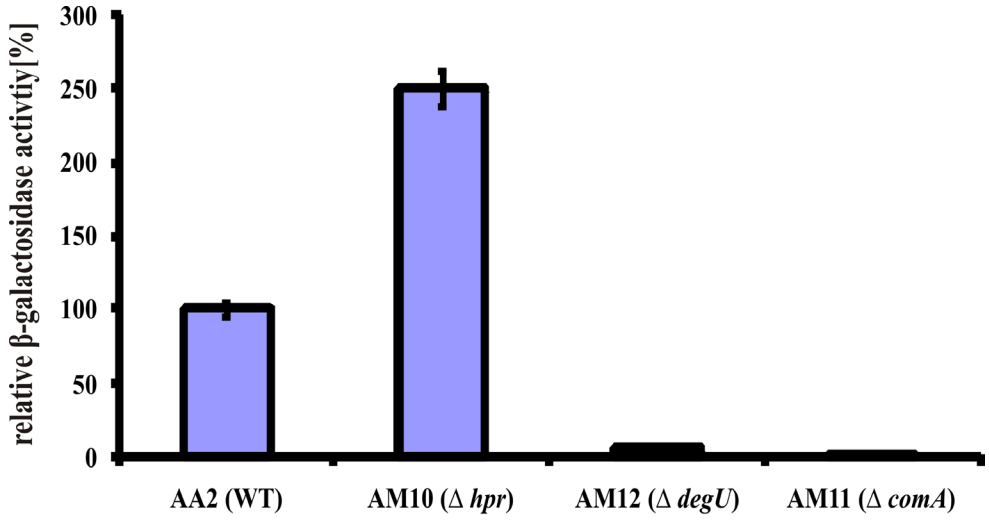


Figure 13: β-Galactosidase activity in mutants. All the cultures were grown in GA medium. AA2 was the wild type strain compared to its mutant. AM10 has higher activity compared to wild type indicating its role as a negative regulator of bacilysin, whereas, AM12 and AM11 are mutant strains of *degU* and *comA* with low or no β-galactosidase activity.

The *comA* mutant , AM11, containing the longest promoter fragment showed no expression compared to the wild type and indicates a positive control of the *bac* promoter (see Fig. 13). There are only few reports so far for the direct binding of *comA* to promoter region [93]. It is not clear in case of the bacilysin promoter.

Hpr (AM13) acting as negative regulator was also confirmed by *lacZ* expression. The activity was 2 fold higher than that of the wild type. It indicated a negative influence of Hpr over the *bacA* promoter. On the other hand, Hpr, a DNA-binding class of regulatory proteins, could

mediate its effect on the *bac* promoter by direct binding to the promoter region. Therefore, it is confirmed that DegU, ComA and Hpr play a major role in full activation of *bacA* promoter mainly through the upstream region of 150 bp (-671 to +221 bp relative to transcriptional start).

3.3.4 Transcriptional regulators of *bacA* promoter

It was demonstrated in this work that the bacilysin promoter was influenced by several DNA binding transcriptional regulators using β-galactosidase assay. Especially, DegU, ComA and Hpr play a major role in attenuating the transcription of *bac* promoter. Furthermore, in order to confirm the role in activation, DNA - protein interaction studies were conducted using putative regulators. The results of these experiments are explained below in detail.

3.3.4.1 Hpr and DegU directly affect p_{bac} expression

The two-component response regulators DegU and Hpr directly bind to the p_{bac} promoter. In order to confirm that DegU has a role in full attenuation of p_{bac}, (which is also evidenced by antibiotic sensitivity test and transcriptional *lacZ* fusion) the N-terminal His- tagged DegU protein was prepared and purified (see Materials and Methods). The nativity and functionality of the protein were confirmed by SDS-PAGE. The purified protein was used for EMSA and DNase I footprint studies.

It was demonstrated in this work that the *bacA* promoter was significantly influenced by the regulatory protein Hpr using *lacZ* reporter assay. Furthermore, to check, whether the Hpr protein binds to the *bacA* promoter, Hpr protein was prepared using an expression vector in *E. coli* (see Materials and Methods).

3.3.4.2 EMSA indicates direct binding of DegU and Hpr to p_{bac}

The results from *lacZ* fusion and antibiotic sensitivity test indicated the positive role of DegU in the transcription of p_{bac}. The full length fragment (-671 to +221 bp relative to transcriptional start) of the *bacA* promoter was used to study the gel retardation of both DegU and DegU~P proteins. Increasing concentrations of protein were used in each experiment. The result indicated that DegU has specific binding sites on the p_{bac} promoter and is essential for the full activation of transcription of *bacA*. The bands shifted between a concentration of 0.2 μM and

0.5 µM of unphosphorylated DegU (see Fig.14). A delay in migration of the promoter fragment was observed with increasing concentration of DegU and DegU~P reaching a saturation level at 3.0 µM. Binding of DegU was more specific, since the incubation of promoter DNA with the same amount of non-specific protein (poly dI-dC) did not lead to shift under the same conditions.

To investigate, whether Hpr binds to the promoter region of *bacA* to directly control transcription, I applied an electro-mobility shift assay. The experiments indicate binding of Hpr to p_{bac} promoter. This is the same fragment used in constructing transcriptional reporter fusion to monitor *bacA* expression.

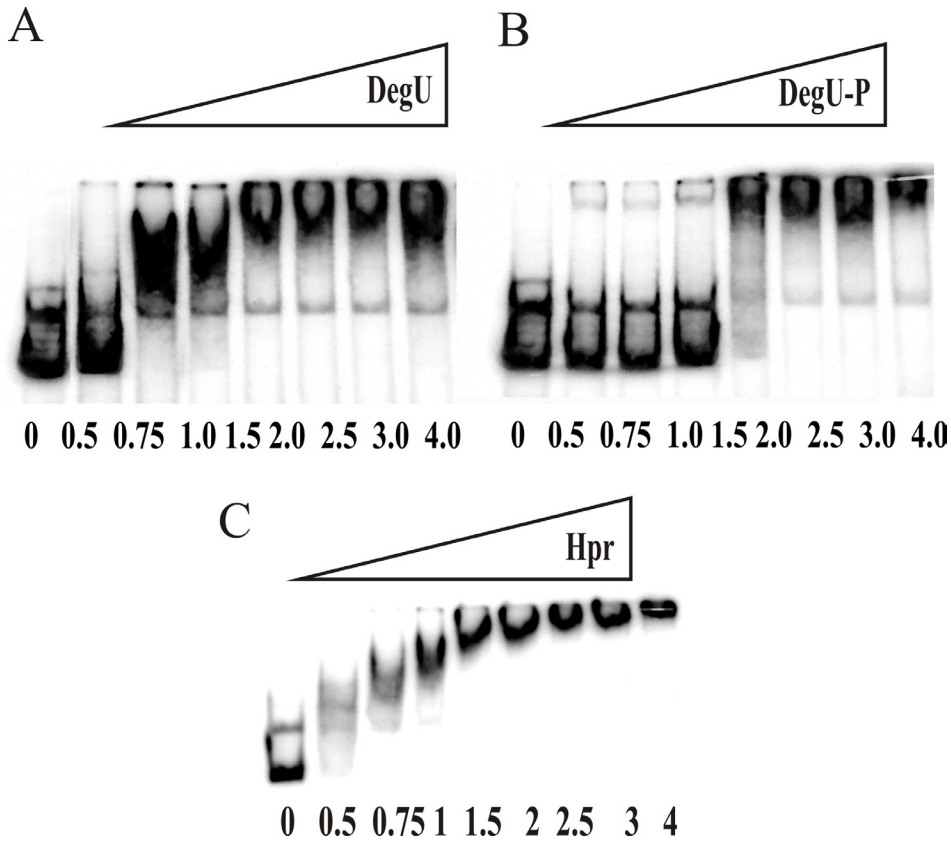


Figure 14: Gel retardation of DegU, DegU~P and Hpr on p_{bac} A) indicates the binding of DegU to $p_{bac.}$ With raising concentrations, the binding of DegU became stronger and reached a saturation level at 3.0 µM. B) DegU~P also caused a visible shift similar to the unphosphorylated form C) Indicates the binding of Hpr. In this case also the saturation level was attained at 3.0 µM.

3.3.4.3 Footprinting of DegU and Hpr binding sites

The binding of DegU to the p_{bac} and p_{hpr} was confirmed by gel retardation assay. Furthermore, the specific binding sites were identified using DNase I footprinting. The fragment (264 bp) of p_{bac} containing the entire length of the promoter was radiolablled and treated with DNase I along with the increasing concentration of regulatory protein, DegU. The protection of *bac* promoter was observed in the footprints. It revealed three different binding sites between -125 to -98, -82 to -74 and -57 to -5 within the coding strand of *bacA* promoter region. These regions were spanned by hypersensitive sites indicated by yellow arrows heads. On the non-coding strand only one protected region (-65 to -35) flanked by hypersensitive sites was found (see Fig.15). Furthermore, the protection indicated that adenine and thymine were the preferred bases for DegU binding. The region of this protection includes promoter elements like -10 and -35 which are essential for the full activation of *bac* genes.

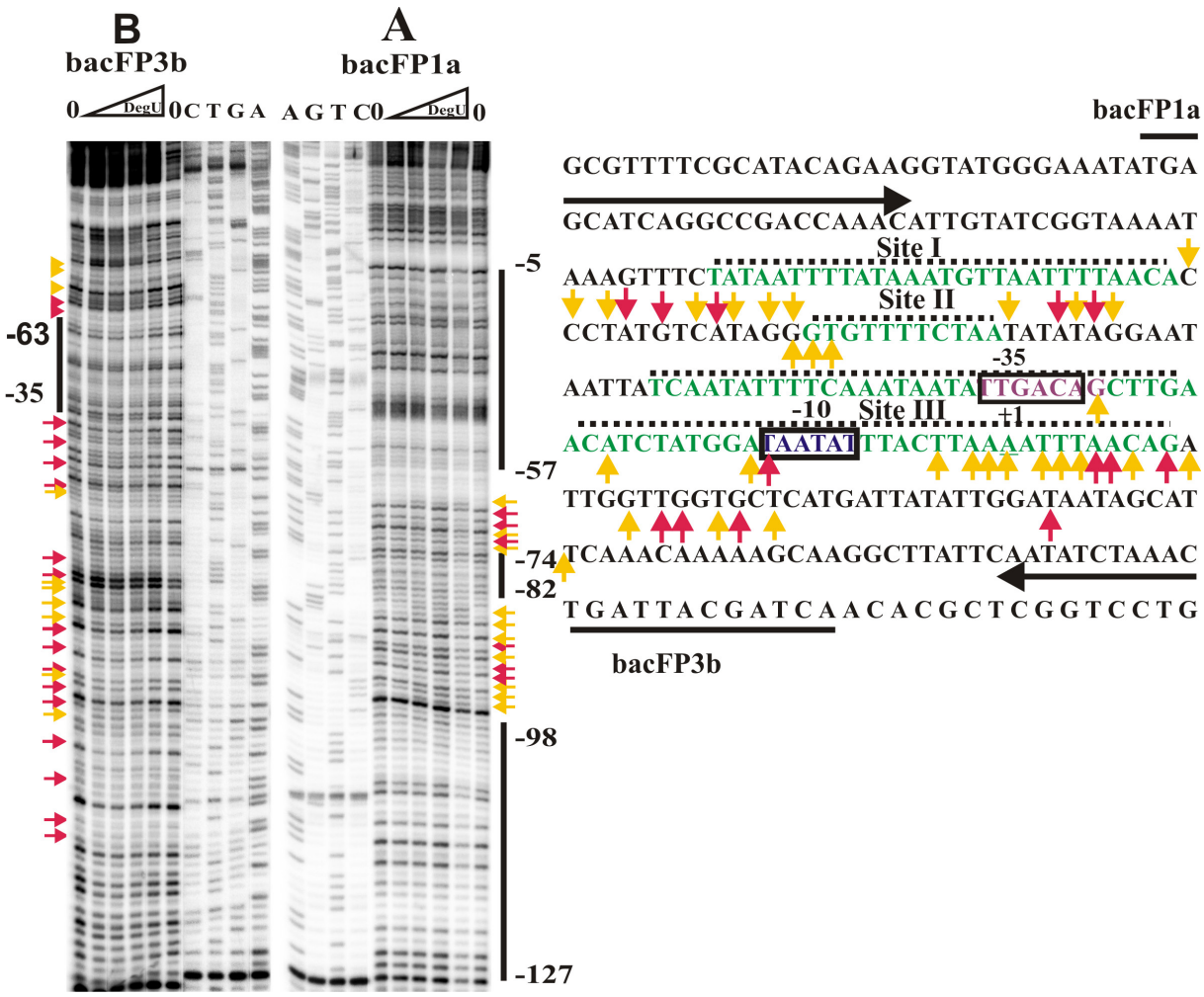


Figure 15: Mapping DegU binding sites. The DNase I footprint represents the binding of DegU to the promoter region of bacilysin. A) P_{32} labelled forward primer (bacFP1a) DNA sequence was digested with DNase I while the whole sequence was PCR synthesized before digestion. The digested product was separated using a 7 M denaturing urea gel. B) The bacFP3b (antisense strand) was labelled and digested along with DegU. Binding of DegU to the antisense strand was not so strong as to the sense strand; however, there were few hypersensitive bands indicating the dynamics between DegU and DNA strand. Sequencing ladders are marked as C, T, G, and A. 0 indicates the control with DegU protein and undigested DNA. The DegU concentrations raise (1.0 µM, 2.0 µM, 5.0 µM, and 10.0 µM) from left to right. The hypersensitive strands are marked by yellow arrows in both footprints and pink arrows representing disappearance of bands with increasing concentration of DegU.

It was demonstrated in this study that Hpr acts as a negative regulator of *bac* genes using transcriptional fusion assay. The electrophoretic mobility shift assay indicated the binding of Hpr to the *bac* promoter region. The P^{32} labelled forward primer bacFP2a was used to map the Hpr binding site on *bacA* (see Material and Methods). The labelled fragment was digested with DNase I along with Hpr. The footprint revealed three different binding sites on the *bacA* pro-

moter. Site I (TTATCAATATTTTCAAATAATATTGACAG) included the region between -60 to -31.Protections which covered the -35 region was also demonstrated in DegU footprint.

This overlapping of regulators is the clue to how bacilysin might be controlled tightly during the vegetative growth phase. Site II (TATGGATAATATTTACTTAA) spanning between -20 to +6 revealed the protection of -10 and +1 regions of the promoter (see Fig.16). Site III was deep inside the translational region of *bacA*. The protection spans between +26 to +47. Once again, Hpr is proved to be a DNA binding protein regulator.

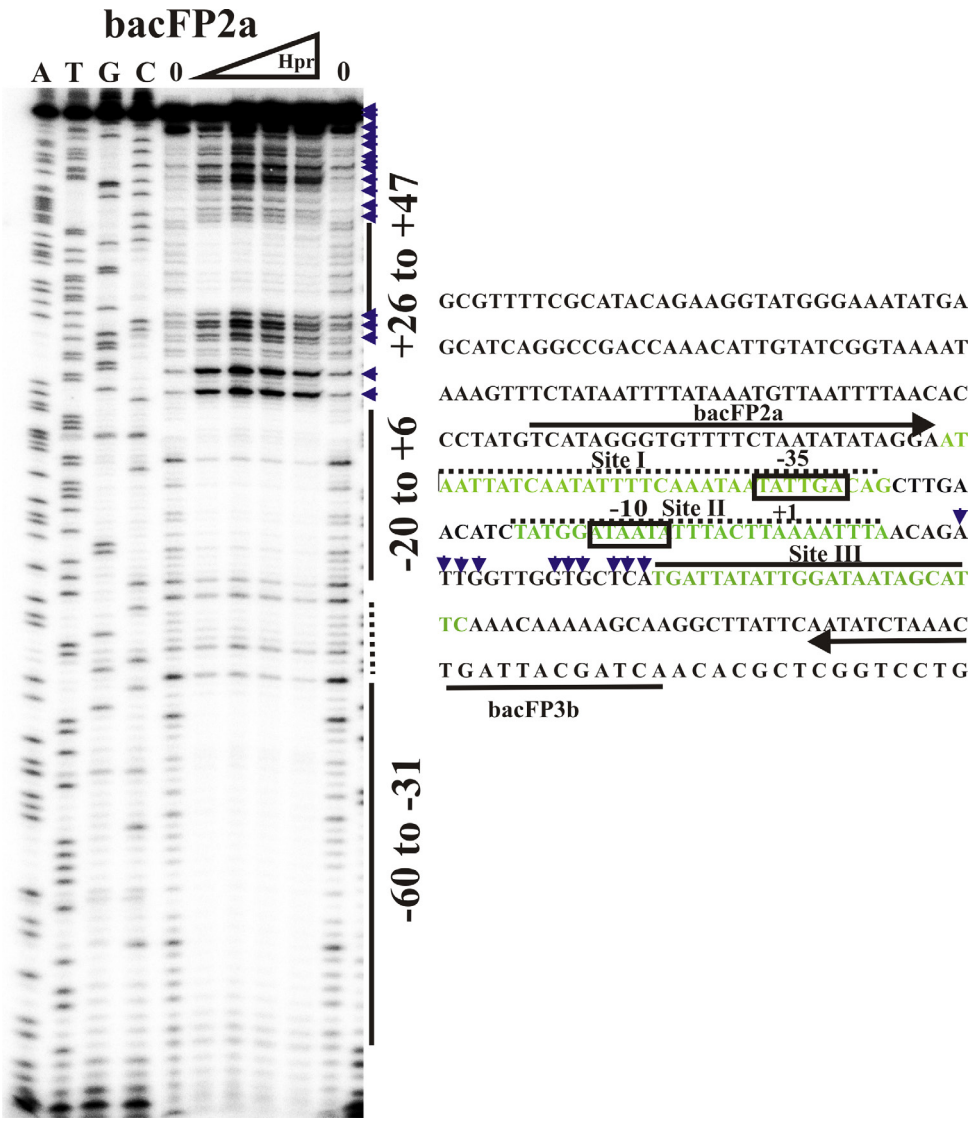


Figure 16: Hpr-DNase I digestion of p_{bac}**.** Mapping of Hpr binding site on p_{bac} promoter. The sequencing lanes are marked as A, T, G, and C. The concentration of Hpr raises (1.0 µM, 2.0 µM, 4.0 µM, 8.0 µM) from left to right. The Hpr binds to the promoter of *bacA* and induces conformational changes which can be inferred through hypersensitive bands which are marked as blue arrows. The fragment between -90 to + 90 relative to the transcriptional start point was used for DNase I digestion. The primers used for this fragment were indicated in table.2

3.3.5 Transcriptional regulation of *ywfH* in FZB42

The *ywfH* is a monocistronic gene located downstream of the *bac* operon. It has been reported that YwfH has a putative nicotinamide- dependent reductase or dehydrogenase activity. It was shown to be involved in the reduction of H_2HPP 5 to H_4HPP 6 which is the precursor of anticapsin [75].

In case of p_{ywfH}, the promoter region of ~600bp was fused to *lacZ* and transformed into FZB42. The resulting strain was referred as AA6. The expression of the *ywfH* gene was monitored over the complete growth cycle. The ß-galacotosidase activity was measured. The activity reached its maximum after 11 hours, an hour or two later when compared to *bacA* gene which can be assumed that the protein of *ywfH* is needed later for the synthesis of bacilysin. Thereafter, the activity decreased (see Fig.17).

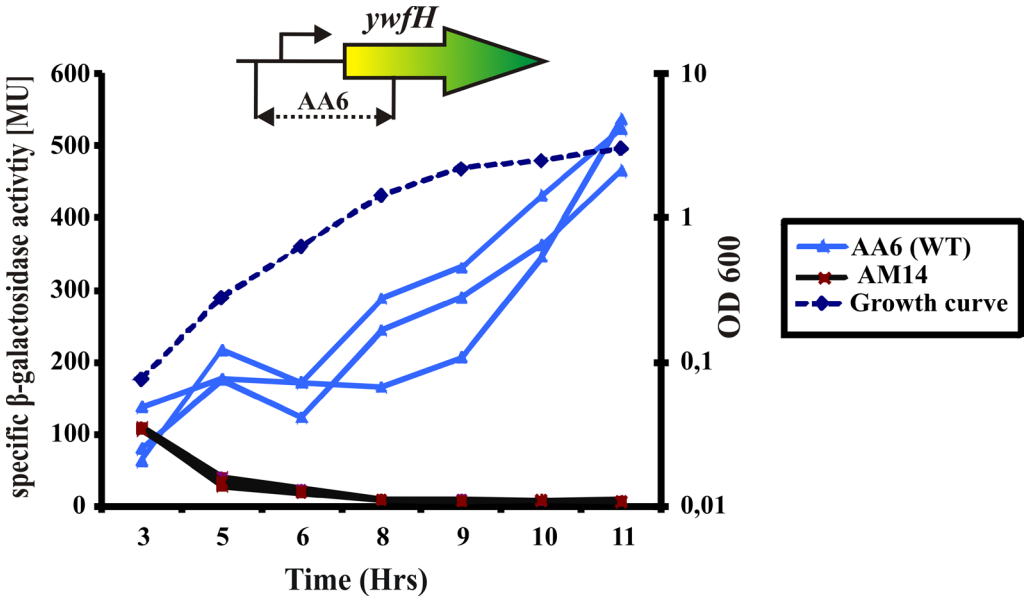


Figure 17: The *in vivo* transcriptional activity of p_{ywfH}. A) The gene map indicates the region used for analysis. It was not drawn to scale. B) A fragment of 600bp was fused with *lacZ* and transformed into AA0 resulting AA6. AA6 was grown in GA medium and β-galactosidase activity was assayed. In order to obtain DegU mutant (AM14), the chromosome of TF1, the *degU* mutant, was isolated and transformed into competent AA6 cells. β-galactosidase activity was measured for both wild type and DegU mutant (AM14). It clearly indicated that DegU plays a major role as a positive indicator in the case of *ywfH* promoter.

3.3.5.1 Two-component response regulator DegU binds to p_{ywfH}

Interestingly, the gel retardation assay indicated a visible delay in the migration of *ywfH* promoter when incubated with DegU regulator protein, clearly indicating that DegU plays a positive role in attenuating the *ywfH* gene expression. It was also shown in the transcriptional fusion assay (see Fig.17). A fragment containing the whole promoter region was used to study

the binding of DegU. It represents +40 to -500 relative to the transcriptional start. Increasing concentrations of DegU were used in order to find the saturation level.

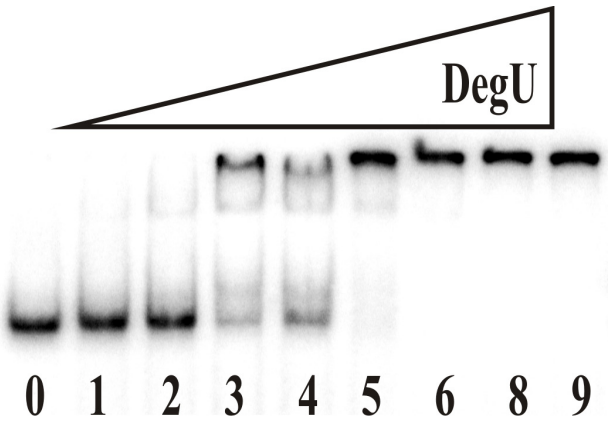


Figure 18: EMSA indicated the binding of DegU to the *ywfH* promoter. The fragment started to shift at 3.0 µM and saturated at 6.0 µM. Increasing concentrations of DegU were used. Amounts are indicated in the figure.

The binding of DegU to *ywfH* is important in the biosynthesis of bacilysin. EMSA revealed direct binding of DegU to DNA sequences of p_{ywfH}, but the exact binding regions cannot be assessed using this technique. They were detected by DNase I footprinting.

3.3.5.2 Mapping DegU binding sites on p_{ywfH}

Mapping of DegU binding sites in *ywfH* promoter was performed using DNase I. The promoter fragment covering -119 to +39 relative to transcriptional start was used for this study. Labelling of this fragment was performed using P^{32} radiolabelled primers ywfHFw-FP and ywfHRw-FP. As a result, the PCR synthesized fragment was radiolabelled. Figure 19 represents the DNase I digestion of *ywfH* promoter.

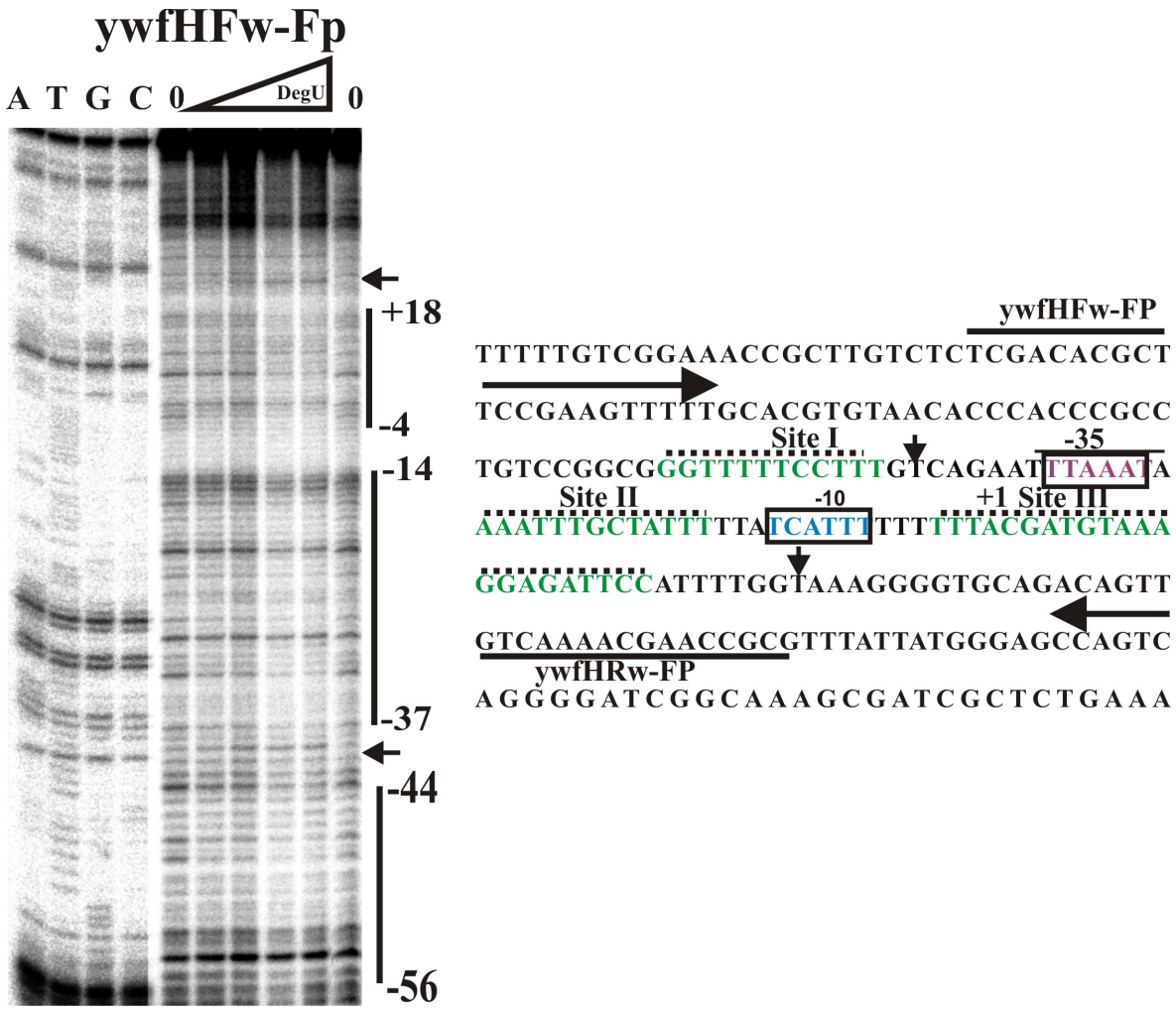


Figure 19: Mapping of DegU binding sites on p_{ywfH}. Hypersensitive sites are shown as arrow mark. Binding region can be divided into two different sites although there is no clear boundary to detect. The concentration raises (1.0 µM, 2.0 µM, 5.0 µM, 10.0 µM) from left to right. The primers used for this study are indicated in table 2.2.3. The Sequence ladders used for this study are marked as A, T, G and C.

From the footprinting patterns, it is clear that DegU binds to p_{ywfH} and the bound region can be seen as decreasing band intensities, when the concentration of DegU increased. The un-phosphorylated DegU binds two distinct sites at the *ywfH* promoter inducing bends and local changes in the DNA architecture adjacently to these sites (seen as hypersensitive bands). In detail, the preferred bases of DegU binding include adenine and thymine. From the Figure 19, it is evident that the site I is composed of A and T's (between -57 to -45) and has good consensus to the reported sequence [94]. Site II ranges from -28 to -16. The promoter element -10 is protected and also the transcriptional start (+1). Binding of DegU to such important promoter elements indicates that it is an essential protein regulator for the expression of bacilysin.

3.4 Transcriptional regulation of *hpr* (p_{Hpr}) in *B. amyloliquefaciens* FZB42

Hpr is a transition state regulator expressed by the *hpr* gene. It is a 23,7 kDa protein involved in the expression of neutral and alkaline proteases. Characterization and insertional inactivation experiments have shown that Hpr is a negative regulator of proteases [95]. Hpr appears to regulate not only proteases, but also other functions like alkaline phosphatase, motility and glucose mediated repression of sporulation [6]. Additionally, Hpr has shown to be a DNA binding protein in the regulation of proteases. It was demonstrated in this study that Hpr also acts directly on *bacA* promoter. While this report was in preparation, Inaoka *et al*. 2009 reported the function of Hpr in bacilysin regulation. The footprint experiments in this work revealed the exact binding sites of Hpr being the first report of Hpr footprinting in an antibiotic gene.

3.4.1 β-Galactosidase assay for p_{Hpr} (*hpr*)

In order to understand the expression of *hpr* at the transcriptional level, the promoter of *hpr* was fused to a *lacZ* reporter gene. The region selected encompassed the entire promoter in addition to extended upstream sequences (see Appendix 6.3 for sequence). PAK9 plasmid was used to construct the fusion. It is a vector which contains the flanking region of *amyE* and helps to integrate a single copy of construct into FZB42 chromosome (see Materials and Methods). Primers Hpr HindIII and Hpr SalI were used for the synthesis of PCR fragments. The fragments were digested using restriction enzymes (HindIII and SalI) and ligated into PAK9 vectors, referred as pAHN1. Plasmid pAHN1 was transformed into competent AA1 strain (see Materials and Methods). Colonies were checked for the transformants which were named as AA7. Cells were grown in Difco sporulation medium for the complete growth cycle and the cells were collected. β-galactosidase activity was calculated and the gene expression pattern was indicated in the Fig.20. The activity was less compared to bacilysin assay. The reason behind this phenomenon is discussed in the last section.

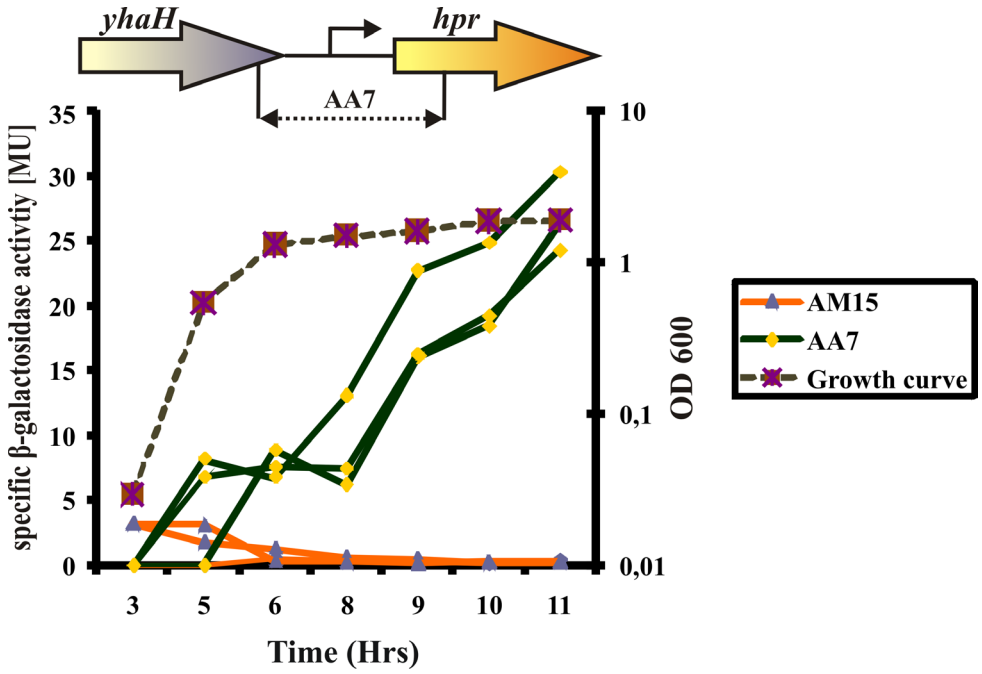


Figure 20: β-galactosidase activity of *hpr* promoter. A) The fragment used for *lacZ* fusion was shown and not drawn to scale. B) Entire upstream and downstream region of *hpr* promoter was fused to *lacZ* and transformed into AA0, termed as AA7. Further, chromosome was isolated from AM07 and transformed into AA7 to obtain AM15 harbouring a mutant of *abrB*. Both cultures were grown in GA medium and β-galactosidase activity was measured. The mutant strain AM15 has completely lost its activity, when compared to the wild type strain AA7.

The expression of *hpr* gene in the regulatory mutant strain of *abrB* (which was identified using *in silico* analysis) was studied. The *hpr* promoter was fused to *lacZ* gene and obtained the strain AA7 by transforming the FZB42 using this construct. The chromosome was extracted from mutant AM07 (Δ*abrB*) and transformed into AA7. The obtained mutants was referred as AM15. β-galactosidase activity was calculated (see Fig.20). The disruption of *abrB* gene severely affected the expression of *hpr* gene indicating the importance of AbrB. Obviously, the protein is required for the full activation of *hpr* promoter.

3.4.2 Global regulator AbrB is controlling the expression of p_{Hpr} (*hpr*)

Since it was evident from the *in vivo* transcriptional studies that AbrB plays a role in *hpr* expression, gel retardation assay was performed to narrow down the region that binds AbrB. A P^{32} radiolabelled fragment of ~600bp (PCR fragments synthesised with primers Hpr FPF1and Hpr FPF4) was used to study the shifting. The experiments revealed hindrance in the migration of promoter region indicating that *hpr* contains specific binding sites for AbrB. It is also evident from Figure 21 that higher concentrations caused shift of the DNA fragments.

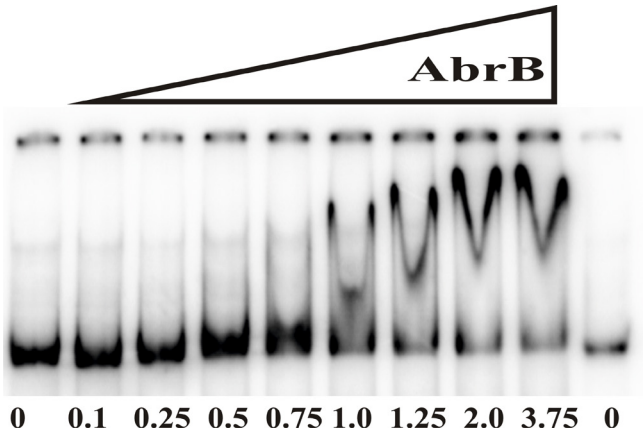


Figure 21: EMSA indicated shifting of the *hpr* promoter with increasing concentrations of AbrB. The incubation of the *hpr* promoter with nonspecific polydI-dC did not cause migration of DNA fragments.

Additionally, another interesting phenomenon was observed. It was known from previous experiments in our lab that AbrB has the ability to bind large DNA sequences (Neubauer, Pers.comunication). I designed primers in such a way that two fragments with overlapping regions were synthesized and radiolabelled using P^{32}. They were subjected to EMSA. Surprisingly, both the fragments shifted at the same concentrations indicating that the fragments harbour AbrB binding sites.

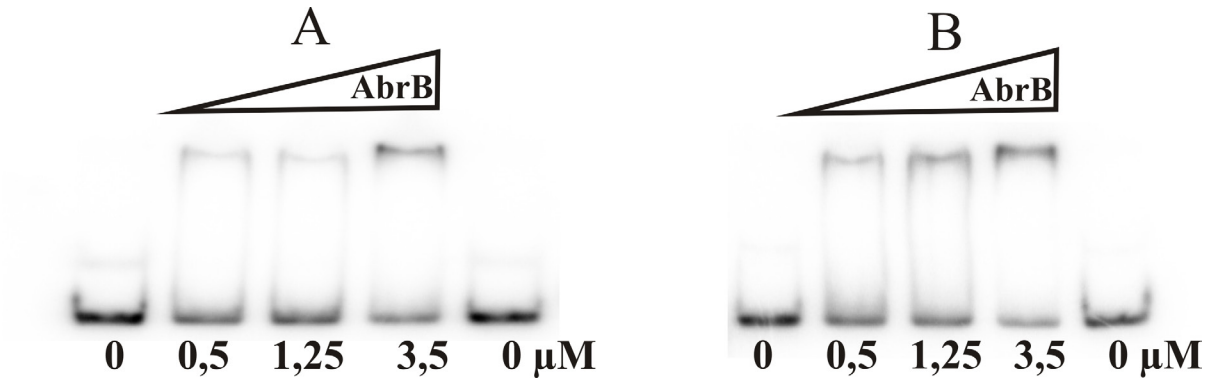


Figure 22: Binding of AbrB to *hpr*. A) Fragment F1 showed partial binding of AbrB indicating that a sequence in the upstream region is essential for AbrB binding. B) Fragment F2, the downstream sequence of F1, indicated a shift even though the affinity of AbrB to the fragment was not so strong as that of the whole fragment (see Fig. 21)

Both these fragments exhibited affinity towards the AbrB protein. This can indicated that binding of a single AbrB molecule to the promoter might induce a cooperative binding of more AbrB molecules and change the promoter architecture.

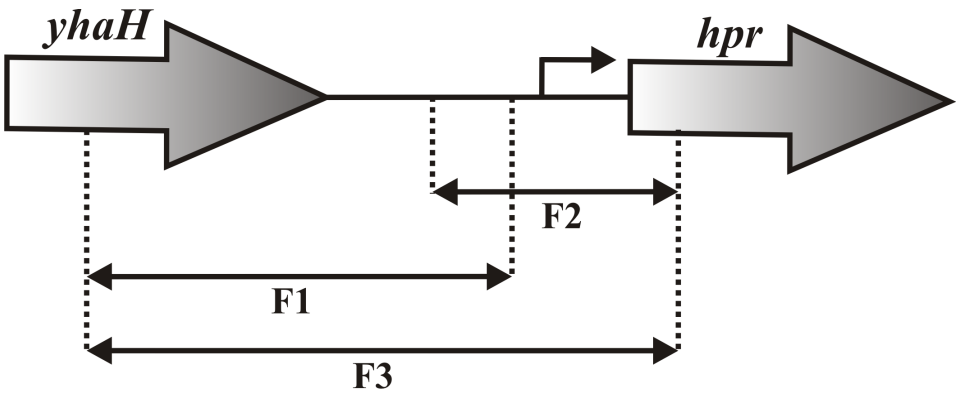


Figure 23: Representation of different fragments used in EMSA and footprint. The primers used to obtain these fragments are indicated in the table 2.2.3. The fragment F3 was used for the footprint.

Furthermore, the DNase I footprint was performed in order to identify the exact location of AbrB binding. The footprinting obtained using F1 and F2 fragments did not indicate significant binding of AbrB, while EMSA indicated a shift. The reason for this could be the sensitivity of the footprint. In order to solve this problem, a new fragment encompassing the whole fragment similar to the fragment used in EMSA (see Fig.21) was synthesized. The primers Hpr FPF1 and Hpr FPF4 were radiolabelled to generate PCR fragments. The complete fragment was digested with DNase I. The resulting fragments were separated using 7M urea gel. The result obtained is shown in Figure 24. The footprinting obtained contains both the coding and non-coding strand leading to a similar conclusion that AbrB binds to the entire region of the promoter DNA sequence.

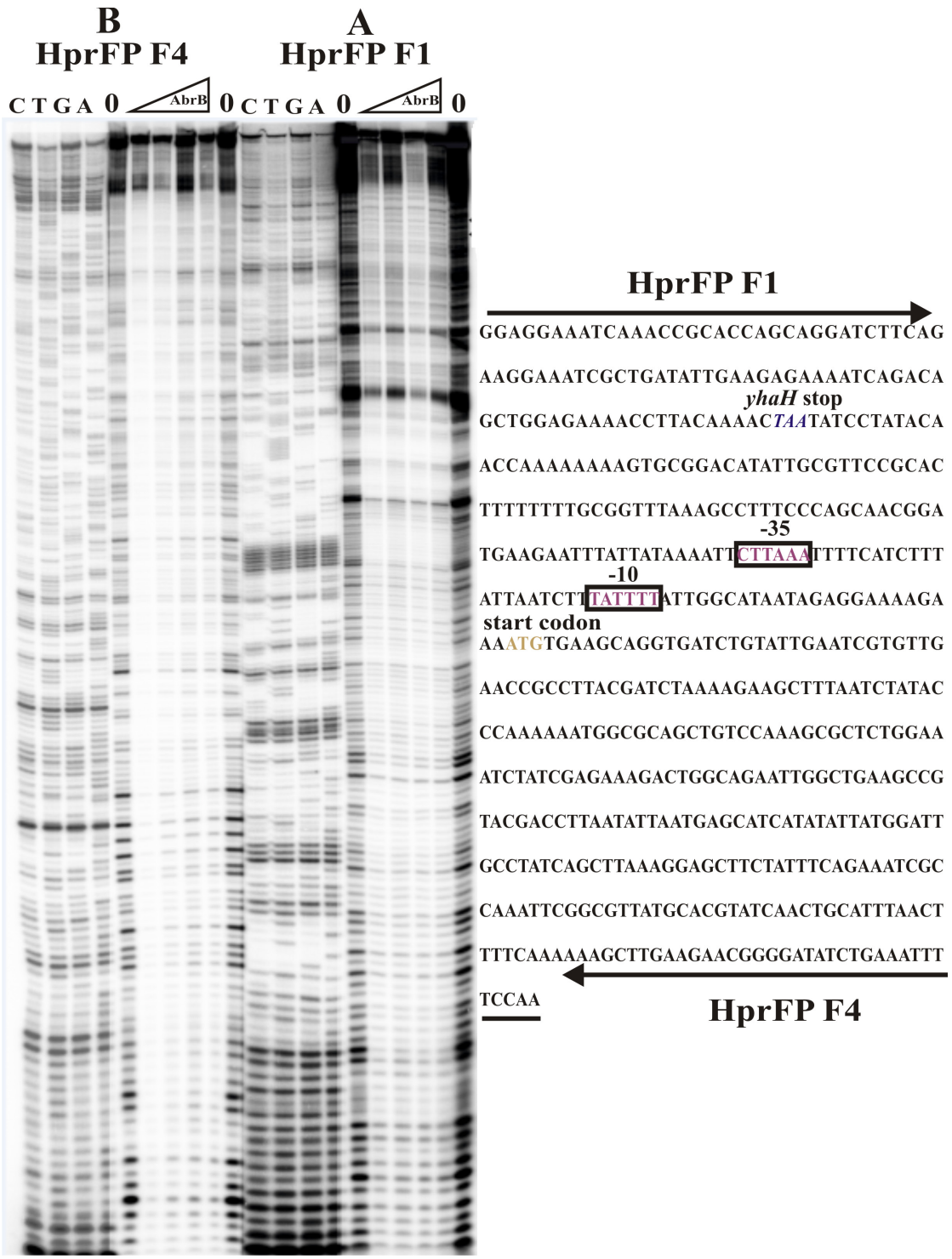


Figure 24: Footprinting of the *hpr* promoter with AbrB. The complete fragment of ~550bp was digested with DNase I and separated on a polyacrylamide gel. A) The forward primer fragment shows clear regions of the binding of AbrB to the *hpr* promoter. B) Represents the reverse strand; digested fragments indicate clear binding of AbrB to the entire region. The triangle represents the increasing concentration of AbrB. 0 indicates control. The concentrations of AbrB used are 1.25 µM, 3.75 µM, 7.5 µM, and 12.5 mM. The sequence used for footprint was indicated on the right side of the diagram. The important promoter elements are marked in different colours.

Based on this experiment, I am not able to locate exact sequences, where AbrB binds, since the protection was observed in the complete promoter region. The possible mechanism of binding is discussed in the last chapter of this thesis.

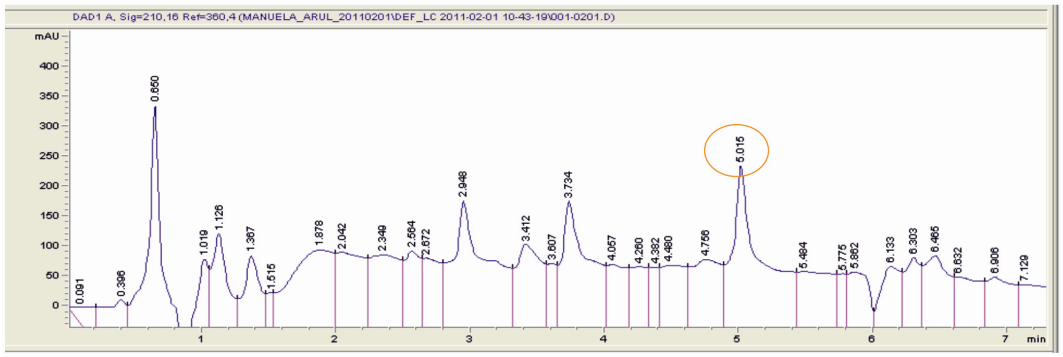
3.5 Analysis of bacilysin synthesis in wild type and mutant strains of FZB42

In order to quantify and check the production of bacilysin, HPLC and HPLC-MS was performed in wild type and mutants. The culture was grown overnight in GA medium. The samples for HPLC and HPLC-MS were collected and the supernatant was used for analysis. A C_{18} reverse phase column was used to separate bacilysin (see Material and Methods). The bacilysin peak appeared after 5 minutes of the total run of 25 minutes. It was detected at 210nm.

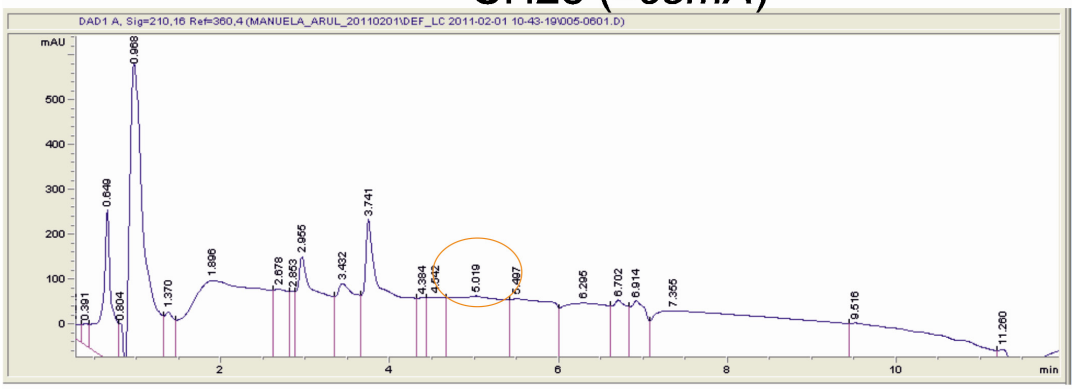
I have used RS06 (Δ*bacA*, Δ*sfp*), a double mutant as a negative control for bacilysin production. Regulatory mutants were checked for their production of bacilysin. As expected, mutants of *degU* (TF1) and *comA* (CH23) were affected negatively in their ability to produce bacilysin (see Fig. 25). On the other hand, *hpr* (AM10) mutant has similar or little higher activity compared to the wild type FZB42. Once again, the role of regulatory mutants has qualitatively been proved to be essential for bacilysin biosynthesis.

We were expecting a higher production of bacilysin in case of *hpr* mutant (AM10) but, surprisingly the amount of bacilysin detected was similar to the wild type. To find a reason for this phenomenon, we have checked the production of bacilysin using protease inhibitor mixture which contains alkaline protease inhibitor (as supplied by Sigma) since alkaline protease has been associated with degradation of bacilysin [96]. Always after using the protease inhibitor in culture medium, there was no marked increase in bacilysin production. It was indicated in the earlier part of this thesis that the *ywfH* gene is essential for the complete biosynthesis of bacilysin. We hypothesize that the other reason could be the negative control of protein regulators which are acting on *ywfH* gene. So far, there is no clear evidence of these negative regulators acting on this gene. However, *in silico* analysis indicated the presence of nitrogen regulators such as CodY and TnrA on the promoter region. To a certain extent, AbrB has also been shown to shift the *ywfH* promoter region but not in the range of biologically available amount of regulator in the cell during growth.

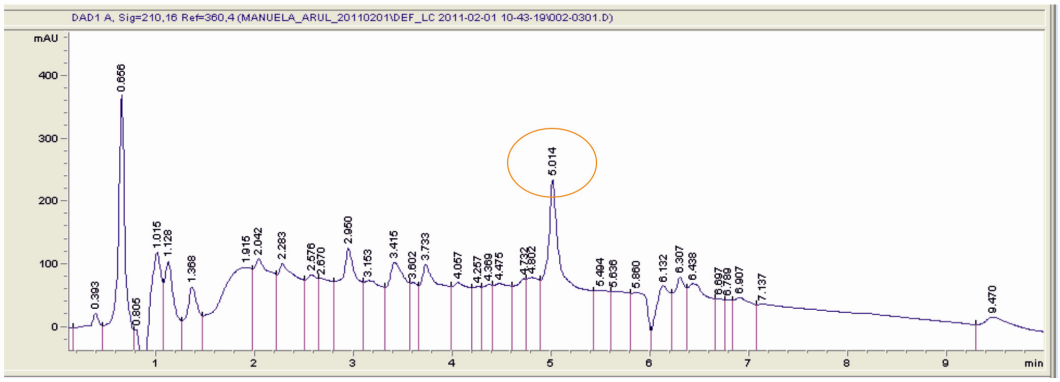
FZB42



CH23 (△*comA*)



AM10 (△*hpr*)



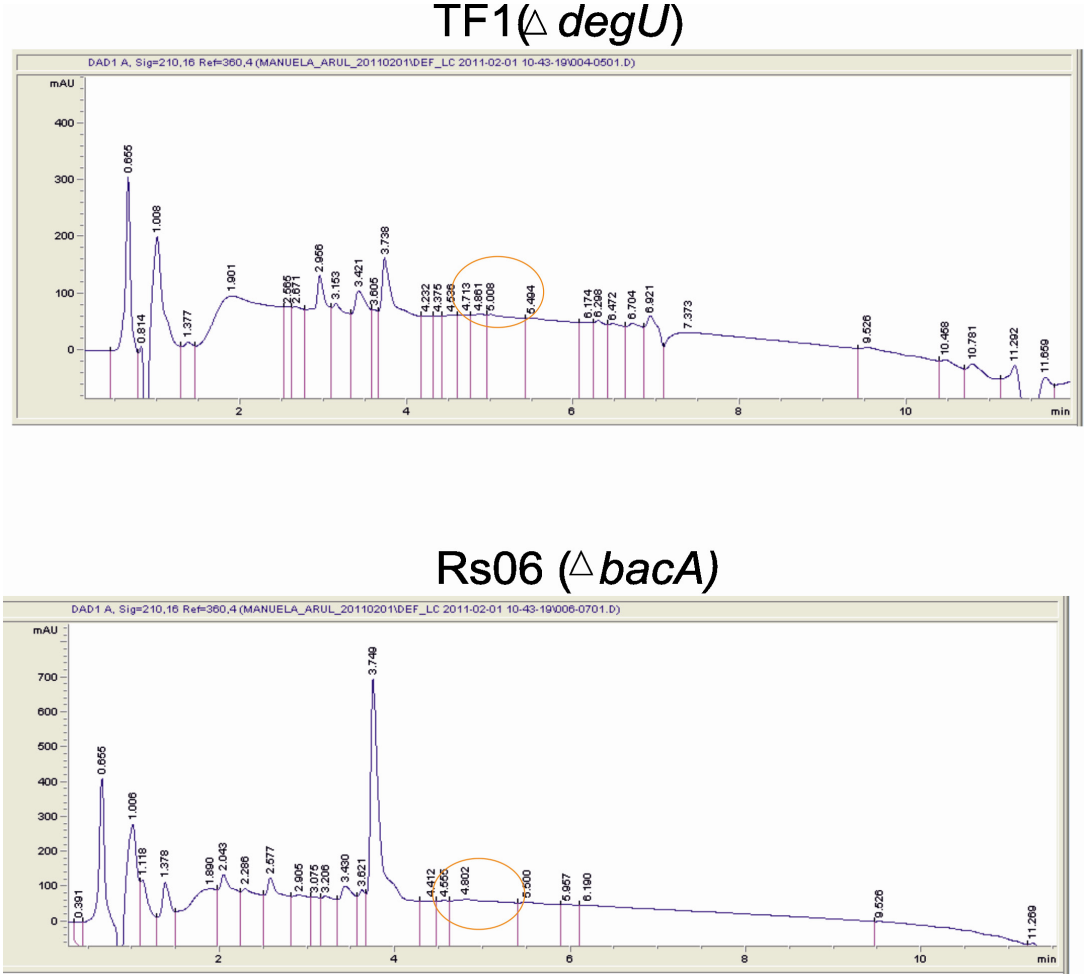
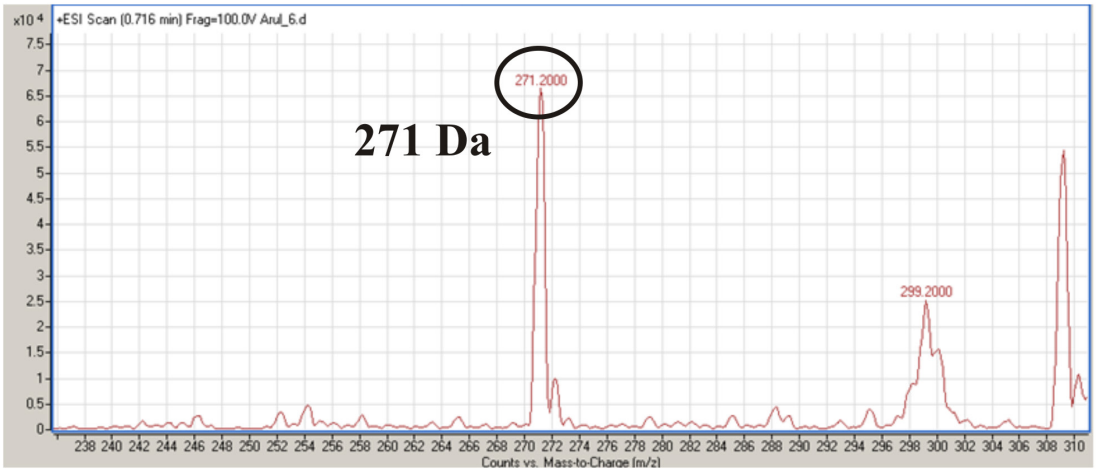


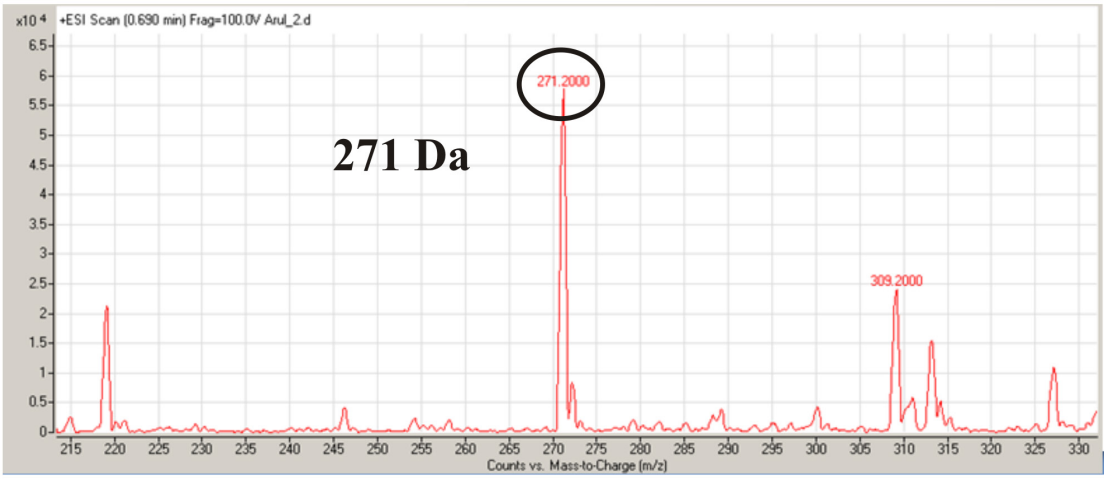
Figure 25: HPLC analysis of bacilysin production. The wild type and mutants were grown in GA medium overnight and the supernatant was collected and analyzed using HPLC. The samples were marked above. In each chromatogram the bacilysin peak appeared at 5th minute of 25 minutes of total run. Bacilysin was indicated by red round on each sample.

High performance liquid chromatography (HPLC) combined mass spectrometry was also performed. Extracted ion chromatograms (EIC) were shown in the Figure 26. The analysis revealed the confirmation of previous results that DegU and ComA positively regulate bacilysin, while Hpr negatively controls this dipeptide antibiotic. In case of HPLC-MS, the chromatogram from mutants (TF1 and CH23) did not indicate a complete loss of bacilysin, whereas the HPLC chromatograms indicate loss of the bacilysin peak in these mutants. It was shown earlier in this study that *B. amyloliquefaciens* FZB42 retained some basal activity. Apart from this phenomenon, the sensitivity of the column and the method might be the reason for differences in the chromatogram.

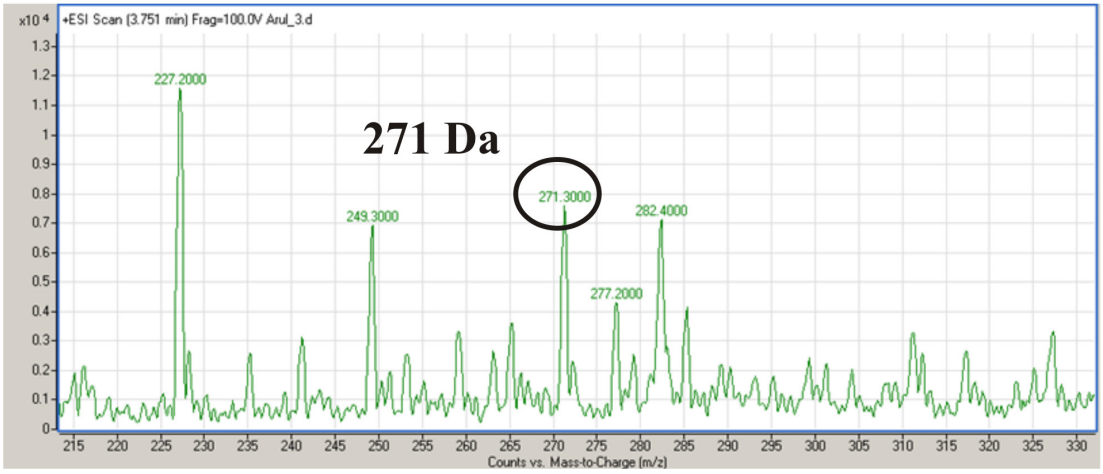
FZB42



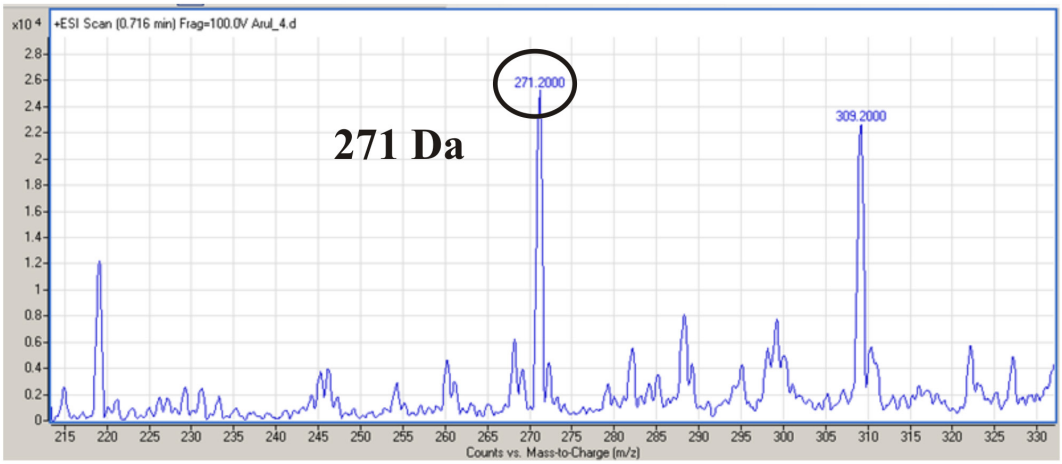
AM10 (Δ*hpr)*



TF1 (Δ*degU)*



CH23 (Δ*comA)*



RS06 (Δ*bacA, sfp)*

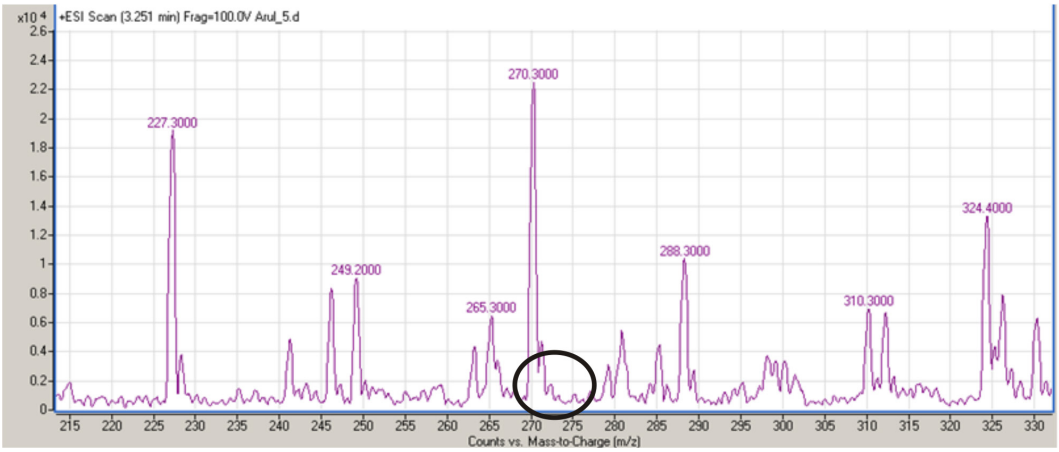


Figure 26: HPLC-MS of bacilysin. Different regulatory mutants were grown overnight in GA medium, the supernatant was collected and analyzed using HPLC-MS of Thermo scientific make. The extracted ion chromatograms (EIC) indicate the mass of bacilysin which is 271.0 Dalton. In the wild type, the mass appears in large amount whereas in regulatory mutants (*comA* and *degU*) only little of bacilysin was available. RS06 was used as a control since it is a knock-out mutant of *bacA* and *sfp*. The orange round indicates the abundance of bacilysin in different samples.

The analytical analysis of bacilysin using HPLC and HPLC-MS indicate the importance of regulators in bacilysin biosynthesis. I have tried to quantify bacilysin; however it was not successful due to the unavailability of pure bacilysin to compare as standard. HPLC-MS chromatogram was further analysed by expanding the region around m/z 271 kDa. The regulatory mutants indicated a lower abundance of bacilysin compared to its wildtype. The chromato-

gram of RS06 (Δ*bacA* and Δ*sfp*) indicated the absence of bacilysin and it was used as a negative control.

4 Discussion

The results reported here provide new insights into the regulation of bacilysin genes at the molecular level, which has been a subject of study for more than a decade. The *bac* operon and the monocistronic gene *ywfH* for bacilysin production were studied at the promoter level. I have also demonstrated here that DegU and Hpr act together in the expression of bacilysin. The DegU plays the role of a positive regulator, while Hpr negatively controls bacilysin production in FZB42. On the other hand, *ywfH* has also been found to be controlled by DegU. There was no negative control from Hpr. The overall regulation of bacilysin production exemplifies transcriptional regulation of a gene involved in this process during vegetative and the transient growth stage of FZB42.

4.1 Promoter analysis of bacilysin genes and the *ywfH* gene

The promoter of bacilysin genes can be recognised as a well organised and utilized promoter by protein regulators of DNA binding class. The structural elements of the promoter, especially -35 (5'TTGACA3') and -10 (3'TAATAT5') are well conserved and correspond to the reported consensus [97]. Apart from these regular hexamers, there are -16 (TATG) and putative UP element which are present in the promoter of bacilysin. It was proposed more than a decade ago that optimal promoter activity is achieved by different combination of promoter elements which include not only the -10 ad -35 hexamers which are recognized by the σ^{70} subunit of RNAP, but also upstream and downstream sequences [98-100].

In addition, it was recognized in *E. coli* and *B. subtilis* that RNAP protects regions of both upstream and downstream of -10 and -35 hexamers in the footprint [101-103]. The *in vitro* transcriptional studies indicate binding of RNAP accelerated by A+T rich upstream regions of -35 hexamer [104]. UP elements, components located upstream of -35 help in transcription by increasing the binding affinity of RNAP α-subunit [105]. UP elements are not highly conserved as -10 and -35 elements [106]. However, A+T rich regions were identified as prominent features of a subset of *E. coli*, *B. subtilis* and *Clostridium* promoters. Gorse *et al.* reported a study based on the comparison of consensus UP sequence. The study reveals that there

are several bases upstream of promoter function as UP elements which have different effect on varied promoters [107].

An UP element was identified upstream of -35 region in the bacilysin promoter. The UP element (-54 TATTTTCAAATAAT -41) of bacilysin shares nine out of 10 nucleotides to the reported consensus [107], and overlaps the DegU binding site. Apart from the hexamers and the UP element, the ribosome binding site (RBS) of the bacilysin promoter is not well conserved. It is mainly composed of T+G bases, whereas adenine bases are commonly found in RBS, however, in *B. subtilis* 168 RBS is well conserved. The reason for the differences in RBS between *B. subtilis* and *B. amyloliquefaciens* FZB42 is still unknown. Nevertheless, the RBS of FZB42 can be utilized and substituted by conserved bases in order to increase the production of bacilysin in the host cell.

Similarly, the promoter of *ywfH* has been analysed in this study. Essential promoter elements like -10 (TCATT) and -35 (TTAAAT) are not well conserved. It contains no conserved UP element. At the same time, the RBS of *ywfH* is highly conserved, when compared to *bacA* promoter.

4.2 Transcriptional regulators of bacilysin

The promoter of bacilysin could be envisaged as a well utilized promoter directed by several DNA binding regulators. Several studies have already revealed that bacilysin is influenced by global and metabolic regulators [80, 108]. Table.8 represents regulators reported from different studies.

Table 8: Regulators involved in bacilysin production.

Regulators	Effect on bacilysin	Reference
ywfA	+	[78]
thyA	+	[81]
opp	+	[80]
spo0A	+	[80]
spo0B	+	[80]

comQ/comX	+	[80]
comA and *phrC*	+	[80]
srfA	+	[80]
codY	-	[78]
abrB	-	[80]

However, the effects of these regulators were not studied at transcriptional level. Most of them are indirectly involved in the regulation of gene expression. For example, it has been shown that bacilysin production and ComA are linked, while it has been understood from our study that ComA is indirectly regulating bacilysin through other mediators, like DegQ [66]. The sections below will describe extensively the global and other kind of regulators involved in the production of bacilysin.

4.2.1 DegU positively regulates bacilysin and *ywfH*

DegS-DegU controls various processes that characterize the transition from the exponential to the stationary phase in *B. subtilis*. It includes induction of extracellular degradative enzymes, expression of late competence genes and down-regulation of σ^D regulon [109]. Changes in the cell environment are often sensed by bacterial two-component systems consisting of a sensor kinase and its cognate response regulator. The autophosphorylating histidine protein kinases sense the stimulus, and transfer their phosphoryl group to a conserved aspartate residue of response regulator [110]. DegU has been shown to be a master regulator of multicellular behaviour and the genes within the regulon respond in a different manner to the level of DegU~P [111-112]. In addition, It has been reported that DegU controls swarming and biofilm formation [113]. Furthermore, DegU has been associated with salinity tolerance in *B. subtilis* [114].

DegU belongs to the LuxR-FixJ family whose members have a helix-turn-helix (HTH) structure at their C-terminus [115]. DegU has been attributed to two modes of action: the phosphorylated form of DegU~P acts directly on degradative enzyme biosynthesis (*aprE* and *nprE*), whereas unphosphorylated DegU binds to the *comK* promoter enhancing transcription [116]. On the other hand, DegU is also influenced by two smaller polypeptides of 46 and 60

amino acids referred as DegQ and DegR, respectively. The expression of these genes also depends on the DegSU system [109]. It has been reported that DegQ stimulates phosphotransfer from DegU~P to DegU *in vivo* and DegR helps in stabilization of phosphorylated DegU [109, 115, 117]. In addition, *degQ* expression was shown to be regulated by the ComP-ComA two-component system. Recently, it was reported that DegU expression is controlled by three different promoters. The first is located upstream of the *degS* gene, the second promoter downstream of *degS* and third in front of *degU*. It has been shown that the third promoter of *degU* is controlled by nitrogen limitation regulators such as GlnA and TnrA [118-119].

In my case, DegU activates bacilysin expression at vegetative and transition growth stages. The experiments in this study were conducted using unphosphorylated DegU. Parallel experiments were performed with DegU phosphorylated using acetyl phosphate. The results obtained were similar in both cases. Initial experiments, like *lacZ* reporter fusion, have clearly demonstrated that in absence of DegU FZB42 failed to produce bacilysin (see Fig. 13).

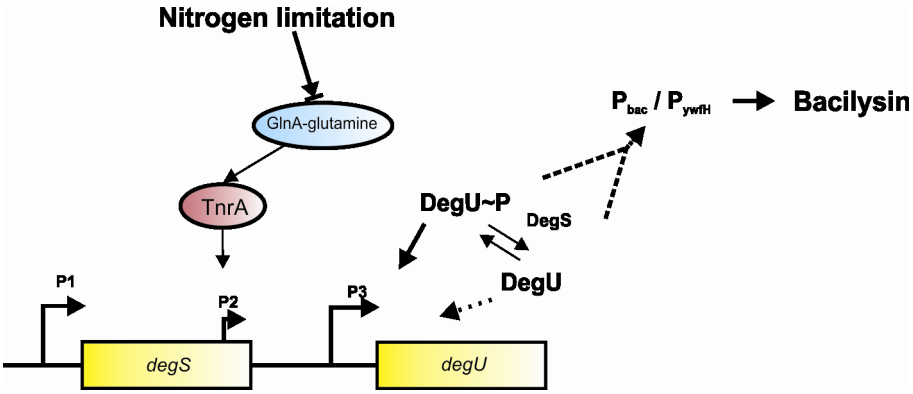


Figure 27: Schematic representation of DegU regulation on bacilysin production.

Furthermore, DNA-protein interaction studies were carried out so that the effect of DegU on p_{bac} can now be deciphered. EMSA indicated that DegU binds to the p_{bac} directly and a small quantity (0.5 µM) of protein was enough to shift the promoter fragments. The fragment used for the EMSA covered the entire promoter region (-170 to +22 relative to the transcriptional start).

DNase I protection studies revealed new insights into DegU binding sites. They revealed two important binding sites, in case of p_{bac} -90 to -61 represents site I and -30 to +22 forms site II

(see Fig.15). It agrees with several other studies of DegU footprinting [94, 113, 120]. The pattern of DegU binding to p_{bac} raised important questions regarding the mechanism of such classes of DNA binding proteins. Interestingly, despite differences in promoter structure similar binding effects of DegU were observed on p_{ywfH} and p_{bac}. Both possess two binding sites, at -64 to -15 and at -4 to +22 relative to the transcriptional start site. So the nature of DNA binding can be derived from this and previous studies of *bmyD* promoter by Koumoutsi et al. It seems that several molecules of DegU are required to activate the promoters (see Fig.28) [66].

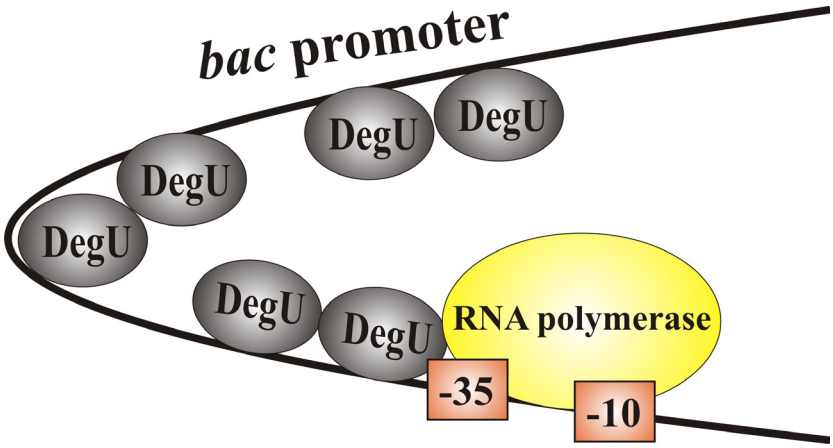


Figure 28: The interaction of DegU and RNA polymerase with the *bac* promoter.

DegU mainly prefers to bind at the A/T rich base region which was demonstrated in this thesis, but the binding of DegU to the bac promoter is weaker compared with Hpr. This could be due to DegU binding sites which are also similar in case of bacilomycin D, where DegU acts positively [66]. Nevertheless, this study indicates, how DegU might activate the bacilysin promoter. The two DegU binding sites in p_{bac} and p_{ywfH} are clues to the function of DegU. It could be possible that DegU bends the DNA and alters its structure in order to the binding of RNA polymerase, a mechanism which is common for the activation by protein regulators [88].

Apart from this regular mechanism, it could be possible that multimerisation of DegU plays a role in DegU binding at the target sequence, as it has been found in the case of *phyC* promoter reacting with the global regulator AbrB [121]. Meanwhile, a study was published about the nature of DegU~P and DegU binding, indicating that both forms prefer binding at either a

tandem repeat of a 5-nucleotide sequence (TAAAT) or an inverted repeat of (ATTTA-N7-TAAAT) [120, 122]. Such a phenomenon of DegU binding was also observed in our case (see Fig.15). However, in order to find the exact binding sites more elaborated studies, like site-directed mutagenesis and hydroxyl foot printing, have to be performed.

Finally, it was clearly demonstrated that DegU plays a major role in the expression of bacilysin by positive regulation. The initial signals perceived by DegU are still unclear. Our experiments demonstrate that unphosphorylated DegU is more efficient in *bac* promoter binding than the phoshorylated one. According to our results, unphosphorylated DegU is also controlling cellular activities, but at this stage it is not clear, whether unphosphorylated DegU acts during the vegetative growth cycle especially to control special genes, like bacilysin [123]. However, unphosphorylated DegU has been shown to directly stimulate competence through the *comK* promoter [113]. DegU has an opposing effect on the DNA uptake gene cascade, it coactivates and represses *comK* and the *srf* operon, respectively. Therefore, it inhibits *comS* which is residing inside the *srf* operon [114]. A genome-wide transcriptional profiling using the *degU* regulon (along with *degS* mutant) indicates that a delicate balance between the amounts of phosphorylated and unphosphorylated DegU is important in controlling the genes [124].

Hpr negatively regulates the *bac* promoter

Hpr encoded by *hpr* (*scoC*) is a transition state regulator of DNA binding proteins. It belongs to the MarR family of regulators referred to their control of multiple antibiotic resistance genes in Gram-negative bacteria [125]. Homologs of MarR family are distributed throughout the bacterial and archaeal domains. It has been suggested that the MarR family serves as sensors of changing environments. ScoC (Hpr) was first identified by mutations leading to increased synthesis of alkaline (*aprE*) and neutral (*nprE*) protease in *B. subtilis* [126].

Characterization of mutants and insertional activation has shown that Hpr acts as a negative regulator. It acts directly on the transcriptional level, while in case of subtilisin it can be considered as a temporal regulator indirectly involved in gene regulation. Recently, it was shown that *phoPR*, which mainly regulates the majority of the genes induced during phosphate starvation was directly controlled by Hpr at the transcriptional level [127]. In addition, investiga-

tions performed with multicopy *senS* and mutants of *salA* indicate that the product of both these genes control *hpr* (*scoC*) expression [128-129]. In most of these studies, Hpr was shown to act as negative regulator either directly or indirectly affecting gene expression.

This study is the first which is analysing the effect of Hpr on an antibiotic gene by deciphering the binding site using DNase I footprint (see Fig.16). I have demonstrated that Hpr binds to the promoter of bacilysin in order to negatively regulate the bacilysin production during the active growth stage of bacteria. It is still unclear what makes the expulsion of Hpr from the DNA recognition helix and the binding of DegU to this region. It was observed that DegU and Hpr bound at similar elements of the DNA recognition helix. The DNA binding domains of MarR proteins adopt a conserved winged helix-turn-helix. The winged helix DNA binding motif is defined topologically by secondary structure elements arranged in the following order: H1-S1-H2-H3-S2-W1-S3-W2, where “H” represents α-helix, “S” represents β-strand and “W” represents a loop [130].

However, a report suggested a possible mechanism of DNA recognition by the MarR family of regulators using molecular-dynamics-guided-mutation analysis. It was shown that the amino acid at position W20 from the N-terminal helix and R80 from helix 3 serve as a scaffold for the DNA recognition helix [131]. Here in this study of bacilysin, I have demonstrated binding of Hpr to the p_{bac} promoter at three different sites. It was not clear, why Hpr footprinting indicated three distinct binding sites, while the study on the protease promoters footprints indicate two binding sites for Hpr [6]. We postulate that Hpr belongs to the family of winged helix-turn-helix proteins acting due to the nature of available cognate DNA binding motif. The sites on the p_{bac} promoter which were protected by Hpr covered nucleotide from -60 to +47 relative to the transcriptional start site. It protects important promoter elements, like -35 and -10. DegU and Hpr share common elements of DNA binding motifs. Therefore, the interaction between Hpr and DegU is essential for the modulation of bacilysin expression.

4.3 Indirect control mechanisms of bacilysin production

Several global regulators were found to indirectly influence bacilysin production at transcriptional level. These regulators are discussed below.

4.3.1 ComA positively controls the expression of *bac* genes through DegQ

ComP-ComA belongs to the family of two-component systems activated by cell-density signals [132]. It is known to control several central developmental processes in the cell. The phosphorylated ComA activates the *srfA* operon, a first step towards the competence development [93]. *srfA* encodes an enzyme complex catalyzing the synthesis of the lipopeptide antibiotic, surfactin along with the competence regulatory gene *comS* which lies within the *srfAB* gene but out of frame. In addition, ComA stimulates the expression of *degQ, rapA* and *rapC* [133]. Moreover, ComA destabilizes the MecA-ClpC/ComK ternary complex by releasing ComK which is degraded subsequently [134].

We have found in our study that ComA influenced bacilysin production in a positive manner. The *lacZ* reporter fusion studies indicated that the activity of the β-galactosidase is completely reduced in the absene of ComA , implying that ComA is strictly controlling bacilysin biosynthesis in *B. amyloliquefaciens* FZB42. However, we have not studied the DNA-protein interaction, because ComA mainly acts indirectly [66]. It was shown in this study that DegU bound to the promoter of p_{bac} at -91 to +22. Therefore it is not possible that many regulators are involved at the same time in gene expression due to space constraint. Simultaneously, ComA regulates DegQ which in turn regulates DegU. So, ComA exerts its effect mainly *via degQ* expression [7]. In addition, the consensus for *comA* promoter has already been identified. It consists of a palindromic sequence, named ComA box, i.e. TTGCGG-N_4-CCGCAA [135]. Searching for consensus in p_{bac} does not yield any of such motifs within the promoter sequence corroborating our postulation that ComA acts indirectly.

4.3.2 AbrB negatively controls *bac* gene through expression of Hpr

The suboptimal environmental conditions of bacteria push them into different lifestyles. One of such transitions takes place with the help of global regulator proteins which are controlling gene expression during the exponential growth stage. The transition state protein AbrB is one of the global regulators controlling several genes that commence expression at the end of vegetative growth and the onset of stationary phase [136-138]. The range of genes includes biofilm formation, antibiotic production motility, expression of degradative enzymes, DNA up-

take genes, competence and sporulation. More than 20 genes have been attributed harbouring specific binding sites for AbrB [139-140]. However, the *hpr* promoter did not reveal any sequence that can be assigned to an AbrB conserved binding site [141]. AbrB is a DNA binding protein of 10.4 kDa composed of two domains. The stable DNA binding site of AbrB is formed through two N-terminal domains and a dimer of the C-terminal region [142-143]. The N-terminal region of AbrB forming a single binding domain is referred as a swapped-hairpin barrel. Furthermore, AbrB orthologs and paralogs have been found in genomic sequences of all *Bacillus*, *Clostridium* and *Listeria* species, and AbrB have been shown to be involved in induction of virulence factors in *B. cereus* and *B. anthracis* [144].

At the onset of sporulation and stationary phase, many genes that are under the control of AbrB are relieved through the action of phosphorylated Spo0A [145] The phosphorylation of Spo0A is achieved through a multicomponent phosphorelay involving five histidine autokinases (KinA, KinB, KinC, KinD and KinE) and two phosphorelay proteins Spo0B and Spo0F [146]. Once the concentration of Spo0A increases, the amount of AbrB depletes through the negative regulation of the former protein.

Surprisingly, we found that AbrB positively regulates the *hpr* promoter by direct binding. The *lacZ* reporter fusion study has revealed that AbrB is a positive regulator of Hpr. The promoter fragment, similar to that used for *lacZ* fusion, was used in EMSA assay. Experiments revealed direct binding of AbrB. Since we knew from earlier studies that AbrB binds to a large promoter region (Neubauer pers. communication), I also analysed the promoter region by subjecting two smaller DNA promoter fragments to EMSA. Surprisingly, both of the fragments showed affinity to AbrB.

Based on these results, I performed DNase I footprint for the two shorter fragments, however, it did not reveal any AbrB binding sites using this method. To solve the problem, I have used the whole fragment (see Fig. 23). In this case binding of AbrB was observed. On the other hand, AbrB bound to the whole region without specifying any consensus sequence. Although, the protein has been studied extensively both biochemically and genetically, it is still unclear, how AbrB binds to the target by selecting binding sites on DNA. To date, examination of more than 60 chromosomal sites of AbrB binding has failed to decipher the consensus se-

quence. Alternatively, it has been hypothesized that AbrB requires a specific three-dimensional structure of DNA for binding [142, 147].

Recently, a nuclear magnetic resonance (NMR) study of AbrB yielded a new insight into the complex between the N-terminus of AbrB and the DNA-binding sequence. The flexibility of loop regions LP1and LP2 are considered to be important for the binding of AbrB to various DNA targets which are thermodynamically favourable [148-149]. Similarly, in our study we demonstrated direct binding of AbrB to the *hpr* promoter. Binding occurred at the entire promoter and also in the adjacent upstream sequence of the nearby gene *yhaH*. It might be possible that an AbrB monomer initially bound somewhere at the promoter region and is initiating formation of a long stretch of multiple proteins covering large parts of the promoter and its adjacent regions.

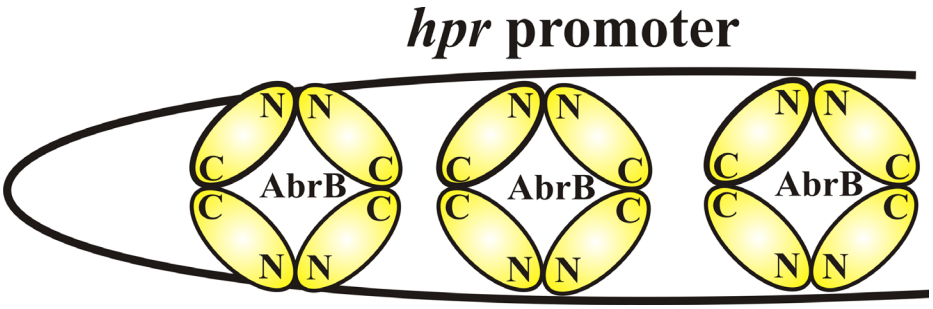


Figure 29: Model AbrB binding to the *hpr* promoter. Several molecules of AbrB are necessary in order to bind the whole promoter region.

Distribution of AbrB binding sites *in vivo* over the entire *B. subtilis* chromosome has been reported by Ishikawa *et al*. [150]. The study includes not only AbrB, but also its paralog, Abh, whose overall identity is 58% to AbrB. It indicates several new insights into the genes controlled by AbrB on a global scale and also its relation towards Abh. It was shown that almost all Abh-binding sites overlapped with the sites for AbrB. Further, *in vivo* cross-linking indicated the existence of AbrB-Abh complexes in wild type cells. Consequently, the Abh would form monomers, when the cellular levels of AbrB decrease. At this stage, AbrB and its ortho- and paralogs need more studies in order to assign their role in the bacterial cell.

4.4 Comparing the regulation of bacilysin and bacillomycin D in FZB42

Bacilysin and Bacillomycin D are synthesized at different growth stages of FZB42. Both are nonribosomally synthesized antimicrobial peptides, but synthesis of bacilysin is Sfp inde-

pendent. Bacilysin consists of two amino acids which are synthesized through few reaction steps involving reductase and ligase activities, whereas bacillomycin D is synthesized using the multicarrier thiotemplate model. A search for similarity on nucleotide level did not yield any conserved promoter elements between these two promoters. However both *bmy D* (bacillomycin D) and *bacA* promoters utilize sigma A (σ^A) dependent RNA polymerase for the transcription. At the level of transcription both promoters require the common global regulator DegU. For full activation DegU prefers A/T rich bases in both promoters.

The DegU has two forms of activity i.e. phosphorylated and unphosphorylated. It was shown in case of bacillomycin D that the phosphorylated form of DegU has a higher affinity to the bacillomycin D promoter region. In case of bacilysin, both forms of DegU were investigated and I found that the unphosphorylated form of DegU has higher affinity to both, the *bac* and the *ywfH* promoter. The reason for such differences could be that syntheses of these peptides occur at different growth stages. Bacillomycin D is synthesized during the stationary phase of FZB42. During this stage, the phosphorylated form of DegU is predominant and the phosphorylation state is maintained through DegQ and DegR. In the case of bacilysin the unphosphorylated form of DegU is predominant which is synthesized during the active growth stage. It has a stronger activity compared to the phosphorylated form. The mechanism by which DegU acts on its DNA target is similar for the promoters of both *bacA* and *bmyD*. It is characterized by bending of DNA, creating local conformation changes in order to support RNA polymerase. This idea is based on the hypersensitive bands which are observed in DNase I footprinting.

Finally, ComA, a competence stimulating factor, affects both *bacA* and *bmyD*. While DegU binds directly to these promoters, ComA affects indirectly *via* DegQ. This assumption was supported by the absence of ComA boxes in these promoters. However, in case of DegQ there was an evidence for the presence of such ComA boxes occurring in *B. subtilis* as well as in *B. amyloliquefaciens* FZB42.

4.5 Summary of the control of bacilysin synthesis in FZB42

In this work, it was demonstrated that the expression of bacilysin genes is influenced by several transcriptional regulators such as DegU, Hpr, ComA and AbrB. In order to understand the

competition between Hpr and DegU, I tried to perform some competition studies involving both DegU and Hpr indicating that DegU bound to the *bac* promoter sequence more intensely than Hpr. Since I performed footprint studies (see Fig.15 and Fig.16), it was clearly understood that the arrangement of binding sites on the *bac* promoter may induce a space competition between these regulators. The DegU protein was identified to contain three binding sites within the *bac* promoter which clearly indicates that binding of a single DegU molecule might induce the recruitment of many DegU proteins to the promoter, so that it creates a space competition with Hpr, eliminating this negative regulator. However more detailed studies have to be carried out.

There are several regulators mediating expression of the bacilysin dipeptide indirectly and some of them were found influence either DegU or Hpr. We propose here an overall scheme describing our present knowledge of the control of bacilysin production (see Fig.30). Initial signals were perceived under nitrogen limitation and mediated by CodY and TnrA/ GlnR whose functions are to control several metabolic genes [151-152]. Recently, it was shown that GlnA mediates DegU synthesis *via* TnrA [66, 118-119]. In our study we have demonstrated that DegU is a major factor in regulating *bac* genes expression. Furthermore, I searched for CodY and TnrA/ GlnR binding sites on the promoter of the *com-QXPA* operon. It revealed the presence of binding motifs to the regulators. Expression of this operon is essential for the competence development and synthesis of surfactin through ComA [93]. We have also shown here that ComA is essential for biosynthesis of bacilysin.

A similar search for binding motifs of AbrB within *hpr* promoter revealed the presence of such motifs. We confirmed binding of Hpr by performing DNA-protein interaction studies (data not shown). So, we propose that AbrB mediates the expression of *hpr* gene, which in turn controls the bacilysin production. Then, activation of Spo0A negatively controls AbrB, relieving its effect on the target genes which are under the repressive effect of this global regulator [153]. Hpr which is under the positive control of AbrB is now lowered in the cell, and DegU can fully activate the bacilysin promoter. Unfortunately, the data obtained from HPLC analysis of the *hpr* mutant (AM10) did not indicate a higher production of bacilysin. While Hpr negatively controls several steps of bacilysin synthesis catalysed by the *bac* genes, the

reaction catalysed by the *ywfH* gene product remains still a bottle neck in synthesizing the mature bacilysin dipeptide, since expression of that gene is not relieved by removing Hpr.

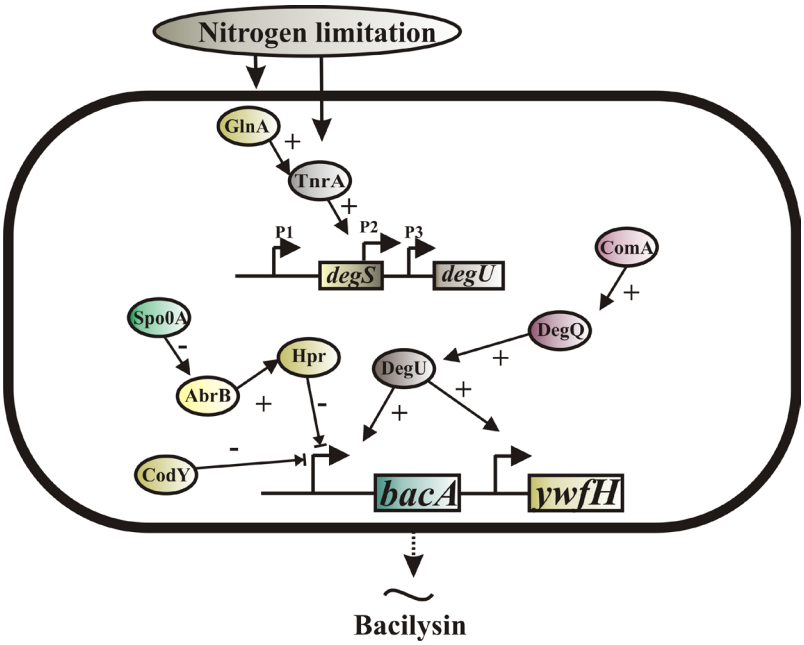


Figure 30: Regulatory network of bacilysin production. Several global regulators are directly or indirectly regulating bacilysin. However, DegU and Hpr are directly acting on the *bacA* promoter.

Consequently, our bacilysin study opened some new details concerning the overall regulation of an antibiotic synthesis. The key mechanisms that control the bacilysin biosynthesis by global protein regulators were studied. In the future, using these results the production of bacilysin in *B. amyloliquefaciens* FZB42 can be increased. A strain with increased antibiotic activity might act as a good candidate for PGPR

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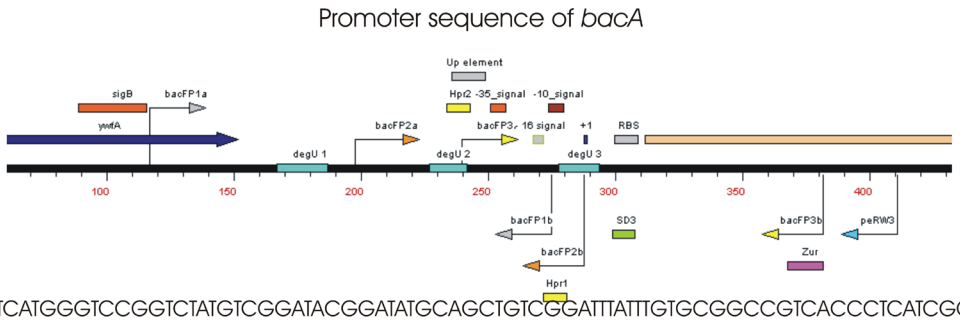
6 Appendix

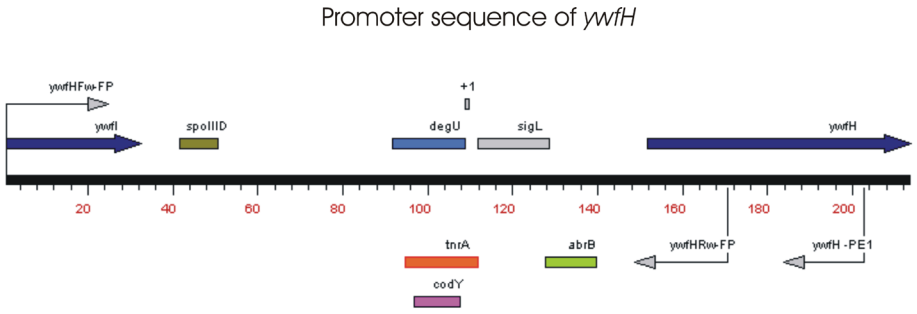
6.1 Promoter sequence of *bacA* promoter



5'TGTCATGGGTCCGGTCTATGTCGGATACGGATATGCAGCTGTCGGATTTATTTGTGCGGCCGTCACCCTCATCGGCTT
TGTCCTCAGCGTTTTCGCATACAGAAGGTATGGGAAATATGAGCATCAGGCCGACCAAACATTGTATCGGTAAAATAAA
GTTTCTATAATTTTATAAATGTTAATTTTAACACCCTATGTCATAGGGTGTTTTCTAATATATAGGAATAATTATCAATATTTTCAAATAATAT
TGACAGCTTGAACATCTATGGATAATATTTACTTAAAATTTAACAGATTGGTTGGTGCTCATGATTATATTGGATAATAGCATTCAA
ACAAAAAGCAAGGCTTATTCAATATCTAAACTGATTACGATCAACACGCTCGGTCCTGAAGGGACAAGTAGTGAGTACG
CAGCAAAAAACTTCATCACCAATTTTACCCTTCTCCAAGGGGTGAACAGCAAATTATCACTGCATGATACATTTGAATCGT
GCAAAGCTTATATAAAT3'

Figure 31: Sequence of the *bacA* promoter used in this study is given above. The primers used for footprint are indicated. Different regulators acting on the promoter were also indicated.

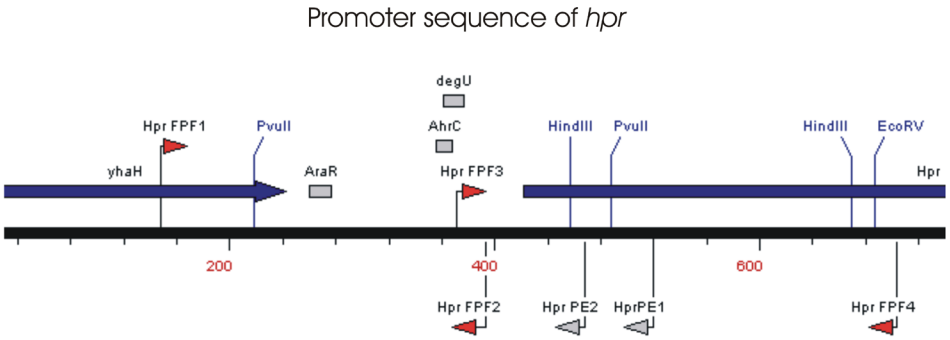
6.2 Promoter sequence of *ywfH* promoter



5'TCGACACGCTTCCGAAGTTTTTGCACGTGTAACACCCACCCGCCTGTCCGGCGGGTTTTTCCTTTGTCAGAATTTAAAT
AAAATTTGCTATTTTTATCATTTTTTTTTACGATGTAAAGGAGATTCCATTTTGGTAAAGGGGTGCAGACAGTTGTCAAAACGAAC
CGCGTTTATTATGGGAGCCAGTCAGGGGATCGGCAAAGCGATCGCTCT3'

Figure 32: Represents the entire promoter region used for the study of *ywfH* gene. Primers used for footprint were indicated.

6.3 Promoter sequence of *hpr* promoter



5'GGAGGAAATCAAACCGCACCAGCAGGATCTTCAGAAGGAAATCGCTGATATTGAAGAGAAAATCAGACAG
CTGGAGAAAACCTTACAAAACTAATATCCTATACAACCAAAAAAAAGTGCGGACATATTGCGTTCCGCACTTTTTTTT
GCGGTTTAAAGCCTTTCCCAGCAACGGATGAAGAATTTATTATAAAATTCTTAAATTTTCATCTTTATTAATCTTTATTTTATTG
GCATAATAGAGGAAAAGAAAATGTGAAGCAGGTGATCTGTATTGAATCGTGTTGAACCGCCTTACGATCTAAAAG
AAGCTTTAATCTATACCCAAAAAATGGCGCAGCTGTCCAAAGCGCTCTGGAAATCTATCGAGAAAGACTGGCA
GAATTGGCTGAAGCCGTACGACCTTAATATTAATGAGCATCATATATTATGGATTGCCTATCAGCTTAAAGGAGCTTC
TATTTCAGAAATCGCCAAATTCGGCGTTATGCACGTATCAACTGCATTTAACTTTTCAAAAAAGCTTGAAGAACGGG
GATATCTGAAATTTTCCAA3'

Figure 33: Indicates the promoter region of *hpr* gene. The primer used for the footprint was represented as Hpr FPF.

6.4 Purification of DegU protein

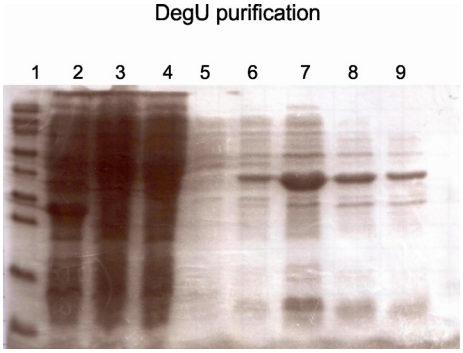


Figure 34: DegU was purified as given in materials and methods. On the SDS gel, 1 = protein marker, 2 = cell extract, 3 = flow through, 4 = wash1, 5 = wash 2, 6 = elution 1, 7 = elution 2, 8 = elution 3, 9 = elution 4. The elution indicates extraction of protein from the column (see Materials and Methods) using elution buffer containing imidazole.

6.5 Purification of Hpr protein

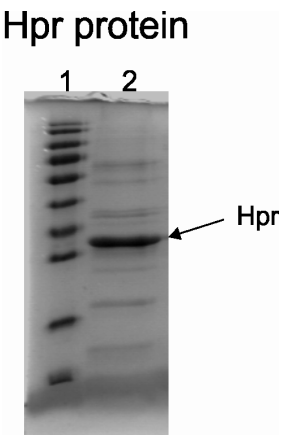


Figure 35: Hpr is a 27 kDa protein. The SDS gel represents the purified Hpr. 1= protein marker, 2 = Hpr protein eluted using elution buffer (see Materials and Methods).

6.6 Comparison of P_{bac} in FZB42, *B. subtilis* 168, and DSM 7

Multiple sequence alignment of *bacA* promoter

SeqA	Name	Length	SeqB	Name	Length	Score
1	FZB42	161	2	DSM7	305	91.0
1	FZB42	161	3	B.subtilis 168	154	81.0
2	DSM7	305	3	B.subtilis 168	154	80.0

CLUSTAL 2.1 Multiple sequence alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: FZB42 161 bp
Sequence 2: DSM7 305 bp
Sequence 3: *B.subtilis* 168 154 bp
Start of pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 91
Sequences (1:3) Aligned. Score: 81
Sequences (2:3) Aligned. Score: 80
Guide tree file created: [clustalw2-I20110209-160427-0832-42372623.dnd]

There are 2 groups
Start of Multiple Alignment

Figure 36: The above data clearly indicate the identity of the p_{bac} promoter in three different closely related strains. FZB42 and *B. subtilis* 168 are rhizosphere colonizers, whereas DSM 7 has no such attributes. However, the *bacA* promoter sequence has higher similarity with *bacA* of DSM 7, indicating its close relationship with FZB42.

```
CLUSTAL 2.1 multiple sequence alignment


FZB42           -AATAAAGTTTCTATAATTTTATAAATGTTAATTTT-AACACCCTATGTCATAGGGTGTT 58
DSM7            AAATAAAGTTTCTATAATTTTGTAAATGTTAATTTTTAACACCTTATCTCATAAGGTGTT 60
B.subtilis 168  -AACAAAGTTTCTAAATTCCTATAA---------TTAAAAACCCT-TTTTAAAGGGTTTT 49
                 ** ********** * *  * ***         ** ** *** * * * * * *** **

FZB42           TTCTAATATATAGGAATAATTATCAATATTTTCAAATAATATTGACAGCTTGAACATCTA 118
DSM7            TTCTATTATATAGGAATAATTATCAATATTTTCAAGTAATATTGACAGTTTGAATTTCTA 120
B.subtilis 168  TTTGTTTGTATGGGAATAATTATCAATATTTTCAAAAAACATTGACAGTTC-AATTTCCA 108
                **    * *** **********************  ** ******** *  **  ** *

FZB42           -TGGATAATATTTACTTAAAATTT-AACAGATTGGT----------------------TGG 155
DSM7            -TGGATAATATTTACTTAAAATTT-AACAGATTGGTGCTCATGATTATATTGGATAATAG 178
B.subtilis 168  CTGGATAAAATTTACTTAAAATTTTAAAAGATTGGT----------------------TGG 147
                 ******* *************** ** ********                    * *

FZB42           TGCTCA------------------------------------------------------ 161
DSM7            CATTCAGACGAAAAGCAAGGCTTATTCAATATCTAAACTGATTAGATCAACACGCTCGGT 238
B.subtilis 168  TGCTCAT----------------------------------------------------- 154
                   ***

FZB42           ------------------------------------------------------------
DSM7            CCTGAAGGGACTAGCAGTGAGTAAGCAGCAAAAAACTTCATCACCAATTTTACCCTCCTC 298
B.subtilis 168  ------------------------------------------------------------


FZB42           -------
DSM7            CAAGGGG 305
B.subtilis 168  -------
```

Figure 37: Multiple sequence alignment of three closely related strains. From the figure, the first 20 bases were shown to be involved in DegU binding and it can be clearly seen there that *B. subtilis* lacks the conserved domains for such binding. So, FZB42 efficiently controls the synthesis of bacilysin. However, the other promoter elements like -35 and -10 are well conserved among these strains. On the other hand, the SD sequences are completely different among these *Bacillus* strains.

6.7 Comparison of P_{ywfH} in FZB42, *B. subtilis* 168, and DSM 7

Multiple sequence alignment of *ywfH* promoter

SeqA	Name	Length	SeqB	Name	Length	Score
1	FZB42	129	2	DSM7	544	90.0
1	FZB42	129	3	B.subtilis 168	143	84.0
2	DSM7	544	3	B.subtilis 168	143	78.0

CLUSTAL 2.1 Multiple sequence alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: FZB42 129 bp
Sequence 2: DSM7 544 bp
Sequence 3: *B.subtilis* 168 143 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 90
Sequences (1:3) Aligned. Score: 84
Sequences (2:3) Aligned. Score: 78
Guide tree file created: [clustalw2-I20110209-163302-0172-47574629.dnd]

There are 2 groups
Start of multiple alignment

Aligning...
Group 1: Sequences: 2 Score:2230
Group 2: Sequences: 3 Score:2044

Figure 38: The alignment score reveals the distance between the strains for the *ywfH* promoter. FZB42 and DSM 7 have similarity in case of *ywfH,* whereas, the similarity between FZB42 and *B. subtilis* 168 was not very high.

```
CLUSTAL 2.1 multiple sequence alignment


FZB42             ------------------------------------------------------------
DSM7              AAAACTTTTTTATGATTTCATTTGTTTTTTTCATCATTATATCAGGAGCTTTCATTGCAC 60
B.subtilis 168    ------------------------------------------------------------


FZB42             ------------------------------------------------------------
DSM7              TTTCAGTATTTACTCAAACAAAATTACCTTCAATAACTTTTTCTACATTAACGCTTTCAA 120
B.subtilis 168    ------------------------------------------------------------


FZB42             ------------------------------------------------------------
DSM7              TTATAAGCTATGTTCTATGGGCCACCTCCCCATCATTACCATTAAAAAGAGATGAACGAG 180
B.subtilis 168    ------------------------------------------------------------


FZB42             ------------------------------------------------------------
DSM7              GAAAGTTTATTATACAGAAGAGCTTGGCTCATACTGGTATGCTGACTTTCATTTATCTTT 240
B.subtilis 168    ------------------------------------------------------------


FZB42             ------------------------------------------------------------
DSM7              TTATTTTCTTGACTATATTTTATTTTAAATTAATCAACCTGGACAAAGAACAAATTTTAC 300
B.subtilis 168    ------------------------------------------------------------


FZB42             ------------------------------------------------------------
DSM7              TCCTATTTTCCTCGCTAATTATAAGTACAGCGTTTTTATCAATCCTCATTCTGAATAAGA 360
B.subtilis 168    ------------------------------------------------------------


FZB42             ------------------------------------------------------------T 1
DSM7              AATACTAACAATTAATAATTAGAAACCTTCATTATAATTCCAATCTAATGATATTAATCG 420
B.subtilis 168    ------------------------------GTATAAT---------------------- 7


FZB42             AACACCCACCCGCC-TGTCCGGCGGG--TTTTTCC-------TTTGTCAGAATTTAAATA 51
DSM7              AACACCC-CCCGCC-TCTTTGGCGGG--TTTT-CA-------TTTGTCAGAATTTAAATA 468
B.subtilis 168    -ATCCCCTCCCGCCCTATCCGGCGGGAGTTTTTCAATTCTCCTTTATCAGAATTTAAATA 66
                   *  *** ****** * *  ******  **** *        *** **************

FZB42             AAATTTGCTATTTTTATCATTTTTTTTTACGATGTAAAGGAGATTCCATTTTGGT-AAAG 110
DSM7              AAATTTGCTATTTTCGTCATTTTTTTTTACGATGTAAAAGAGATTCCATTTTGGT-AAAG 527
B.subtilis 168    AAATTTGCTATTTTCTTCGTTTTTTCTTACGATGTAGATAATAATCCATTTTTTTCAAAG 126
                  **************  ** ****** ********** *  * * ********  * ****

FZB42             GGGTGCAGACAGTTGTCAA 129
DSM7              GGGTGCAGACAGTTGTC-- 544
B.subtilis 168    GGGTGCAGTTATTTGTC-- 143
                  ********  * *****
```

Figure 39: Most of the essential promoter elements of *ywfH* gene are well conserved among these strains. However, the promoter of DSM 7 is larger compared to FZB42 and *B.subtilis* 168.

6.8 Comparison of P_{hpr} in FZB42, *B. subtilis* 168, and DSM 7

Multiple sequence alignment of *hpr* promoter

SeqA	Name	Length	SeqB	Name	Length	Score
1	FZB42	199	2	DSM7	202	95.0
1	FZB42	199	3	B.subtilis 168	196	81.0
2	DSM7	202	3	B.subtilis 168	196	82.0

CLUSTAL 2.1 Multiple sequence alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: FZB42 199 bp
Sequence 2: DSM7 202 bp
Sequence 3: *B.subtilis* 168 196 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 95
Sequences (1:3) Aligned. Score: 81
Sequences (2:3) Aligned. Score: 82
Guide tree file created: [clustalw2-I20110209-172633-0965-19021713.dnd]

There are 2 groups
Start of multiple alignment

Figure 40: Similarity scores of the different *Bacillus* strains indicate that the promoter of *hpr* is closely related between FZB42 and DSM7.

```
FZB42           -ACAAAACTAATATCCTATACAACCAAAAAAAAGTGCGGAC--ATATTGCGTTCCGCACT 57
DSM7            TACAAAACTAATATCCTATACAACCAAAAAAA-GTGCGGAC--ATATTGCGTTCCGCACT 57
B.subtilis168   -----AACTAATATCCTATTCAAA-AGAAAAAAATGCGGGCCAAAATTG-GACCCGTATT 53
                     ************** ***  * *****  ***** *  * **** *  *** * *

FZB42           TTTTTTTGCG-GTTTAAAGCCTTTCCCAGCAACGGATGAAGAATTTATTATAAAATTCTT 116
DSM7            TTTTTT-GCGTGTTTAAAACCTTTCACAGCAATGGATTAAGAATTTATTATAAAATTCTT 116
B.subtilis168   TTTTTTGGCCGA--AAAACCCTTTCGTCGCAATGGTTTGTGAATTTATTAAAAAATTCTT 111
                ****** **      *** ******   **** ** *   ********** *********

FZB42           AAATTTTCATCTTTATTAATCTTTATTTTATTGGCATAATAGAGGAAAAGAAAATGTGAA 176
DSM7            AAATTTTCATCTTTATTAATCTTTATTTTATTGGCATAATAGAGGGAAAGAAAAGGTGAA 176
B.subtilis168   ACATTTTCATCTTTATTAATCTTTATTTTATTGGCATAATAGAGAAAAAGAAAAAGAGAA 171
                * ****************************************  ******** * ***

FZB42           GCAGGTGATCTGTATTGAATCGT---- 199
DSM7            GCAGGTGATCTGTATTGAATCGTGTT- 202
B.subtilis168   GCAGGTGAC--GTAATGAATCGAGTGG 196
                ********   *** *******
```

Figure 41: Sequence comparison indicates the conserved nature of promoter elements between the closely related strains of *Bacillus*. Essential promoter elements like -35 and -10 are highly conserved.

Selbständigkeitserklärung

Hiermit versichere ich, die vorliegende Dissertatoin selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben.

Berlin,

Aruljothi Mariappan

Veröffentlichungen

- **Aruljothi Mariappan**, Xiao-Hua Chen, Oliwia Makarewicz Alexandra Koumoutsi and Rainer Borriss. *Two- component response regulator DegU controls the expression of bacilysin in PGPR - Bacillus amyloliquefaciens FZB42,* Submitted for publication.

- **Aruljothi Mariappan,** Oliwia Makarewicz and Rainer Borriss. *AbrB positively regulates hpr (scoC) by directly binding to the hpr promoter of Bacillus amyloliquefaciens FZB42.* Manuscript in Preparation.

- Wukun Liu, Kerstin Bensdorf, Ulrich Abram, Ben Niu**, Aruljothi Mariappan**, Ronald Gust. *Synthesis and biological studies of silver N-heterocyclic carbene complexes derived from 4,5-diarylimidazole*. Eur J Med Chem. 2011 Dec; 46(12):5927-34.

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