İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

THE EFFECT OF GLOBAL CONTROL ELEMENTS ON THE EXPRESSION OF *ywfH* GENE IN *B.subtilis*

M.Sc. Thesis by
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Department : Advanced Technologies

Programme : Molecular Biology-Genetics&Biotechnology

JUNE 2009

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JUNE 2009

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

GLOBAL KONTROL ELEMANLARININ *B. subtilis'TE YER ALAN ywfH* GENİNİN İFADESİ ÜZERİNDEKİ ETKİLERİ

YÜKSEK LİSANS TEZİ

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FOREWORD

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June 2009

Orkun PİNAR
Engineer, MSc.

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ABBREVIATIONS

AHL : N-achylhomoserine Lactone
Amp : Ampicillin
bp : Base pair
Cat : Chloramphenicol
CSF : Competence and Sporulation Factor
dH_2O : Distilled water
DNA : Deoxyribonucleic acid
DSM : Difco's Sporulation Medium
EDTA : Ethylenediaminetetraacetic acid
Erm : Erythromycin
EtBr : Ethidium bromide
Kan : Kanamycin
kb : Kilobase
Ln : Lincomycin
µl : Microliter
LB broth : Luria Bertani broth
OD : Optical density
ONPG : 2-Nitrophyl β-D-galacto pyranoside
PA : Perry and Abraham Medium
PCR : Polymerase Chain Reaction
QS : Quorum Sensing
Rpm : Revolution per minute
Spc : Spectinomycin
TAE : Tris acetate EDTA
Tris : Hydroxymethyl aminomethane

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THE EFFECT OF GLOBAL CONTROL ELEMENTS ON THE EXPRESSION OF *ywfH* GENE IN *B. subtilis*

SUMMARY

In *Bacillus subtilis* species, sporulation, genetic competence, and antibiotic production are controlled by quorum-sensing global regulatory mechanism. Bacilysin is a simple and small sized (125kDa) dipeptide antibiotic which is produced extracellularly by certain species of *Bacillus subtilis* that consists of L-alanine residue at N-terminus and non proteinogenic L-anticapsin residue at C terminus which is produced extracellularly by certain species of *Bacillus subtilis.* In previous studies, we showed that the biosynthesis of bacilysin is under the control of quorum sensing global regulatory pathway through the action of ComQ/ComX, PhrC (CSF), ComP/ComA in a Spo0K (Opp)-dependent manner. Recently, It was found that a polycistronic operon (*ywfBCDEFG*) and a monocistronic gene (*ywfH*) are required for the biosynthesis of bacilysin in *Bacillus subtilis*. The main purpose of the present study is to monitor the effects of previously-identified genes *srfA, oppA, comA, phrC, phrF, phrK, comQ* (*comX*), *comP*, *spo0H*, *spo0A*, *abrB, codY, degU and sigB* on the expression of *ywfH* gene. Firstly, to analyze *ywfH* expression, a *B. subtilis* strain, NAO1, containing *ywfH-lacZ* fusion at the *ywfH* gene region was constructed. Then, each of the above-mentioned genes of cell density signaling was insertionally inactivated or deleted in NAO1. The resulting mutant strains and NAO1 as control were cultured in PA medium at 37^{o}C and *ywfH*- directed β-galactosidase activities were monitored.

Mutations in *codY, comP, comA, comQ(comX), phrC, phrK ,phrF, srfA, spo0H, and spo0A* genes completely abolished *ywfH-lacZ* expression. Moreover, *abrB* null mutation gradually relieved the repression of *ywfH* during exponential phase. Complete inhibition of *ywfH* expression in Δ*spo0A* strain was not restored by *abrB* mutation. On the other hand, *ywfH* expression severely decreased but did not become completely eliminated during the stationary phase in codY mutant strain. However, *abrB-codY* double mutations resulted in an increase in *ywfH* expression in exponential phase. In this study, we also found that *ywfH* expression is subject to nutritional repression mediated by Casamino acids. The effect of a transition regulator gene d*egU* and general stress control element *sigB* gene on the expression of *ywfH gene* were also investigated and we found that *ywfH* expression is positively regulated by DegU. On the other hand, *sigB* deletion mutation didn't cause a considerable difference in *ywfH* expression.

GLOBAL KONTROL ELEMANLARININ *B. subtilis'TE YER ALAN ywfH* GENİNİN İFADESİ ÜZERİNDEKİ ETKİLERİ

ÖZET

Bacillus subtilis türlerinde, sporulasyon, genetik kompetans ve antibiyotik üretimi, hücre yoğunluğu sinyal mekanizması ile kontrol edilmektedir. Basit ve küçük boyutlu bir dipeptit antibiyotik olan bacilisin, L-alanin ve L-antikapsinden ibaret olup, belli başlı bazı *Bacillus subtilis* türleri tarafından ekstrasellüler olarak üretilmektedir. Basilisin biyosentezinin, ComQ/ComX, PhrC (CSF), ComP/ComA'nın etkisinde hücre yogunluğu sinyali mekanizmasının kontrolü altında olduğunu ve bunun Spo0K (Opp)'ye bağlı bir biçimde gerçekleştiğini daha önceki çalışmalarımızda göstermiştik. Yakın zamanda, basilisin biyosentezi için *bacABCDE* olarak yeniden isimlendirilen *ywfBCDEFG* operonuna ve *ywfH* genine ihtiyaç duyulduğu belirtilmiştir.

Şu an yapılmış olan çalışmamızın ana amacı, daha önceden tanımlanmış olan *srfA, oppA, comA, phrC, phrF, phrK, comQ* (*comX*), *comP, spo0H, spo0A, abrB, codY, degU and sigB* genlerinin, *ywfH* geninin ifadesi üzerindeki etkisini göstermektir. Bu amaçla, *ywfH* gen bölgesinde *ywfH-lacZ* füzyonu içeren bir *Bacillus subtilis* türüne ait NAO1 suşu oluşturulmuştur. Yukarıda belirtilmiş olan hücre yoğunluğu sinyal mekanizması genleri, NAO1suşu içerisinde inaktif edilmiştir. Oluşan mutant suşlar ve kontrol olarak NAO1 suşu, 37^{o}C'de PA ortamında kültür edilmiş ve *ywfH*'a bağlı β-galaktosidaz aktiviteleri gösterilmiştir.

codY, comP, comA, comQ(comX), phrC, phrK ,phrF, srfA, spo0H, and spo0A genlerinde meydana gelen mutasyonlar *ywfH-lacZ* ifadesini tamamen ortadan kaldırmıştır. *abrB* mutasyonu, *ywfH* geninin büyüme fazındaki baskılanmasını kademeli olarak azaltmıştır. Durağan faz boyunca, Δ*spo0A* mutant suşunda tamamen engellenen *ywfH* gen ifadesi *abrB* mutasyonuna rağmen eski haline getirilememiştir.

Bunun yanında, *codY* mutant suşunda, *ywfH* gen ifadesi durağan faz sırasında fazla miktarda azalmış fakat tamamen ortadan kalkmamıştır. Ayrıca, *abrB-codY* çift mutasyonunda, exponansiyel büyüme fazında *ywfH* gen ifadesinde artış olduğu görülmüştür. Bu çalışmada, *ywfH* gen ifadesinin kazamino asit varlığında besinsel baskılamaya da maruz kaldığı bulunmuştur. Geçiş evresi düzenleyici gen olan d*egU* ve genel stres kontrol elemanı olan *sigB* geninin *ywfH* geni üzerindeki etkisi araştırılmış ve DegU ürününün *ywfH* geninin ekpresyonunun düzenlenmesinde pozitif rol oynadığı bulunmuştur. Öte yandan *sigB* geninde meydana gelen mutasyonun, *ywfH* geninin ekpresyonu üzerinde kayda değer bir etkisinin olmadığı görülmüştür.

INTRODUCTION

1.1. *Bacillus subtilis*

B. subtilis is an aerobic, endospore-forming, rod-shaped Gram positive bacterium which has ability to secrete many secondary metabolites, as well as over two dozen of ribosomal and non-ribosomal antibiotics that have antibacterial, antifungal and antimetabolic features. *B. subtilis* are obtained especially in terrestrial and aquatic environments such as soils, plant roots, gastrointestinal system of animals and water sources.

In a situation of nutritional limitation or other environmental stresses, *Bacillus subtilis* cells create many adaptative responses in order to survive. Their highly resistant dormant endospores are also a response to these situations. Additionally, *B. subtilis* can grow under anaerobic conditions if nitrate is added as the terminal electron acceptor to the environment (Earl *et al.* , 2008, Harwood *et al.*, 1996, Glaser *et al.*, 1995, Ramos *et al.*, 1995, Stein *et al.*, 2005).

B. subtilis became a very studied model organism owing to its metabolic diversity, non-pathogenicity, ability to produce hydrolytic enzymes (e.g. alkaline proteases, amylases), polypeptide antibiotics (e.g. bacitracin), biochemicals (e.g. nucleosides for conversion to flavour enhancers) and insecticides (e.g. 8-endotoxins) especially after the sequencing of its genome (Kunst *et al.*, 1997, Harwood *et al*., 1996).

On the other hand, *B. subtilis* shows health-beneficial properties. Especially, its probiotic property, which is primarily found in spore form, helps to prevent gastrointestinal disorders. Also, it is clearly confirmed that *B. subtilis* can alternate many antibiotics by being a novel prophylactic, therapeutic and growth promoting agent (Hong *et al.*, 2004, Williams, 2007, Fujiya *et al.*, 2007).

Bacillus subtilis is a chemoorganotroph that has the ability to survive when exposed to very simple growth conditions such as simple salt medium containing glucose or other sugars as carbon and energy source, inorganic nitrogen and adequate supply of oxygen (Nicholson and Setlow, 1990).

As similar to many other members of *Bacillus* genus, *B. subtilis*, is mesophilic and can grow as normal-sized colonies within a day at 37^{o}C (Harwood *et al.*, 1990). If nutrients become limiting for optimal growth at the end of the exponential phase, *B. subtilis* develops a motility and chemotaxis system to be able to search for nutrients in order to prevent this limitation for the ultimate purpose of surviving. If there is still a limitation at the stationary phase, these cells start to secrete antibiotics and enzymes such as proteases to reach nutrients from alternative resources which are normally hard to reach. Furthermore, *B. subtilis* exhibits genetic competence development by uptaking exogenious DNA and sporulation when nutritional limitation continues. All of these properties cause *B. subtilis* to earn advantage of surviving and eliminate other bacteria for nutrient competition (Hamoen *et al.*, 2003).

Genome of *Bacillus subtilis* is 4.214.810 base pair long including 4106 protein-coding genes (Kunst *et al.*, 1997, Kobayashi and Ogasawara, 2002). Also 86 tRNA genes, 30 rRNA genes and three small stable RNA genes are annotated on 4215 kb genome beside these 4106 protein-coding genes. When the sequence was published, possible function was assigned to about 58% of all genes (2379 genes) but the number of the genes with assigned function has increased to 63% (2562 genes) in the current database. (Kobayashi and Ogasawara, 2002). On the other hand, 4% of essential genes display unknown functions (Kobayashi *et. al.*, 2003).

1.2. Quorum Sensing Mechanism As A Regulatory System of Gene Expression

Bacteria are found in nature as microbial communities more than as individuals and this situation gives them the advantage of having interactions among them and surviving in different ecological habitats (Lazdunski *et al*, 2004). Therefore, generally, species of bacteria are social organisms with intercellular communication (Ruzheinikov *et al.*, 2001). This communication consists of chemical signal molecules that gives them ability of sensing the presence of other bacteria and generally cause cooperative production for the benefit of population which is not efficient for an individual to perform by itself (von Bodman *et al.*, 2008).

When these signal molecules accumulate and a quorum (e.g. certain threshold concentration) is reached, bacteria cooperationally behave in order to survive by

using chemical signal molecules in response to environmental alterations and this process is called ‘’Quorum Sensing(QS)’’(Miller *et al.*, 2001, Gobbetti *et al.* 2007).

In QS mechanism, different signalling molecules, signal detection systems and signal transduction mechanisms are used by both Gram negative and Gram positive bacteria (Lazdunski *et al*, 2004).

Gram-negative LuxIR circuits and Gram-positive oligopeptide two-component circuits are two general types of bacterial quorum sensing mechanisms. (Taga *et al.*, 2003). In Gram-negative bacteria, signalling molecules are typically acylated homoserine lactones (AHLs) and in Gram-positive bacteria, peptides are generally the signalling molecules (Lazdunski *et al.*, 2004; Fuqua and Greenberg, 1998). There are known cases of bacterial regulation of gene expression in response to cell-cell signaling. For example, bioluminescence in *Vibrio fischeri* , plasmid conjugation in *Agrobacterium tumefaciens*, biofilm production and virulence gene expression in *Pseudomonas aeruginosa*, expression of factors necessary for symbiosis in *Sinorhizobium meliloti*, antibiotic production in *Erwinia carotovora* and *Streptomyces spp.*, conjugation in *Enterococcus faecalis*, genetic competence in *Bacillus subtilis* and *Streptococcus pneumonia* (Miller *et al.*, 2001; Taga *et al.*, 2003; Sturme *et al*., 2002; von Bodman *et al.*, 2008).

In *B. subtilis*, QS contributes different processes such as genetic competence development, sporulation, production of antibiotics and degradative enzymes (Griffith and Grossman, 2008). Also, sporulation and the development of genetic competence are stimulated as cells grow to high density. Sporulation regulation at high cell density needs several extracellular/environmental signals and this regulation is controlled certainly via a quorum sensing mechanism (Lazazzera *et al.*, 1997; Miller *et al.*, 2001).

The dependence of sporulation and competence on both an extracellular signal and an oligopeptide permease operon model suggests that after accumulation of signal peptide, this signal can move through the oligopeptide permease and function intracellularly in the target cell. In a *Bacillus* population, this signal acts as a quorum sensor and helps individuals of population to sense other cells under limited nutritional conditions or to get prepared for DNA-uptake (Solomon *et al.*, 1996; Magnuson *et al.*, 1994).

In *Bacillu subtilis* QS system, there are two peptides called ComX and CSF (competence and sporulation factor) which mediate QS control of competence and regulate the activity of the transcription factor ComA (Figure1.1) (Solomon *et al.*, 1995 and 1996; Lazazzera *et al.*, 1997). ComX and CSF are both secreted and accumulated as cell density increases (Miller *et al.*, 2001). ComX peptide, a 10 amino acids (ADIPITRQWGD) long peptide which has a hydrophobic modification on tryptophan residue that is required for signaling activity, is the major extracellular signaling peptide. ComX interacts with unmodified pentapeptides, known as Phr peptides, that are internalized to inhibit the activity of their target proteins, known as Rap proteins (Lazazzera, 2001; Perego and Brannigan, 2001; Stein, 2005).

B. subtilis encodes a family of 8 Phr peptides (PhrA, PhrC, PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK) and a family of 11 Rap proteins (RapA to RapK) (Table 1.1) Each Phr peptide is encoded in an operon with a Rap protein, and each characterized Phr inhibits the activity of its Rap protein (Auchtung *et al.*, 2006).

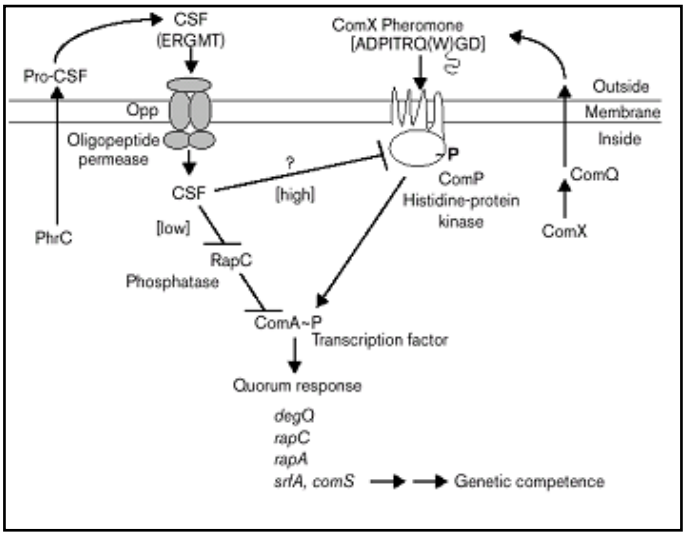


Figure 1.1: Regulation mechanism of quorum responses triggered by environmental signals and initiation of signal transduction cascade through ComX and CSF diffusible peptides (Lazzazera, 2000).

Table 1.1: Processes regulated by rap proteins and phr peptides in *B. subtilis* (Auchtung *et al*., 2006).

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

Additionally a protein called ComQ is required for production of ComX peptide but the specific role of this protein is unknown. The accumulation of ComX signal is detected by two regulators called ComP and ComA (Magnuson et al., 1994; Solomon *et al.*, 1995). In this system, firstly ComX binds to and activate a protein kinase, ComP . Then ComP donates phosphate its phosphate group to ComA, a phosphorylation-dependent response regulator transcription factor. After that, by the generation of ComA~P, the quorum response genes, *degQ*, *rapC*, *rapA* and *srfA* are activated (Figure 1.1). Nonetheless, ComA is inactive when the ComX signal peptide concentration is low and if only the signal peptide concentration increases, ComA gets activated (Magnuson *et al.*, 1994; Solomon *et al.*, 1995 and 1996; Weinrauch *et al*., 1990; Griffith and Grossman, 2008).

The expression of *degQ* is involved in the regulation of degradative enzyme synthesis. Other quorum sensing response genes *rapA* and *rapC* encode phosphatases involved in the control of competence development and sporulation. The *srfA* operon encodes a small protein ComS and the surfactin biosynthetic enzymes (Nakano *et al.*, 1991).

ComA~P activates the production of ComS protein. ComS inhibits the proteolytic degradation of a transcriptional activator, ComK protein, which drives its own transcription. After ComS transcription, ComK concentration increases in the cell quickly and structural genes of competence are expressed resulting DNA-uptake into the cell (van Sinderen *et al*. 1995; Turgay *et al*., 1997 and 1998).

There are also other peptides that stimulate ComA activity such as ComX, the CSF, PhrF, PhrK, PhrH. In addition to master extracellular signaling peptide ComX, the second key quorum sensing peptide in *Bacillus subtilis* is a competence and sporulation factor, CSF peptide, which is a diffusible pentapeptide. CSF (ERGMT) is encoded from *rapC-phrC* operon which encodes both CSF and its cytoplasmic receptor RapC. The CSF signal molecule is formed by cleavage of five amino acids at the C-terminus of the precursor peptide, PhrC. The mechanism of the CSF peptide on ComA regulation is more complex than ComX peptide. The increase of cell density stimulates the accumulation of CSF peptide extracellularly. CSF peptide is transported back into the cell after it reaches its critical concentration by oligopeptide permease *Opp*, which is an ATP-binding casette transporter belonging ABC-type oligopeptide transporter family. Following the intake of this peptide, there are two

different intracellular receptors to which CSF peptide binds to regulate the activity of the ComA transcription factor. (Pottahil *et al.*, 2008; Solomon *et al.*, 1995 and 1996; Lazazzera and Grossman, 1998; Lazazzera 1997; Perego, 1997).When the intracellular concentration of CSF is low (1-5 nM), CSF binds to and inhibits RapC which is ComA-specific aspartylphosphate phosphatase. Low levels of intaken CSF promote competence development because of the inhibition of RapC helps the increase of phospho-ComA which is a regulator controlling the expression of competence genes (*Solomon et al.*, 1996). At higher concentrations (>20 nM), CSF inhibits the expression of *comS* that cause ComK proteolysis which is necessary in the decision of competence development. Also, at high concentration condition, CSF inhibits an aspartyl-phosphate phosphatase, RapB that dephosphorylates a response regulator (Spo0A) which is important at the initiation of sporulation (Figure 1.2). Inhibition of the RapB phosphatase activity increases the levels of phospho-Spo0A. Therefore, on both case, it is obvious that CSF stimulates sporulation at high internal concentration (Miller *et al.*, 2001).

The initiation of sporulation in *Bacillus subtilis* is regulated by a phosphorylation-mediated signal transduction pathway, known as phosphorelay (Stephens, 1998).

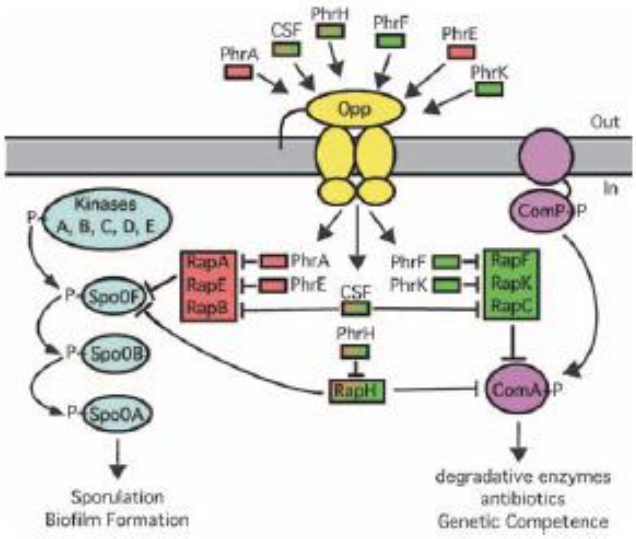


Figure 1.2: Sporulation initiation in *B.subtilis* (von Bodman *et al.*, 2008).

In the quorum sensing mediated sporulation mechanism of *Bacillus subtilis*, not only CSF (PhrC), but also a peptide, ARNQT (PhrA) which is encoded by *phrA* gene takes part and transported into the cell by Opp. These peptides are responsible for inhibition of phosphotases RapB and RapA respectively, which dephosphorylate

Spo0F~P. Additionally, KinA, KinB and KinC also has specific roles in the production of Spo0A. Beside other response regulators, phosphate transfer to Spo0A is not direct from kinases. In Spo0A phosphorylation, firstly, phosphate is transferred from kinases to Spo0F protein and Spo0F~P is formed. Then phosphate is transferred to Spo0B and Spo0B~P is formed. After that phosphate transferred to Spo0A and Spo0A~P protein is formed finally (Figure 1.3) (Stephens, 1998; Perego *et al.*, 1994).

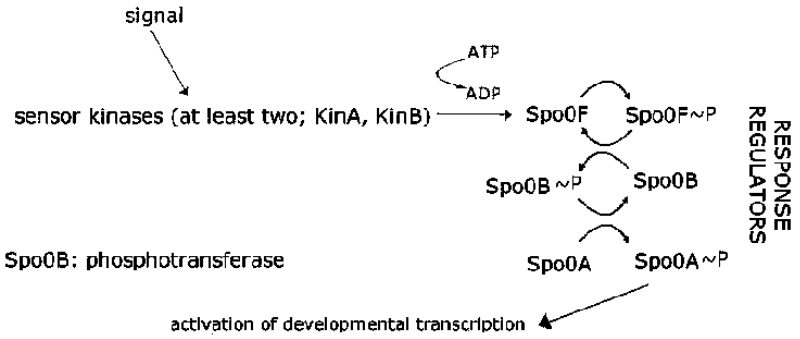


Figure 1.3: Phosphorelay signal transduction system

1.3. Bacilysin : A Dipeptide Antibiotic

The potential of *B. Subtilis* to produce antibiotics has been recognized for 50 years. Peptide antibiotics represent the predominant class. They exhibit highly rigid, hydrophobic and/or cyclic structures with unusual constituents like D -amino acids and are generally resistant to hydrolysis by peptidases and proteases (Stein, 2005).

Bacilysin [L-alanine-(2.3-epoxycyclohexanone-4)-L-alanine] is a simple and small sized(125kDa) nonribosomally synthesized dipeptide antibiotic that consists of L-alanine residue at N-terminus and non proteinogenic L-anticapsin residue at C terminus (Figure 1.4) (Walker and Abraham, 1970). This antibiotic is active against a wide range of bacteria and fungi especially *Candida albicans* (Steinborn *et al.*, 2005).

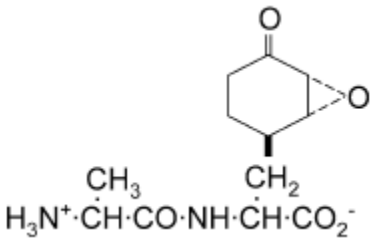


Figure 1.4 : The structure of bacilysin (Walker and Abraham, 1970).

The anticapsin moiety of bacilysin is important at the antibiotic activity which is released by peptidases after bacilysin uptake into susceptible cells by a distinct peptide permease system. After its uptake, the intracellular anticapsin blocks glucosamine synthetase that is responsible for bacterial peptidoglycan or fungal mannoprotein biosynthesis (Kenig *et al*., 1976; Perry and Abraham 1979, Chmara *et al.*, 1981). This situation leads to cell protoplasting and lysis. The antibiotic activity of anticapsin becomes specifically antagonized by glucosamine or N-acetylglucosamine depending on its metabolic target (Walton and Rickes 1962; Kenig and Abraham, 1976).

The synthesis of anticapsin branches from prephenate of the aromatic acid pathway which is the primary precursor of bacilysin (Hilton *et al.*, 1988). The peptide bound with L-alanine proceeds in a non-ribosomal mode, catalysed by bacilysin synthetase which is an amino acid ligase (Sakajoh *et al*., 1987).

Bacilysin production of *B. subtilis* is connected with the active growth in a synthetic medium and is inhibited by certain growth conditions, especially in the presence of certain nutrients, such as glucose or casaminoacids, and/or physiological factors, like pH, temperature (Özcengiz *et al*. 1990; Özcengiz and Alaeddinoglu 1991; Basalp *et al*. 1992).

Bacilysin biosynthesis is under or a component of the quorum sensing pathway which is responsible for the establishment of sporulation, competence development and onset of surfactin biosynthesis. In this pathway, besides ComQ/ComX, PhrC (CSF), ComP/ComA, also their unique transporter Spo0K (Opp) and products of *srfA, spo0A, spo0H* and *abrB* genes are defined as parts of quorum sensing mechanism and have key roles on regulatory circuit of bacilysin biosynthesis (Karataş *et al*., 2003).

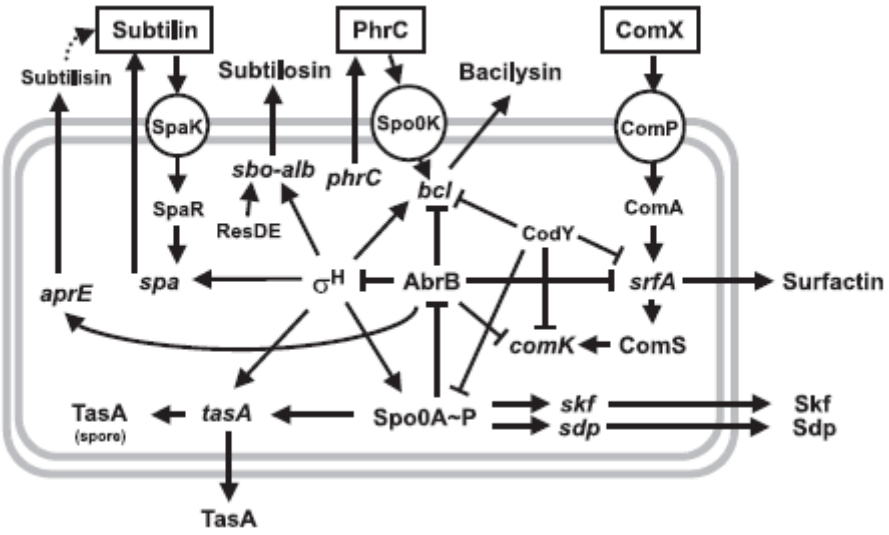


Figure 1.5: Biosynthesis pathways of subtilin, subtilosin, bacilysin, surfactin antibiotics, the killing factor Skf and the spore-associated anti-microbial polypeptide TasA in *B. subtilis* (Stein, 2005).

Bacilysin production is regulated on different levels negatively by GTP via the transcriptional regulator CodY and AbrB (Inaoka *et al*., 2003; Yazgan *et al.*, 2003). In *B. subtilis*, the production of antibiotics and resistance to them are under the control of the transition state regulator AbrB. As a typical example, *tycA* operon which encodes the enzyme tyrocidine synthetase catalyzing the synthesis of a cyclic decapeptide is directly repressed by AbrB which interacts with sequences upstream and downstream of the promoter and controls stationary-phase expression of *tycA* (Guespin-Michel, 1971; Furbah *et al*., 1991).

A polycistronic operon (*ywfBCDEFG*) renamed as *bacABCDE*, and a monocistronic gene (*ywfH*) are required for the bacilysin biosynthesis **(Figure 1.6)** (Steinborn and Hofemeister 1998/2000; Inaoka *et al.*, 2003). Each gene of the operon has specific functions; *bacABC* (*ywfBCD*) encode proteins functioning in the biosynthesis of anticapsin, *bacD* (*ywfE*) in the (amino acid) ligation of anticapsin to alanine and *bacE* (*ywfF*), in self-protection from bacilysin. *ywfB* and *ywfG* encode prephenate dehydratase and an aminotransferase, respectively, which are responsible for anticapsin production from prephenate of the aromatic amino acid pathway. The function of *ywfH* gene is still unkown but YwfH can be assigned to be involved in the alanine-anticapsin ligation. On the other hand, the direct evidence to

involvement in bacilysin synthesis was displayed for *ywfH* by gene disruption experiments or by missense allele analysis (Inaoka *et al.*, 2003).

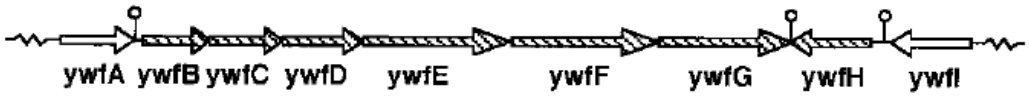


Figure 1.6: Organization of the bacilysin gene cluster *ywfABCDEFG* and *ywfH* gene of *Bacillus subtilis* 168 (Inoaka *et al.*, 2003).

While the ribosome system is universal, other machineries specific for certain peptides are also known, including nonribosomal peptide synthetase (NRPS). NRPSs are composed of repetitive units called as modules, each about 1.000 – 1.500 amino acids in length, which are capable of incorporating one amino acid constituent at a time into peptide chain. The modules can be subdivided into domains, each responsible for catalyzing the three basic reactions: substrate recognition, activation as acyl adenylate, and covalent binding as thioester. (Mootz and Marahiel, 1997; Schwarzer and Marahiel, 2002; Tabata *et al.*, 2005).

It was thought that formation of bacilysin was carried out by multiple-carrier thiotemplate mechanism until recently. But its biosynthesis mechanism was not fully coupled to non-ribosomal peptide synthetase (NRPS) mechanism because of the fact that adenylation and thiolation were obvious only for L-alanine, but not for L-anticapsin (Yazgan *et al.*, 2001).

Bacilysin production is regulated on different levels of positive and negative regulations. Positive regulation is conducted by guanonsine 5'-diphosphate 3'-diphosphate (ppGpp) (Inaoka *et al.*, 2003) and by a quorum-sensing mechanism through the peptide pheromone PhrC (Yazgan *et al.*, 2003). Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) plays a crucial role in transcription of the *ywfBCDEFG* operon and that the transcription of these genes is dependent upon the level of intracellular GTP which is transmitted as a signal via the CodY-mediated repression system. The CodY regulon encodes extracellular degradative enzymes, catabolic enzymes, transporter proteins, genetic competence factors, chemotaxis proteins antibiotic synthesis pathways, sporulation proteins. Genes and/or operons in this regulon are under negative regulation of CodY in the presence of excessive glucose or casamioacids (Ratnayake-Lecamwasam *et al.*,2001; Shivers and Sonenshein 2004).Otherwise, the expression of *ywfH* gene is dependent upon the level of intracellular GTP rather than ppGpp (Inaoka *et al.*, 2003).

Bacilysin production is negatively regulated by GTP via the transcriptional regulator CodY (Inaoka *et al*., 2003) and AbrB (Yazgan *et al*., 2003). In wild-type *B. subtilis* cells, a forced decrease of intracellular GTP increases the expression of these genes. By the disruption of *codY* which regulates stationary phase genes by detecting intracellular level of GTP results in an enhancement in their transcription and increase of bacilysin production only in wild-type cells. On the other hand, CodY is important for the regulation of *spo0A* and it also represses *srfA* operon. Therefore, when *codY* gene is deleted, there is an increase in bacilysin production in wild-type cells and the repression of *oppA*, *srfA* and *spo0A* disappears. In addition if intracellular level of AbrB is under critical threshold rate, AbrB-dependent repression on the production of the various antimicrobials, antibiotic and the other stationary phase-associated products decreases (Strauch 1993; Strauch an Hoch, 1993; Inaoka *et al.*, 2003).

srfA operon encodes the enzyme complex which catalyses the nonribosomal sythesis of the lipopeptide antibiotic, surfactin. Expression of *srfA* operon is induced following the onset of stationary phase and regulated by specific regulatory genes *comP*, *comA*, and *spo0K*. Transcription of *srfA* is directly activated by phosphorylated form of response regulator ComA and activity of ComA is controlled by a membrane-bound histidine protein kinase, ComP and an aspartyl-phosphate phosphatase, RapC. RapC is a negative regulator to remove phosphate from ComA for inactivation of ComA. ComA~P is produced in response to the accumulation of cell-derived two extracellular peptide pheromones ComX(ComQ) and CSF(PhrC). ComQ is proposed to activate ComP while CSF is transported back into the cell by the oligopeptide permease Opp (Spo0K) where it is proposed to interact with and inhibit the activity of RapC *srfA* also contains the competence regulatory gene *comS* and its product, ComS causes the release of a competence-specific transcription factor, ComK which is responsible for transcription of the late competence genes (Karataş *et al*., 2003; Nakano et al., 1991; van Sinderen *et al.*, 1995; Weinrauch et al., 1990).

phrC is partly controlled by sigma factor σ^H (*spo0H* gene product)-dependent promoter (P2) located at upstream of *phrC* and internal to *rapC*. The expression of *spo0H* is repressed by AbrB, the transition state regulator of late-growth gene transcription which in turn is repressed by the phosphorylated active form of Spo0A.

Phosphorylation of Spo0A results from a series of reactions which are catalysed by spo0F response regulator and spo0B phosphoprotein phosphotransferase. *spo0H* gene not only has a role in transcription of *phrC*, but also it controls the additional gene(s) involved in the production of mature CSF with Spo0A and AbrB proteins (Karataş *et al.*, 2003).

DegS and DegU are also sensor and effector proteins that form a two-component signal transduction regulatory system, transcribed from *sacU* locus (Stock *et al.*, 1989) and they are involved in the production of many types of commercially valuable degradative enzymes such as extracellular proteases, α-amylase, proteases (Tanaka *et al.*, 1991).

DegS protein kinase also acts as a DegU phosphatase. The genes coding for the DNA uptake and integration machinery are activated by a single transcription factor, the competence transcription factor ComK. ComK stimulates its own expression with response regulator DegU. Phosphorylated form of DegU is necessary for degradative enzyme synthesis and nonphoshorylated form of it required for expression of genetic competence (Dahl *et al.*, 1992; Duitman *et al.*, 2007).

Sigma-B (σ^B) responds through a complex, multibranched signal transduction pathway. Sigma-B control the transcription of operons which are responsible for diverse functions and a particular set of environmental conditions are required for its exppression such as, excessive heat, ethanol, salt, or acid (Price *et al.*, 2002).

1.4. The Aim of the Present Study

Former researches pointed that bacilysin biosynthetic operon *bacABCDE* (former *ywfBCDEFG*) and a monocistronic gene, *ywfH* are required for the biosynthesis of bacilysin. The aim of the present study was focused on the identification of the effects of previously identified global regulatory genes *srfA*, *oppA*, *comA*, *phrC*, *phrF*, *phrK*, *comQ* (*comX*), *comP*, *spo0H*, *spo0A*, *abrB*, *codY*, *degU* and s*igB* on the expression of *ywfH* gene.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Bacterial Strains and Plasmids

B. subtilis PY79, a prototrophic derivative of standart strain *B. subtilis* 168, was used as wild type during this study. The strains and their genotypes that were used in the study are listed in Table 2.1. *E. coli* Top10F' [*lac*Iq TN10 (Tetr)}, *mcrA* Δ (*mrr hsdRMS-mrcBC*), *f80lacZ*Δ*M15* Δ*lacX74, deoR, recA1, araD139* Δ(*ara-leu*)*7697, galU, galK, rsL,(strr), endA1, nupG*) was used as first step as a host strain for further *B. subtilis* chromosomal DNA cloning. As plasmids, pGEM-T Easy Vector (Figure 2.1) for cloning of PCR products amplified. pMutinT3 (Figure 2.2) was used for the construction of *ywfH-lacZ* transcriptional vector. pDR66 (Figure 2.3) was used for co-transformation of *B. subtilis* cell during transformation period to make easy selection of transformants.

2.1.2. Culture Media

Culture media composition and preparation are given in the Appendix A.

2.1.3. Buffers and Solutions

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

2.1.4. Chemicals and Enzymes

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

Table 2.1: Bacterial strains and their genotypes used in this study.

Strain	Genotype	Source
Bacillus subtilis PY79	Wild type BPS cured protothropic derivative of *Bacillus subtilis* 168	P.Youngman
KE10	Δ*srfA*::*erm*	K. Appelman
JMS315	*trpC2 pheA1* Δ*comQ*::*spc*	A. D. Grossman
BD1658	Δ*comP*::*spc*	D. Dubnau
JRL192	Δ*comA*::*cat*	A. D. Grossman
AK3	*oppA*::*Tn10*::*spc*	A. Y. Karataş
BAL373	*trpC2 pheA1* Δ*abrB*::*cat*	A. D. Grossman
TMH307	*trpC2 unkU*::*spc* Δ*codY*	A. D. Grossman
CAL7	Δ*phrK7*::*spc*	A. D. Grossman
JMA163	Δ*phrF163*::*cat*	A. D. Grossman
JMS751	Δ*phrC*::*erm*	A. D. Grossman
CU741	Δ*degu*::*kan*	A. D. Grossman
ML6	Δ*ML6::cat (sigma B)*	A. D. Grossman
NAO1	*ywfH::lacZ::erm*	This study
NAO3	Δ*spo0A*::*spc ywfH::lacZ::erm*	This study
NAO4	Δ*spo0H*::*spc ywfH::lacZ::erm*	This study
NAO5	Δ*abrB::cat ywfH::lacZ::erm*	This study
NAO6	*ywfH::lacZ::erm* Δ*codY::spc*	This study
NAO7	Δ*spo0A::spc* Δ*abrB::cat ywfH::lacZ::erm*	This study
NAO8	Δ*abrB::cat ywfH::lacZ::erm* Δ*codY::spc*	This study
NAO9	Δ*comA::cat ywfH::lacZ::erm*	This study
NAO10	Δ*comP::spc ywfH::lacZ::erm*	This study
NAO11	Δ*comQ::cat ywfH::lacZ::erm*	This study
NAO12	Δ*phrC::erm ywfH::lacZ::erm*	This study
NAO13	Δ*phrF163::cat ywfH::lacZ::erm*	This study
NAO14	Δ*phrK::spc ywfH::lacZ::erm*	This study
NAO15	*oppA::Tn10::spc ywfH::lacZ::erm*	This study
NAO16	Δ*deg U::kan ywfH::lacZ::erm*	This study
NAO17	Δ*ML6::cat (sigmaB) ywfH::lacZ::erm*	This study
NAO18	Δ*srfA::erm ywfH::lacZ::erm*	This study
E. coli Top10F'	*lac*Iq TN10 (Tetr)}, *mcrA* Δ (*mrr-hsdRMS-mrcBC*), *f80lacZ*Δ*M15* Δ*lacX74, deoR, recA1, araD139* Δ(*ara-leu*)*7697, galU, galK, rsL,(strr), endA1, nupG*	M.A. Marahiel

2.1.5. Maintenance of Bacterial Strains

B. subtilis strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates at cool (+4 °C). *E. coli* strains were kept on Luria-Bertani (LB) agar plates (at +4 °C). All cultures were subcultured monthly. 10 % LB glycerol stock was prepared for each strain and kept at -80°C.

2.1.6. pGEM®-T Easy Cloning Vector

pGEM®-T Easy Vector System that is supplied by Promega, is a convenient system for the cloning of PCR products. pGEM®-T Easy Vectors contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. pGEM®-T Easy Vector contains multiple restriction sites within the multiple cloning region. The pGEM®-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI. Therefore, this property provides three single-enzyme digestions for release of the insert.

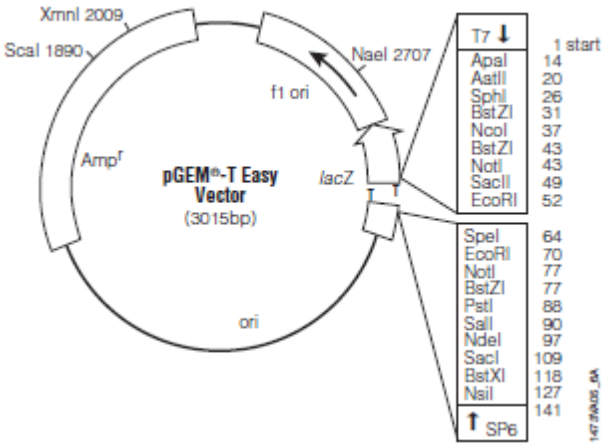


Figure 2.1: pGEM®-T Easy Vector circle map and sequence reference points.

2.1.7. pMUTIN T3 Cloning Vector

pMutin T3 is used for insertional gene inactivation in *B. subtilis* in order to characterize unknown open reading frames and to observe transcripional changes of constructed strains. This vector is a 8834 bp long plasmid and carries a reporter *lacZ* gene allowing to measure gene expression through β-galactosidase enzyme activity. Also pMUTIN T3 plasmid has an inducible promoter, *Pspac*, which is normally

suppressed by product of *lacI* gene, can be induced by IPTG at the same time. Besides, the plasmid carries *amp* and *erm* antibiotic resistance genes expressed in *E.coli* and *B. subtilis*, respectively.

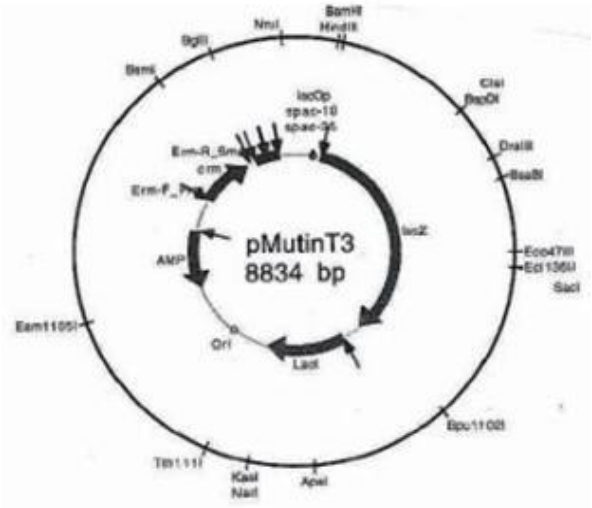


Figure 2.2: Genomic map of pMUTIN T3 vector showing the restriction map and the functional genes.

2.1.8. pDR66 Cloning Vector

pDR66 is a 9,3 kb sized vector including *cm* resistance gene is used for co-transformation of *B. subtilis* cell during transformation period to make easy selection of transformants which have same antibiotic resistance gene with competent cell.

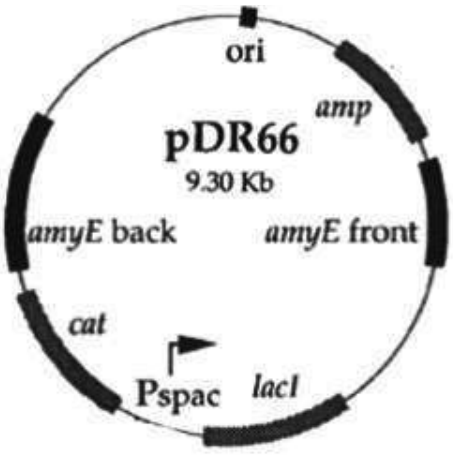


Figure 2.3: Schematic presentsation of pDR66 vector used for transformation due to Cat^R region facilitating the selection of mutant strains.

2.2. Methods

2.2.1. DNA Techniques and Manipulation

2.2.1.1. Plasmid DNA Isolation

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were mostly used for isolation of *E. coli* plasmid DNA as specified by the manufacturers. Bacterial cells were harvested by centrifugation at 13.000 rpm for 5 minutes. After removing supernatant, the pellet was resuspended in 300 µl P1 buffer (Appendix B). The pellet an buffer mixed complately by vortexing. Following step, 300 µl P2 (Appendix B) buffer was added and solution mix was then incubated at room temperature for 5 minutes. After this incubation, 300 µl P3 (Appendix B) buffer was added and mixed through inverting the tubes until the lysate is no longer viscous. The sample was incubated for 15 minutes on ice. Then centrifuged at 13.000 rpm for 15 minutes. After that supernatant was transferred to a new clean 1.5 ml eppendorf tube. Plasmid DNA was precipitated following the addition of 0,7 volume isopropanol and collected by centrifugation at 13.000 rpm for 30 minutes. Acquired pellet was washed with 1 ml of 70% ethanol. Ethanol was dried out of 37 ˚C for 15 minutes after removing the supernatant completely. Finally, the pellet was dissolved in 15 µl elution buffer (EB) at 37˚C and 200 rpm, and stored at -20˚C. The isolated DNA was run on 1 % agarose gel.

2.2.1.2. Chromosomal DNA Isolation

Chromosomal DNA of *B. subtilis* strains was isolated and purified by using a standart procedure designed for *Bacillus* species (Cutting and Horn, 1990). 1.5 ml of overnight culture of *Bacillus subtilis* was harvested by centrifugation at 13000 rpm for 5 minutes. After discarding the supernatant obtained pellet was resuspended in 567 µl of TE buffer (Appendix B) by repeated vortexing. 10 µl of proteinase K (20 mg/ml), 6 µl of RNase (10 mg/ml), 24 µl of lysozyme (100 mg/ml) and 30 µl of 10% SDS were added one by one and well mixed solution mix was incubated for 1 hour at 37°C in a water bath or in a thermomixer. Then, 100 µl of 5M NaCl solution was added and the sample was mixed by inverting the tubes without vortexing until the mucosal white substance can be seen. After that, 80 µL of CTAB/NaCl (Appendix B) (prewarmed to 65^{o}C because of viscosity) solution was added into the mixture and it was incubated for 10 minutes in 65°C water bath or thermomixer.

Freshly prepared phenol/chloroform/isoamyl alcohol (25:24:1) was then added to the mixture with the same volume of solution for extraction and it was centrifuged at 13000 rpm for 10 minutes. After centrifugation, the upper phase was transferred to a new 1.5 ml microfuge tube and 0.7 volume isopropanol was added. After up-down for 5-6 times, the sample was centrifuged at 13000 rpm for 15 minutes. The supernatant was removed and the pellet was washed with 1 ml 70% ethanol and centrifuged at 13000 rpm for 5 minutes. Subsequently, the pellet was dried at 37°C for 1 hour and was dissolved in 10 µl of TE buffer. Obtained chromosomal DNA was stored at 4°C. Finally, the isolated DNA was made run on 0.8% agarose gel.

2.2.1.3. Polymerase Chain Reaction (PCR)

The oligonucleotide primers were purchased from OPERON, Co. (Table 2.2). PCR was performed using Taq polymerase 10x reaction buffer from Fermentas. All cycles lasted for 1 minute. The denaturation temperature was 94^{o}C and the extention temperature was 72^{o}C. The annealing temperature for the first 5 cycles was 50-55^{o}C and 55-60^{o}C for the next 25 cycles dependin on the primer. The concentration of chromosomal DNA was 0.01 to 0.001 ng/µl. The oligonucleotide primers were used at 1 - 10 pM (equimolar) and deoxyribonucleoside 5'triphosphates (dNTPs) were used at 2 mM final concentration. Oligonucleotide primers given in Table 2.2 used for confirmation of deletions.

Table 2.2 : Oligunucleotide Primer Sequences

Primer	**Oligunucleotide Primer Sequences**
ywfH HindIII Forward	5'- GCA AGC TTT TTT CCC TCG TCA TTA ATT -3'
*ywfH Bam*HI Reverse	5'- GCG CAT CCC ATT CAT CAT ACT GTT TGT -3'
srfA Forward	5'-TAT TTG TAC AGG GTC CGC CG -3'
srfA Reverse	5'- AAG CAG CTT CTC TTT CTC CGC -3'
*phrC Pst*I Forward	5'- GCC CTG CAG GCG GTC TCC ACA TTT GAA AGC -3'
*phrC Bam*HI Reverse	5'- CGG GGA TCC TAG AAA GTA GGA AGC AGA CAG -3'

A master mix composed of the materials listed below (Table 2.3) was prepared. Then, the master mix was divided into for each PCR tubes and 1 µl of chromosomal DNA of *Bacillus subtilis* PY79 was added into each tube as template DNA. Finally, 0.5 µl of Taq polymerase was added for each tube.

Table 2.3: List of materials used for preparation of PCR mixture

Content of PCR mixture	**Amount**
Reverse primer	1 µl
Forward primer	1 µl
10x Buffer (-$MgCl_2$)	1 µl
$MgCl_2$	5 µl
dNTP Mix (2 mM)	5 µl
Template DNA	1 µl
Taq polymerase	0,5 µl
dH_2O	35,5 µl

2.2.1.4. Agarose Gel Electrophoresis

According to the purpose of electrophoresis, different concentration of agarose gels were used, which were given in Table 2.4. Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus and in a gel system composed of ~1% agarose gel (different gel concentration for different samples) (Table 2.4) containing 1xTAE buffer (Appendix B) and ethidium bromide of a 0.2 µg/mL finalconcentration. 6X Loading dye was added into the samples. Electrophoresis was performed at 90-120 Volts for 20-30 minutes. The DNA bands were visualized on a shortwave UV transilluminator (UVP) and photographed by using Gel Imaging System. *EcoR*I+*HindIII* digested DNA marker and *HinfI* digested DNA Marker (Appendix D) were used to determine the molecular weights of DNA bands for desired purposes.

Table 2.4 : Agarose gel concentration for different samples.

Sample	Concentration
Chromosomal DNA	0,8 %
Plasmid DNA	1 %
Digestion products of plasmid	1 %
PCR products with	1.5 % - 2 %

2.2.1.5. Gel Extraction

The desired fragments were extracted from the gel by using a Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA). The gel slice containing the DNA band was cut off from the gel and DNA extraction from gel was performed according to the Qiagen's instructions. After obtaining DNA, an aliquot was run on agarose gel to monitor the final DNA concentration.

2.2.1.6. Ligation of PCR Products into pGEM®-T Easy Vector

Ligation of PCR products to pGEM®-T Easy Vector was performed as follows: 5 µl 2X Rapid Ligation Buffer, T4 DNA Ligase, 1 µl (50 ng/µl) pGEM®-T Easy Vector, 2 µl insert DNA (PCR product) and 2 µl dH_2O was added and total volume was completed to 10 µl. Reaction mixture was incubated overnight at 4°C. After ligation was completed, the mixture was used for transformation into *E. coli* Top10F'.

2.2.1.7. Ligation of pMutinT3 Vector

Ligation procedure for cloning into the pMutinT3 vector was carried out using 9.5 µl of *ywfH* PCR products as insert fragments and 0.5 µl of pMutinT3 vector. Vector and fragment were mixed in a clean eppendorf tube and incubated for 5 min at 65°C. Then, the tube was cooled on ice. Following cooling step, 2 µl of ligation 10x buffer, 2 µl of Polyethylene glycol (50% PEG 8000), 2 µl of T4 DNA ligase, 4 µl of dH2O were added into the same eppendorf tube. In last step, the mixture was again centrifuged for a short spin and incubated at 16°C for 16 h.

2.2.1.8. Restriction Enzyme Digestion

Digestion reactions were carried out in a way that the amount of 10X digestion buffer was 1/10 of the total reaction mix. The reaction mix was incubated for 1-4 hours at 37°C, then enzyme denaturation at 65°C for 10 minutes. The sample was stored at -20°C.

2.2.2. Transformation

2.2.2.1. Preparation of *E.coli* Electrocompetent Cells and Transformation of Electrocompetent *E.coli* Top10F' Cells

The overnight culture of *E.coli* Top10F' was diluted 1:100 fold into 400 ml 2xYT medium which contained Tetracyclin (20 μg/ml). Then incubated at 37 ^{o}C with 250 rpm by shaking until reached to 0.6 of OD_{600} (Optical Density at 600 nm). After that incubated cells were stayed on ice for 30 minutes. Cells were harvested by centrifugation at 5000 rpm for 5 minutes. After removal of supernatant, pellet was resuspended in 40 ml of cold distilled water and centrifuged at 5000 rpm for 15 minutes. Supernatant was discard and pellet was resuspended in 20 ml cold distilled water and centrifuged at 5000 rpm for 15 minutes again. After this step, supernatant was discarded and pellet was resuspended in 1 ml of cold steril 10 % glycerol. Finally, it was dispensed into aliquots of 40 μl into eppendorf tubes. These aliquots were frozen immediately by immersing within liquid nitrogen and stored at -80 ^{o}C.

For transformation, competent *E. coli* cells were incubated on ice shortly. 10 μl of ligation products or 0.5 μg of appropriate plasmid DNA was added into the tube of competent cell and were mixed gently by help of finger. Then, mix was transferred into pre-cold electroporation tube. The sample was placed onto electroporator and the process was carried out at 1800V. After addition of 1ml LB broth into the mixture, it was transferred into a 2 ml eppendorf tube. Incubation was made at 37^{o}C for 60 minutes by shaking at 200 rpm. After incubation, the cells were centrifuged at 5000 rpm for 10 min and supernatant was discarded. Obtained pellet was resuspended in 100 μl 8.5 % saline solution (NaCl) (Apendix B). Transformed cells were plated on selective medium containing appropriate antibiotic (100μg/ml ampicillin). For the purpose of blue-white colony selection, they were plated on LB agar media containing 40 mg/ml X-gal, 1 mM IPTG and 100μg/mL ampicillin.

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

Preparation of *B. subtilis* competent cells and transformation were performed as described by Klein *et al.* (1992). HS and LS (Appendix B) mediums were used for the preparation of *B. subtilis* competent cells. Appropriate amount of *B. subtilis* cells were inoculated into 3 ml of HS medium and it was cultured overnight at 37°C by

shaking at 250 rpm. 0,5 ml of this overnight culture was transformed into 20 ml of freshly prepared LS medium and incubated at 30°C by shaking at 200 rpm until OD600 reached to 0,55. Then, 1 ml of competent cells was transferred into 2 ml eppendorf tube in which contained 1 µl of chromosomal DNA or suitable plasmid was added. The cells were then incubated at 37°C for 2 hours by shaking at 200 rpm and incubated cells were harvested by centrifugation at 5000 rpm for 10 minutes. Finally, the pellet was resuspended in 100 µl of sterile saline solution and was spread out on LB agar plates containing selective antibiotic and incubated at 37°C for 16 h.

2.2.2.3. The Selection by MLS Resistance Method

Competent *B. subtilis* cells were incubated in HS medium until reached to 0.55 of OD_{600} by the same conditions given the Section 2.2.2.2 and then incubated for 2 hours. In the overlay step, firstly, 2 µl of 2 mg/ml erythromycin (*erm*) antibiotic solution was added into a small test tube containing 2,5 ml % 0.7 LB agar which was melted and kept in 50°C. Then, immediately, all amount of transformed cells were added into same tube, mixed quickly and were poured onto an antibiotic-free LB agar plate containing 25 ml LB agar. Following incubation at 37°C for 2 hours, a second overlay was performed. This second overlay has contained 2.5 ml of melted % 0.7 LB agar, 20 µl (2 mg/ml) erythromycin antibiotic solution and 20 µl (50 mg/ml) lincomycin antibiotic solution. The agar plate was incubated at 37°C for 1-2 days.

2.3. Beta-Galactosidase Activity Assay

Initially, all strains were grown overnight at 37°C in PA medium (Appendix A). Then, they were used to inoculate 100 ml of PA medium to an initial optical density of about 0.1 at 595 nm (OD595). The cultures were incubated at 37°C shaking at 250 rpm for 30 h. 1 ml of culture was collected as double eppendorf tubes for each hour for checking up of assay. Suitable dilution was made for measurement, if needed.

After each sampling, the culture was centrifuged at 13000 rpm for 5 minutes and the supernatant was discarded. Then, the pellet was washed with 500 µl of ice-cold 25 mM Tris-Cl (pH 7.4) by centrifugation. Following, the removal of the supernatant, the pellet was resuspended in 640 µl Z-buffer by vortex and 160 µl of lysozyme was added. Later, the solution was vortexed for a second and incubated at 37°C for 5 min. Subsequently, the samples were taken on ice and 8 µl of 10% Triton-X100 was

added. After vortexing for a while, the extracts were stored on ice until performing β-galactosidase assay.

β-galactosidase assay was executed in the first place by prewarming the extracts in 30°C water bath for 5 min. Subsequent to this, 200 µl of ONPG solution was added and the solution was watched for the progression of the yellow color. Following, the reaction was stopped by the addition of 1 M Na_2CO_3 and reaction time was recorded. Reaction time refers to a period that was started by adding ONPG and continued until formation of the yellow color, and so this period was recorded. At last, the samples were centrifuged at 13000 rpm for 5 min. and the supernatant was taken to measure OD at A_{420} and A_{550}. Calculations for β-galactosidase activity were carried out according to the formulation (Miller, 1972) below and a graph for both the β-galactosidase activity and log OD_{595} was drawn.

$$Miller\ units = \frac{A_{420} - (1.75 \times A_{550})}{Reaction\ time\ (min) \times OD_{595}} \times 1000 \qquad \textbf{(2.1)}$$

3. RESULTS AND DISCUSSION

3.1 Construction of *ywfH* Insertional Plasmid

For integration of pMUTIN T3 vector, as first step, a *ywfH* gene fragment, 394 bp in lenght, was amplified by PCR using chromosomal DNA of *Bacillus subtilis* PY79 wild type strain as the template DNA. The primers *ywfH* Forward 5'-GCAAGCTTTTTTCCCTCGTCATTAATT -3' and *ywfH* Reverse 5'-GCGGATCCCATTCATCATACTGTTTGT -3' containing extra residues including *Hind*III and *BamH*I site at their 5′ ends (underlined residues) were used for a partial 394 bp gene fragment amplification of 777 bp *ywfH* gene. Hence, a total of 394 bp amplified gene fragment was obtained by PCR (Fig. 3.1).

The amplified PCR product was purified after running on agarose gel. Extracted fragment was ligated into pGEM®-T Easy Cloning Vector (Promega) and used for the transformation of *E. coli* Top10F' electro-competent cells. Suitable plasmid carrying 394 bp insert within pGEM®-T Easy Cloning Vector was selected and the sequence analysis was performed. Nucleotide sequence result of sequence analysis data was compared to *B. subtilis* genome sequence, using BLAST search, at National Center for Biotechnology Information (NCBI) database for further confirmation.

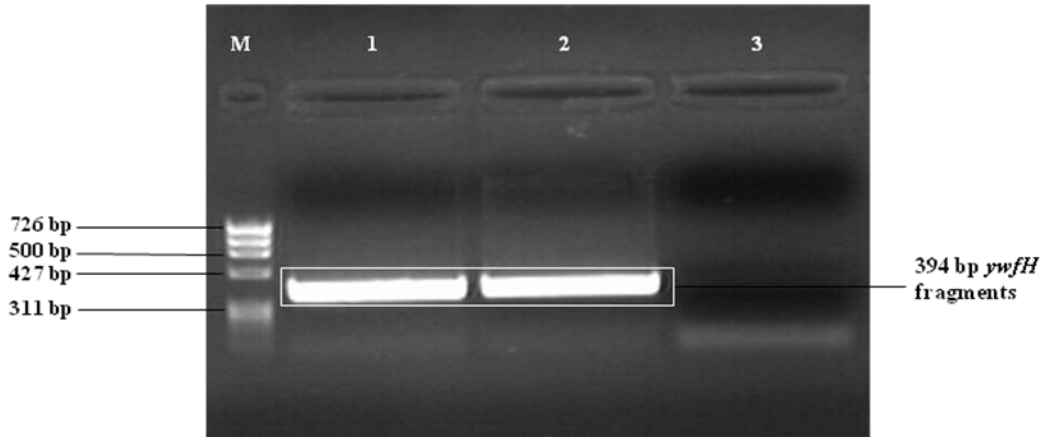


Figure 3.1: 1-2; PCR products of 394 bp *ywfH* gene fragment amplified with specific primers *ywfH* F and *ywfH* R, 3; Negative control of PCR and M; Marker 10: ΦX174 DNA / HinfI (Appendix D).

This recombinant pGEM®-T Easy Vector was firstly digested only with *EcoRI* in order to take out the fragment which included *ywfH* fragment and was run on agarose jel (Fig 3.2).

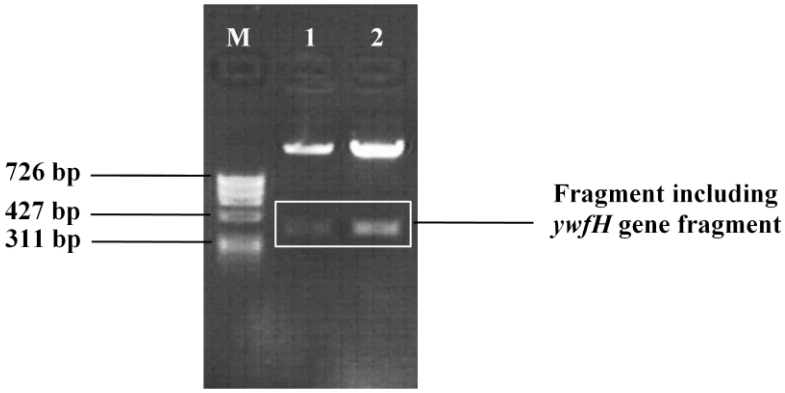


Figure 3.2: Fragment obtained from *EcoRI* digestion of the recombinant pGEM®-T Easy Vector, including *ywfH* gene fragment and M; Marker 10: ΦX174 DNA / HinfI (Appendix D).

Then, *ywfH* gene fragment from pGEM®-T Easy recombinant vector and pMUTIN T3 vector were both digested with *Hind*III and *BamH*I restriction enzymes. Following that digested *ywfH* fragment from pGEM®-T Easy and digested pMUTIN T3 were extracted from agarose gel and they were ligated with T4 ligase overnight at 4^{o}C. After the transformation *E. coli* Top10F' competent cells with this ligation product, transformants were selected on 100 µg/ml ampicillin containing agar plates. Plasmid DNAs were isolated from the selected colonies, subsequently linearized and run on agarose gel in order to verify the expected size (9359 bp). Then, suitable sized

plasmids were selected and digested with *Hind*III and *BamH*I restriction enzymes for confirmation of the existance of *ywfH* fragment in the pMUTIN T3 vector.

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

The selected recombinant pMUTIN T3 plasmid carrying 394 bp *ywfH* fragment was used to transform *B. subtilis* PY79 wild type strain in order to construct *ywfH*::*lacZ*::*erm* transcriptional fusion in its chromosome. This integration, also called Campbell-like insertion, was a result of a single cross-over event at *ywfH* region.

The selection based on the erythromycin resistance on LB plates containing erythromycin (1μg/mL) could not be fully performed because of the generation of the spontaneous erythromycin resistant *B. subtilis* PY79 cells. To eliminate this problem, an alternative selection method, Macrolide-Lincosamide-Streptogramin B-resistance (MLS^R) conferred by *erm* gene, was used. Chromosomal DNA of the resultant MLS^R transformant was isolated and screened through PCR analysis by using primers specific to *erm* resistance gene on pMUTIN T3 in order to confirm the insertion of the plasmid into the *B. subtilis* chromosome. 703 bp *erm* gene was amplified by using the chromosomal DNA of MLS^R *B. subtilis* transformants as template (Fig 3.3). The corresponding MLS^R *B. subtilis* PY79 transformant was designated as NAO1.

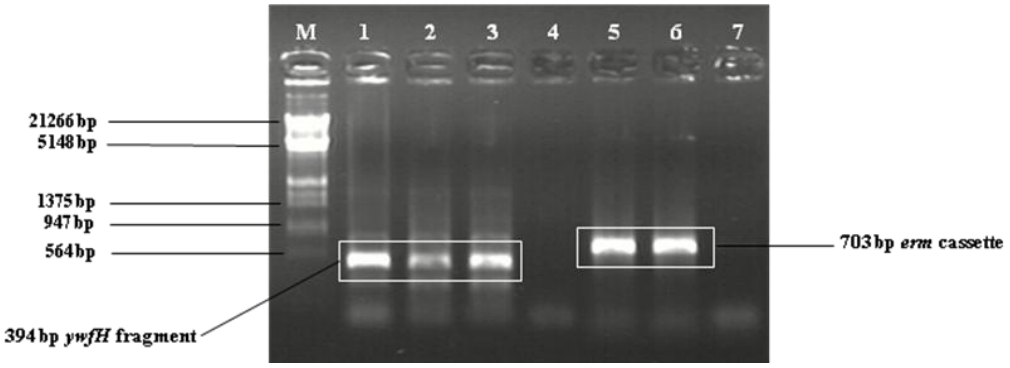


Figure 3.3: The confirmation of *ywfH::lacZ::erm* in *B. subtilis* chromosome. 1: PCR reaction with primers specific to *ywfH* gene by using Bacillus subtilis PY79 genomic DNA as positive control; 2-3: PCR product amplified with specific primers to *ywfH* gene using chromosomal DNA of *ywfH::lacZ::erm* mutant as template; 4: Negative control for PCR product of *ywfH* gene 5-6: PCR product amplified with specific primers to within pMutinT3 vector using chromosomal DNA of *ywfH::lacZ::erm* mutant as template; 7: Negative control for PCR product of *erm* resistance gene; M: Marker 3: Lambda DNA / *Eco*RI+*Hind*III (Appendix D).

3.3. Expression of Transcriptional *ywfH*::*lacZ* Fusion in PA Medium

In order to compare the expression level of *ywfH* both in *ywfH*::*lacZ* mutant strain NAO1 and wild type *B. subtilis* PY79, cells were grown in PA medium, which is a defined medium that stimulates bacilysin production, at 37^{o}C and 200 rpm. Samples for this culture were collected at 1 hour interval for thirty hours, and growth of cells was monitored. Collected cells were assayed for their β-galactosidase activity, using ONPG as substrate and enzyme activity was explored in Miller units. The results of expression of *ywfH*::*lacZ* fusion and *B. subtilis* PY79 wild type strain were compared as can be seen in the Figure 3.5. Growth profiles of both strains were similar (Figure 3.4). Expression of *ywfH* in NAO1 mutant strain was observed to be increased in the exponential phase slowly, then was increased in the transition state between exponential and stationary phase and reached to its maximal level (detected as 86 Miller units) upon entry into stationary phase. (Figure 3.4).

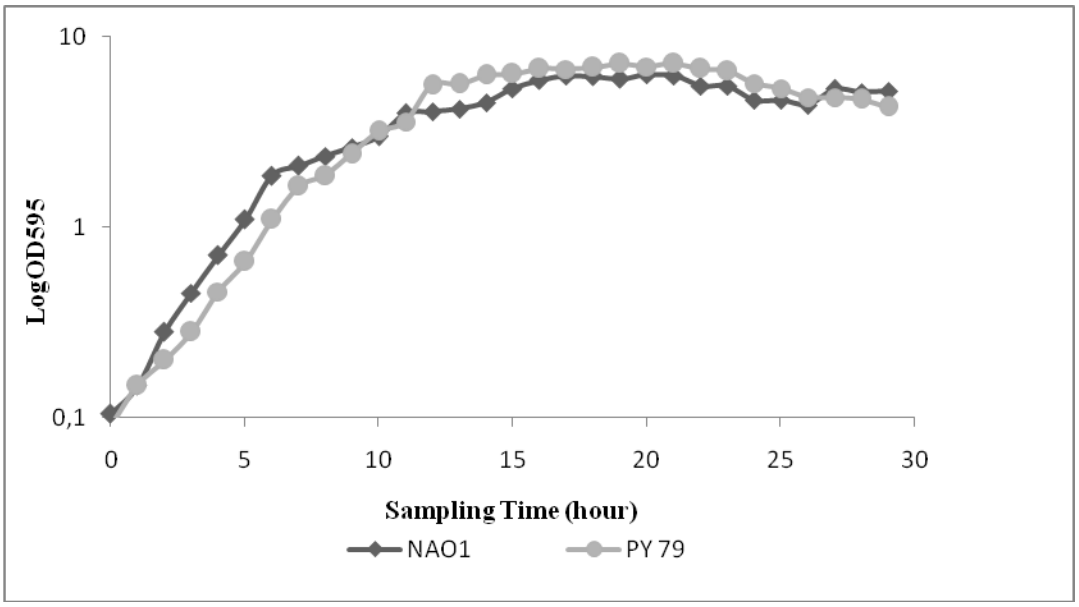


Figure 3.4: Growth profiles of *B. subtilis* PY79 and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (●) PY79 (wild type) and (♦) NAO1 (*ywfH*::*lacZ*::*erm*).

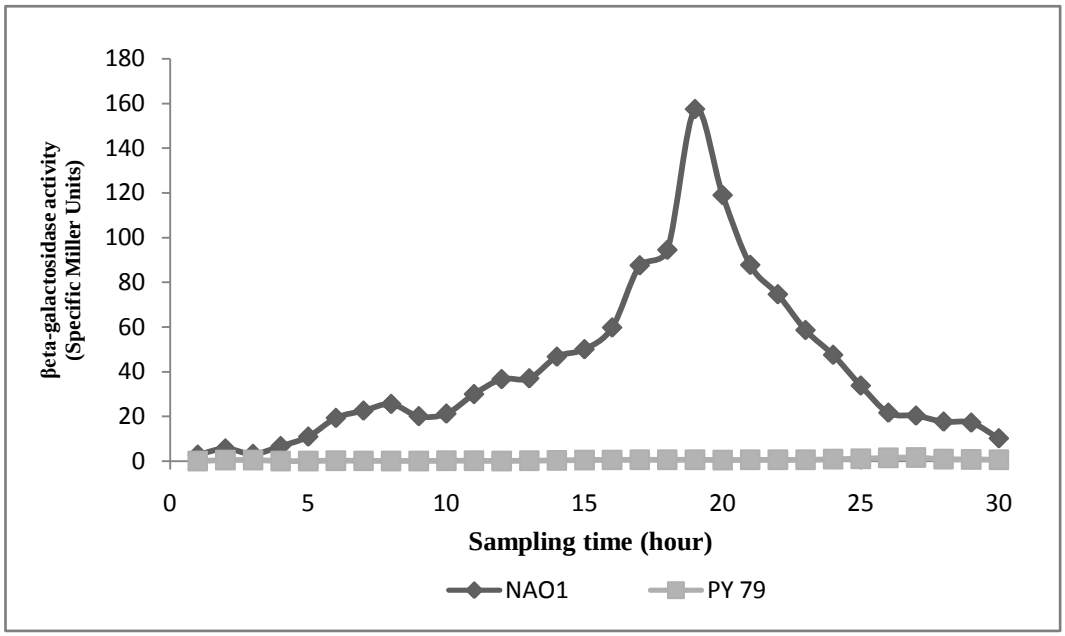


Figure 3.5: β-Galactosidase activities of *B. subtilis* PY79 and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units were calculated using the formula as denoted in Section 2.3. The symbols used for the strains are; (●) PY79 (wild type) and (♦) NAO1 (*ywfH*::*lacZ*::*erm*).

3.4. Deletion of regulatory Genes and Their Effects on the Expression of *ywfH* gene in *B. subtilis*

3.4.1. Deletion of *srfA* Gene and Its Effect on the Expression of *ywfH* Gene in *Bacillus subtilis*

srfA operon is consisted of four genes (*srfAA, srfAB, srfAC, srfAD*) and is necessarily needed for the efficient development of genetic competence which is regulated by ComA response regulatory protein. *srfA* contains the competence regulatory gene *comS* which is nested within and out of frame with the second gene of the operon, *srfAB*. Furthermore, ComS is essential for competence to develop. The *srfA* operon is normally expressed during active growth at a low level, however, just before the transition to stationary phase, its transcription is increased sharply dependent on *comA, spo0K* and *comQ* activity. Phosphorylation of ComA stimulates the binding of ComA~P to the *srfA* promoter, and as such induces *srfA* transcription and surfactin production. Also null mutations of those three genes resulted in a decrease of the expression level of *srfA* (Roggiani & Dubnau, 1993; Cosby *et al.*, 1998; Hamoen *et al.*, 2003; van Sinderen *et al.*, 1990, Hahn and Dubnau, 1991).

In order to monitor the effect of *srfA* gene deletion on *ywfH* profile, *srfA* gene was deleted in the *ywfH*::*lacZ* fusion strain NAO1. NAO1 that was used as competent cell, was co-transformed by the chromosomal DNA of Δ*srfA*::*erm comS* derivative strain, KE10 and pDR66 plasmid DNA. Both NAO1 and KE10 had *erm* antibiotic resistance so using pDR66 made selection of tranformants easier considering the advantage of its *cat* antibiotic cassette region. Selection of transformants on LB agar plates was performed with Erm(1μg/ml), Ln (25μg/ml) and Cat (20 μg/ml) antibiotics and then transformants were screened with PCR using specific primers. Selected mutant was named as NAO18 (Δ*srfA::erm ywfH::lacZ::erm*).

The resulting strain NAO18 and NAO1 as control, were cultured in PA medium and were sampled in every 1 h period for the β-galactosidase assay. Growth profiles were shown in Figure 3.6 and β-galactosidase activities were shown in the Figure 3.7.

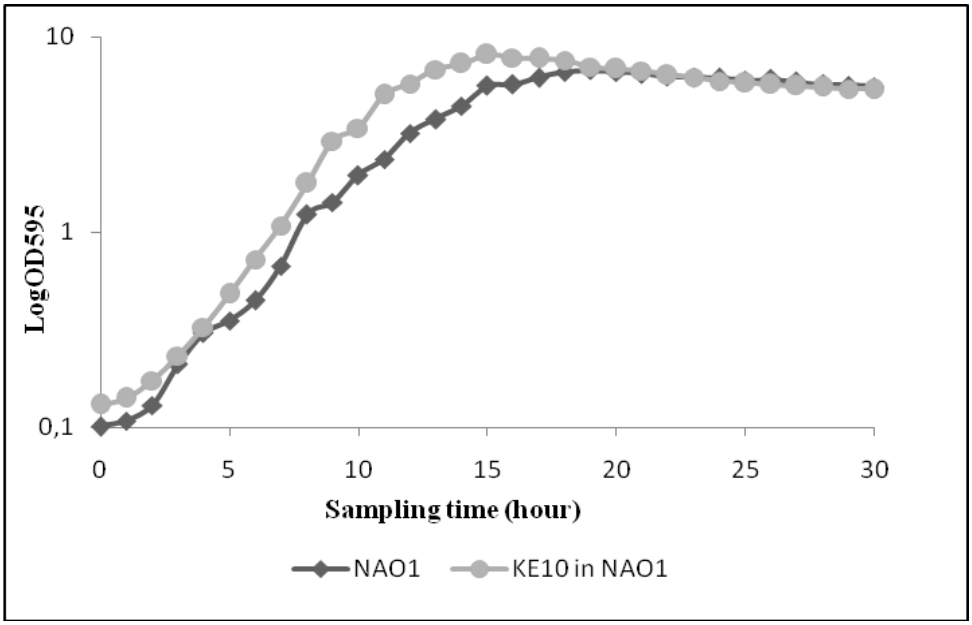


Figure 3.6: Growth profile of NAO18 (Δ*srfA::erm ywfH::lacZ::erm*) and NAO1(*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (●)NAO18 (Δ*srfA::erm ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

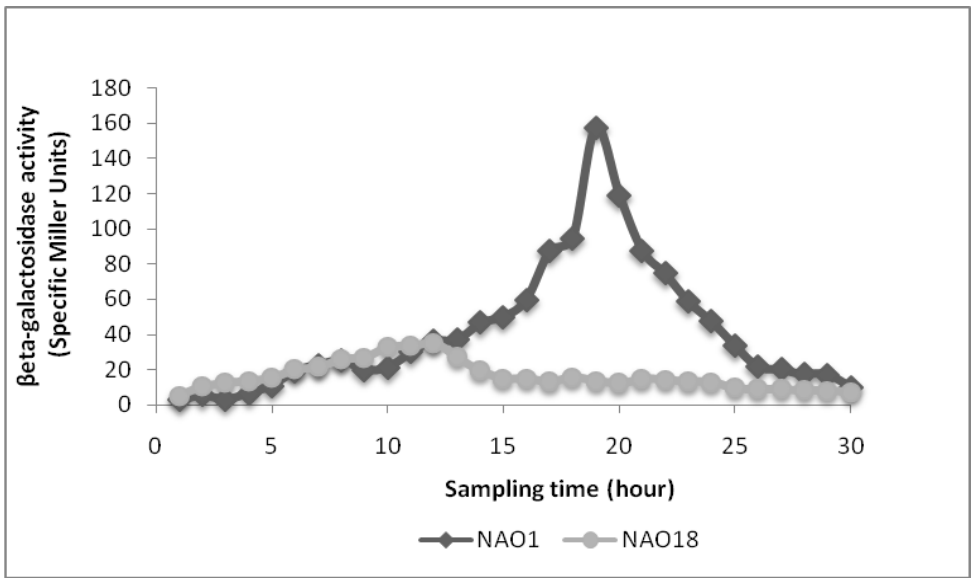


Figure 3.7: β-Galactosidase activities of NAO18 (Δ*srfA::erm ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (●)NAO18 (Δ*srfA::erm ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

Figure 3.7 points out that deletion of *srfA* gene dramatically disrupted *ywfH* gene expression. Previous data indicated that *srf* operon might be an intermediate component of the regulatory pathway and also proposed that *srfA* gene products act

in *B.subtilis* cell specialization and differentiation (Hahn and Dubnau, 1991; Nakano et al., 1991). It was previously shown that *srfA* disturbed mutants were not able to produce bacilysin, so *srfA* might be effective on biosynthesis of bacilysin (Karataş *et al.*, 2003). In this present study, it was suggested that the disruption of *srfA* gene might seriously effect bacilysin production via effecting the expression of *ywfH* gene negatively.

3.4.2 Deletion of *oppA* Gene and Its Effect on the Expression of *ywfH* gene in *B. subtilis*

Oligopeptide permeases are needed for bacteria to utilize peptides as nutrient sources (Solomon *et al.*, 2003). *B. subtilis opp* operon encodes five proteins—OppA, OppB, OppC, OppD, and OppF. OppA binds to the peptide substrate and facilitates its uptake by interacting with and delivering the peptide to the OppBCDF complex. *opp* was first identified as a sporulation operon, named *spo0KABCD*. In CSF pathway, the Opp component plays a key role in the peptide export-import circuit which coordinates timing of competence and sporulation events. Mutations in *oppA*, *oppB*, *oppC*, and *oppD* completely block the ability for oligopeptides uptake into cell (Perego *et al*., 1991; Perego 1997; Rudner *et al*., 1991;).

To monitor the deletion effect of *oppA* gene on *ywfH* expression, *oppA* gene was deleted in NAO1 by transforming NAO1 competent cells by the chromosomal DNA of *oppA*::*Tn10::spc* mutant strain, AK3. Then selection of transformants on LB agar plate containing Spc (100μg/ml) was performed. Selected mutant was named as NAO15 (*oppA*::*Tn10::spc ywfH::lacZ::erm*).

The resulting strain NAO15 and NAO1 as control, were cultured in PA medium and were sampled at 1 h period for the β-galactosidase assay. Growth profiles were shown in Figure 3.18 and β-galactosidase activities were shown in the Figure 3.9.

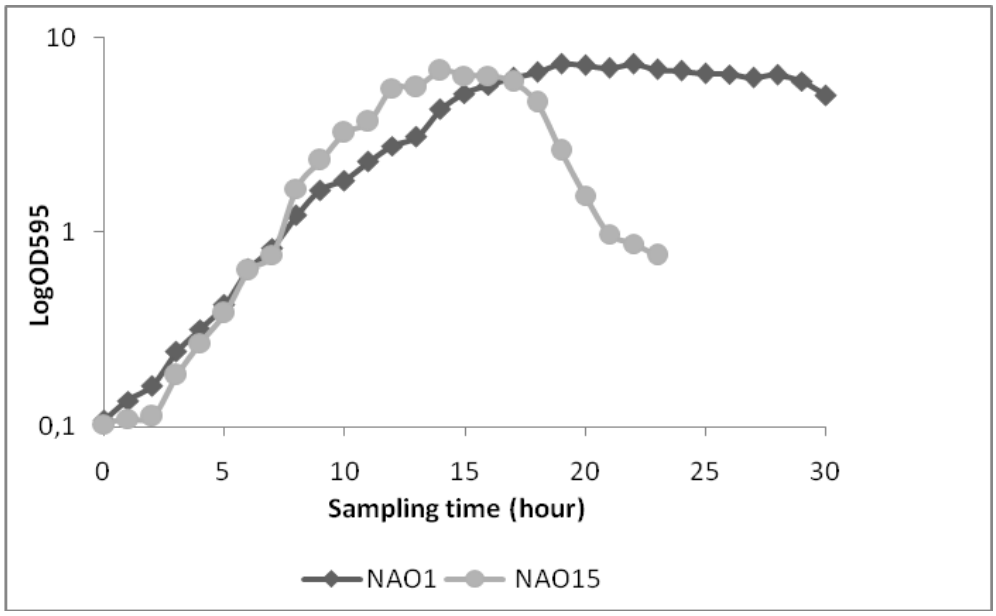


Figure 3.8: Growth profile of NAO15 (*oppA*::*Tn10*::*spc ywfH*::*lacZ*::*erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (●) NAO15 (*oppA*::*Tn10*::*spc ywfH*::*lacZ*::*erm*) and (♦) NAO1 (*ywfH*::*lacZ*::*erm*).

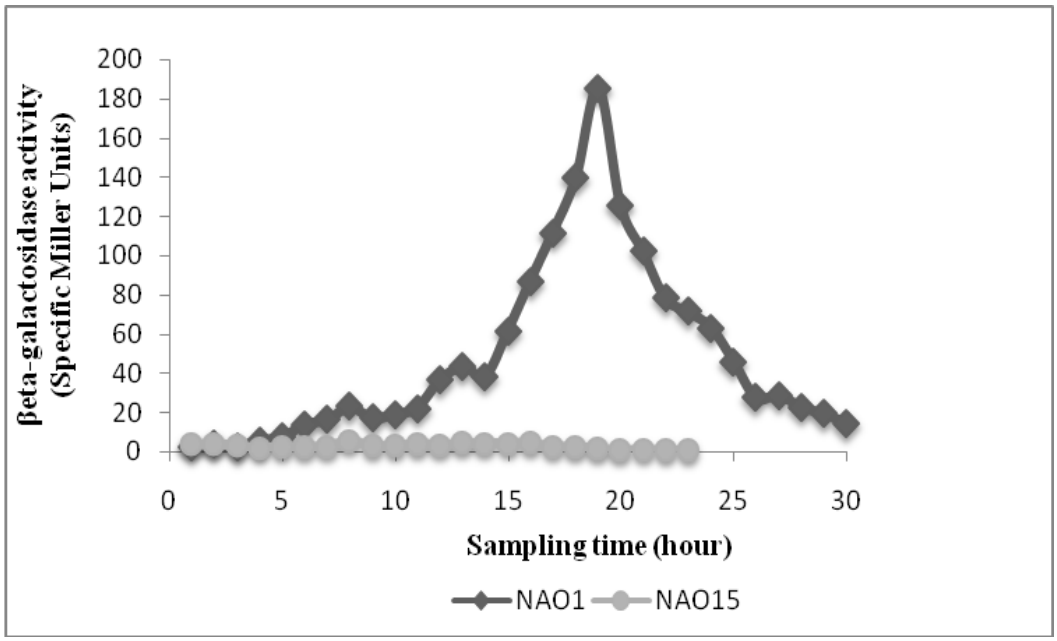


Figure 3.9: β-Galactosidase activities of NAO15 (*oppA*::*Tn10*::*spc ywfH*::*lacZ*::*erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are;(●)NAO15 (*oppA*::*Tn10*::*spc ywfH*::*lacZ*::*erm*) and (♦)NAO1 (*ywfH*::*lacZ*::*erm*).

Figure 3.9 shows that *ywfH*::*lacZ* expression was severely disrupted in the absence of *oppA* gene product both during exponential and stationary phases. Yazgan *et al.* (in 2001) reported that *oppA* mutation created a loss of bacilysin biosynthesis and therefore, it was suggested that bacilysin biosynthesis is under the global quorum sensing mechanism or a component of this mechanism. (Yazgan *et al.*, 2001). In this study, *ywfH* expression was shown to be regulated crucially with *oppA*, as a probable part of quorum sensing mechanism.

3.4.3. Deletion of Phr Peptides Genes (*phrC, phrK, phrF*) and Their Effects on the Expression of *ywfH* gene in *B. subtilis*

B. subtilis encodes a family of 8 Phr peptides (PhrA, PhrC, PhrE, PhrF, PhrG, PhrH, PhrI, and PhrK) and a family of 11 Rap proteins (RapA to RapK) **(Table 1.1).** Each Phr peptide is encoded together with its cognate Rap protein from an unique operon, and each characterized Phr inhibits the activity of its Rap protein. Very recently, it was shown that ComA-dependent gene expression is stimulated by Phr peptides including PhrC, PhrF and PhrK and that all three peptides are required for full expression of ComA-dependent genes (Auchtung *et al*., 2006).

Under the light of the finding that opp deletion resulted in complete repression of *ywfH* expression, Opp-imported pheromones PhrF and PhrK as well as PhrC are considered to have function in *ywfH* expression and thus the expression of *ywfH::lacZ* expression was examined in the PhrC, PhrF and PhrK mutant backgrounds.

Therefore, in order to monitor the effects of blockage of *phrC*, *phrK*, and *phrF g*enes on *ywfH::lacZ* fusion, mutant strain NAO12 (Δ*phrC::erm ywfH::lacZ::erm*), NAO14 (Δ*phrK::spc ywfH::lacZ::erm*) and NAO13 (Δ*phrF163::cat ywfH::lacZ::erm*) mutant strains were constructed separately. Transformation of NAO1 mutant strain with CAL7 (Δ*phrK7*::*spc)* and JMA163 (Δ*phrF163*::*cat)* chromosomal DNAs, respectively. Only for deletion of *phrC* gene in NAO1 (*ywfH*::*lacZ::erm*), the competent cells of JMS751 (Δ*phrC*::*erm)* supplied from A.D. Grossman, was co-transformed with chromosomal DNA of NAO1 and pDR66 plasmid and selection was made depending on the resistance to Erm(1μg/ml), Ln (25μg/ml) and Cat (20 μg/ml) antibiotics.

One of the transformants was selected and named as NAO12 (*ywfH::lacZ::erm* Δ*phrC::erm*). Moreover, *phrC* deletion mutations were confirmed by PCR analysis using specific primers (Table 2.2). Other resulting strains were named as NAO14 (Δ*phrK::spc ywfH::lacZ::erm*) and NAO13 (Δ*phrF163::cat ywfH::lacZ::erm*) mutant strains. The resulting strains NAO12, NAO14, NAO13 and NAO1, as control, were cultured in PA medium and the specific activities of the samples were measured. Also growth profile of mutants was followed (Fig. 3.10, 3.11, 3.12, 3.13).

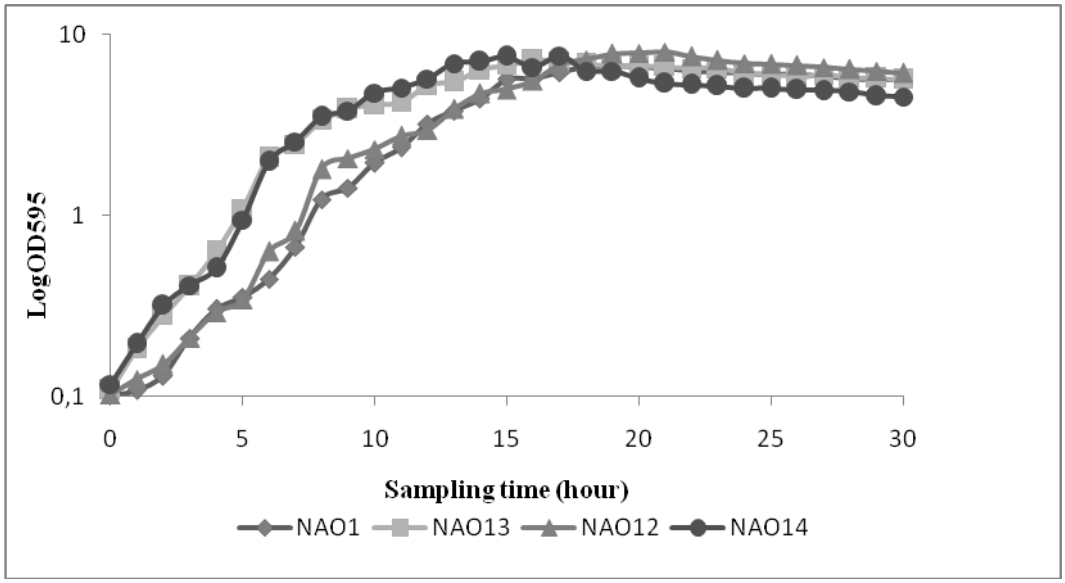


Figure 3.10: Growth profile of NAO12 (*ywfH::lacZ::erm* Δ*phrC::erm*), NAO13 (Δ*phrF163::cat ywfH::lacZ::erm*), NAO14 (Δ*phrK::spc ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (▲)NAO12 (*ywfH::lacZ::erm* Δ*phrC::erm*); (●)NAO14 (Δ*phrK::spc ywfH::lacZ::erm*); (■)NAO13 (Δ*phrF163::cat ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

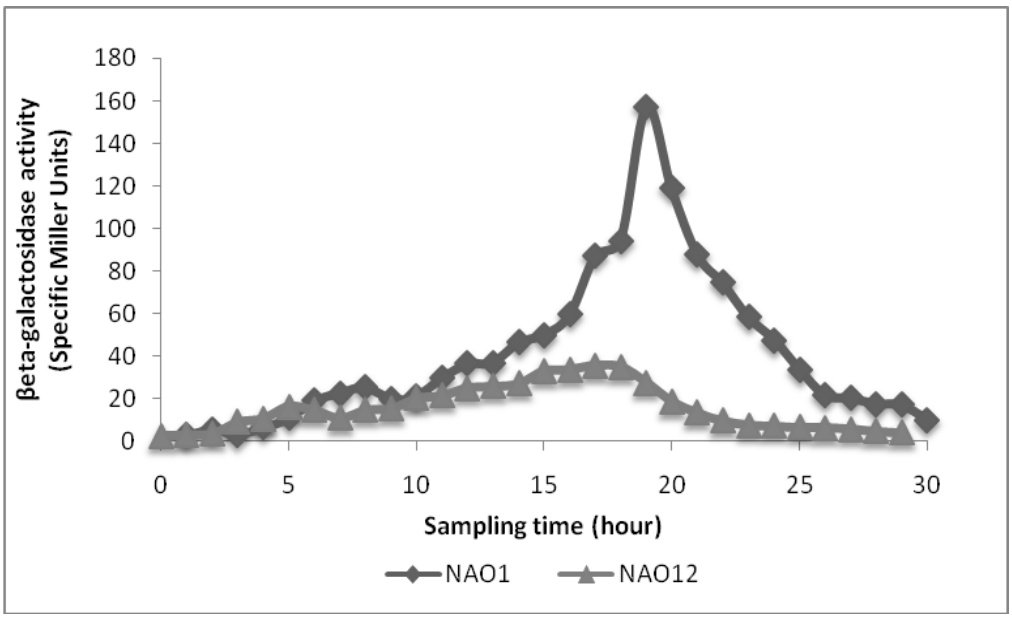


Figure 3.11: β-Galactosidase activities of NAO12 (*ywfH::lacZ::erm* Δ*phrC::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (▲) NAO12 (*ywfH::lacZ::erm* Δ*phrC::erm*) and (♦)NAO1 (*ywfH*::*lacZ::erm*).

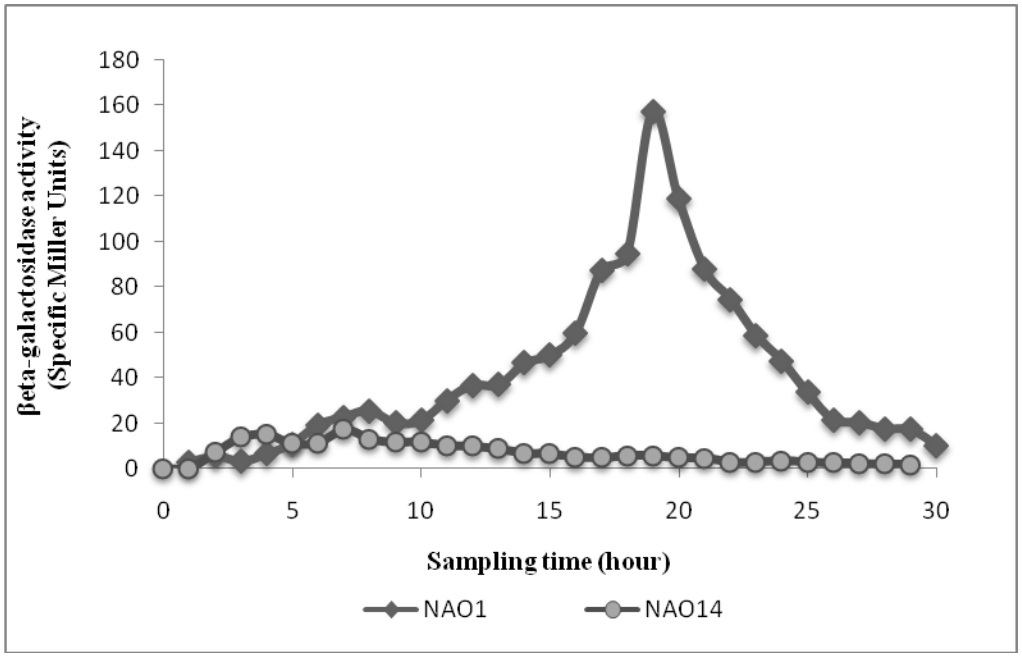


Figure 3.12: β-Galactosidase activities of NAO14 (Δ*phrK::spc ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (●)NAO14 (Δ*phrK::spc ywfH::lacZ::erm*) and (♦)NAO1 (*ywfH*::*lacZ::erm*).

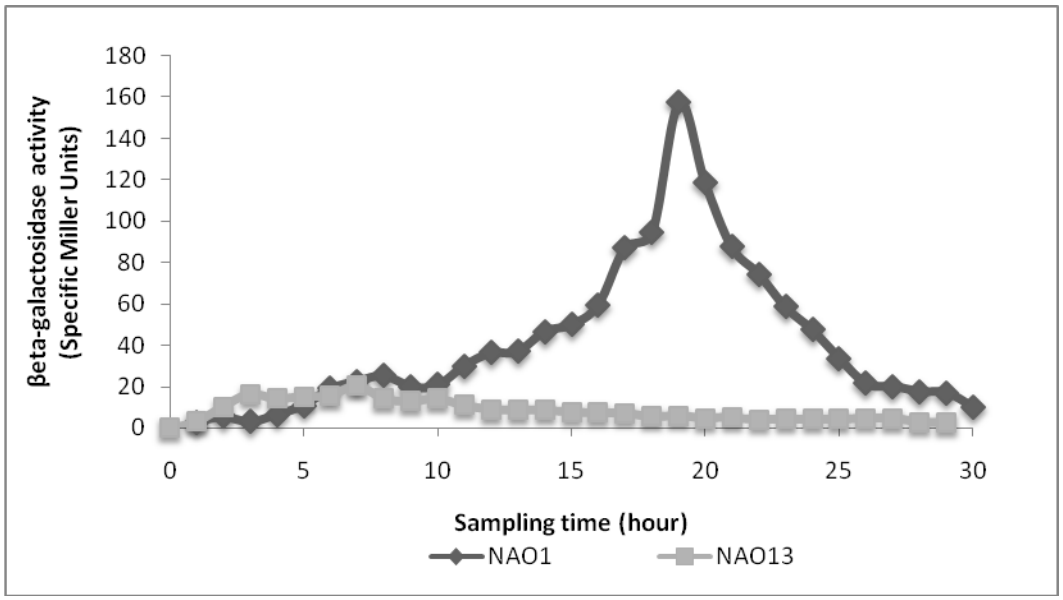


Figure 3.13: β-Galactosidase activities of NAO13 (Δ*phrF163::cat ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (■) NAO13 (Δ*phrF163::cat ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

As seen in Figure 3.11, Figure 3.12 and Figure 3.13 the deletion mutation of *phrC*, *phrF* and *phrK* genes affected *ywfH*::*lacZ* expression severely. Therefore, results in this present study shows that the PhrC, PhrF and PhrK peptides are required for the regulation of *ywfH* expression by enhancing the full activation of transcriptional factor ComA~P in bacilysin biosynthesis.

3.4.4 Deletion of *comQ(comX), comP, comA* and *spo0H* and Their Effects on the Expression of *ywfH* gene in *B. subtilis*

Genes of a two-component regulatory system, *comA* and *comP* are located at downstream of *comX* gene the on the chromosome of *B. subtilis*. The upstream gene *comQ* is needed for producing a modified peptide pheromone in the system. The ComQ-ComX–ComP–ComA signaling pathway controls the quorum response in *B. subtilis.* (Griffith and Grossman, 2008; Kleerebezem *et al.*, 1997; Weinrauch *et al.*, 1990).

ComX, which is a major extracellular signaling peptide, binds to its cognate receptor kinase, ComP, resulting in autophosphorylation of ComP at a conserved histidine residue. Then, phosphorylated ComP donates phosphate to its cognate response regulator, ComA, on a conserved aspartate residue. Once phosphorylated, ComA~P

functions to activate transcription of target genes such as *srfA, degQ, rapA* and *rapC* (Solomon *et al.*,1995 and 1996; Msadek *et al.*, 1999; Griffith and Grossman, 2008).

spo0H gene which encodes σ^H, is involved in many cellular responses. The transcription of *phrC* is partly controlled via sigma factor σ^H dependent promoter (P2) located at upstream of *phrC* and internal to *rapC*. Defects in production of some of signaling peptides in *spo0H* mutants are indicated that several cellular responses are modulated by population density and by controlling effect of sigma H on the expression of the *phr* genes by sensing extracellular peptides (Rudner *et al.*, 1991; Ireton et al., 1993; Lazazzera et al., 1997; McQuade *et al.*, 2001). Beside the role of σ^H in *phrC* transcription, it is indicated that it has at least one other role in production of CSF. Therefore, σ^H, respectively, play a key role in the initiation of sporulation (Lazazzera *et al.*, 1999).

The effects of *comQ, comP, comA* and *spo0H* genes on *ywfH* expression were monitored. For this purpose, *comQ, comP, comA* and *spo0H* genes were insertionally deleted in NAO1 that was used as competent cell and transformed by JMS315 (*trpC2 pheA1* Δ*comQ*::*spc*), BD1658 (Δ*comP*::*spc*), JRL192 (Δ*comA*::*cat*) and JMS175(Δ*spo0A*::*spc*) chromosomal DNAs respectively. Resulting transformants were named as NAO11 (Δ*comQ::cat ywfH::lacZ::erm),* NAO10 (Δ*comP::spc ywfH::lacZ::erm*), NAO9 (Δ*comA::cat ywfH::lacZ::erm*) and NAO4 (Δ*spo0H*::*spc ywfH::lacZ::erm*).

The resulting strains NAO11, NAO10, NAO9, NAO4 and NAO1, as control, were cultured in PA medium and the specific activities of the samples were measured. Also growth of mutants was followed (Fig. 3.14, 3.15, 3.16, 3.17, 3.18).

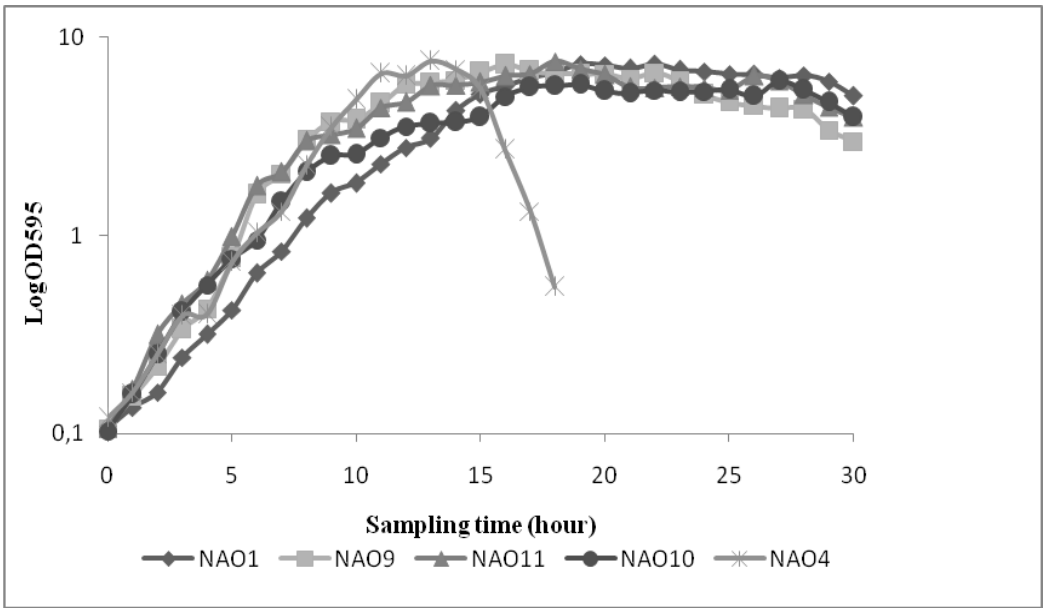


Figure 3.14: Growth profile of NAO11 (Δ*comQ::cat ywfH::lacZ::erm*), NAO10 (Δ*comP::spc ywfH::lacZ::erm*), NAO9 (Δ*comA::cat ywfH::lacZ::erm*), NAO4 (Δ*spo0H*::*spc ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (▲) NAO11 (Δ*comQ::cat ywfH::lacZ::erm*); (●) NAO10 (Δ*comP::spc ywfH::lacZ::erm*); (■) NAO9 (Δ*comA::cat ywfH::lacZ::erm*); (*) NAO4 (Δ*spo0H*::*spc ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

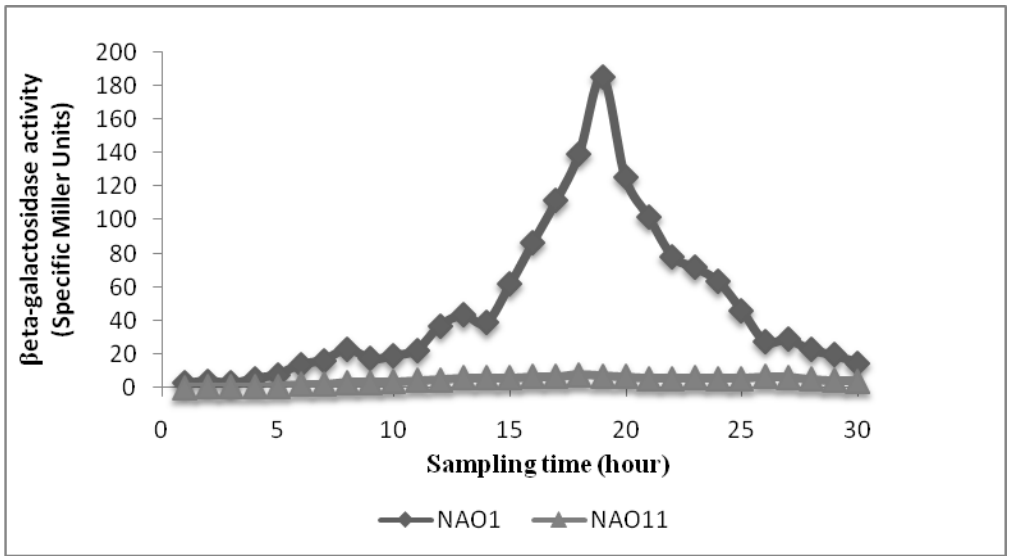


Figure 3.15: β-Galactosidase activities of NAO11 (Δ*comQ::cat ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (▲) NAO11 (Δ*comQ::cat ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

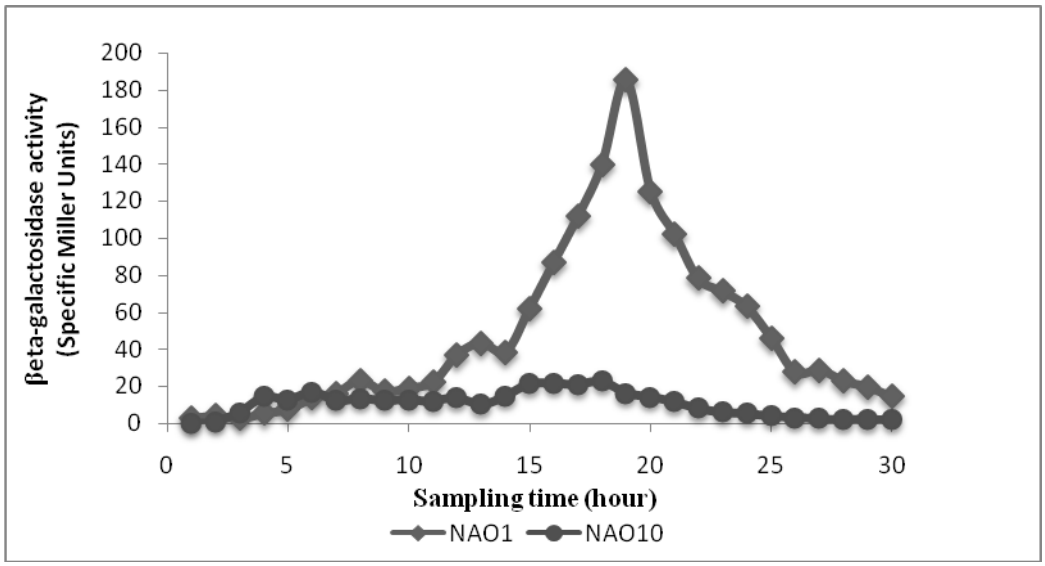


Figure 3.16: β-Galactosidase activities of NAO10 (Δ*comP::spc ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (●) NAO10 (Δ*comP::spc ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

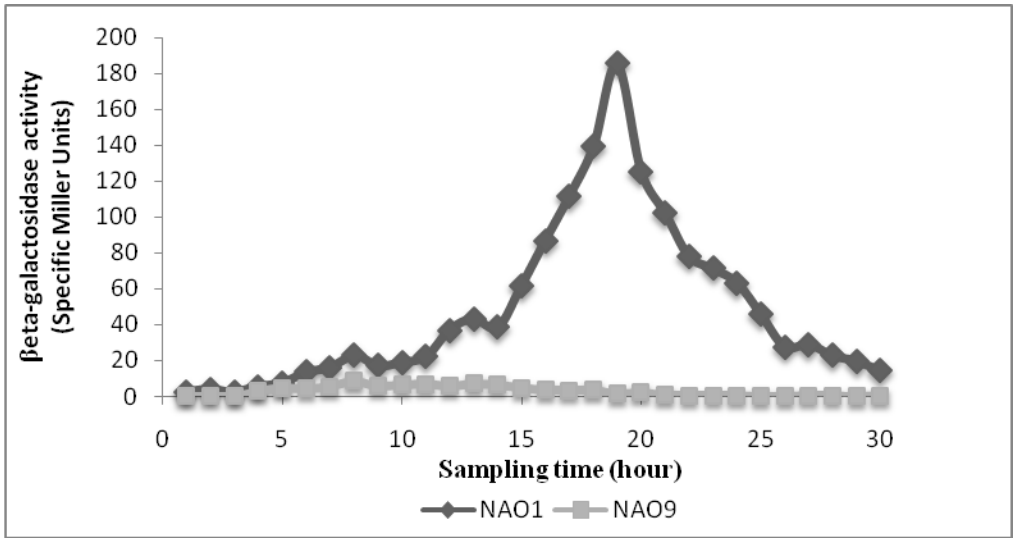


Figure 3.17: β-Galactosidase activities of NAO9 (Δ*comA::cat ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (■) NAO9 (Δ*comA::cat ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

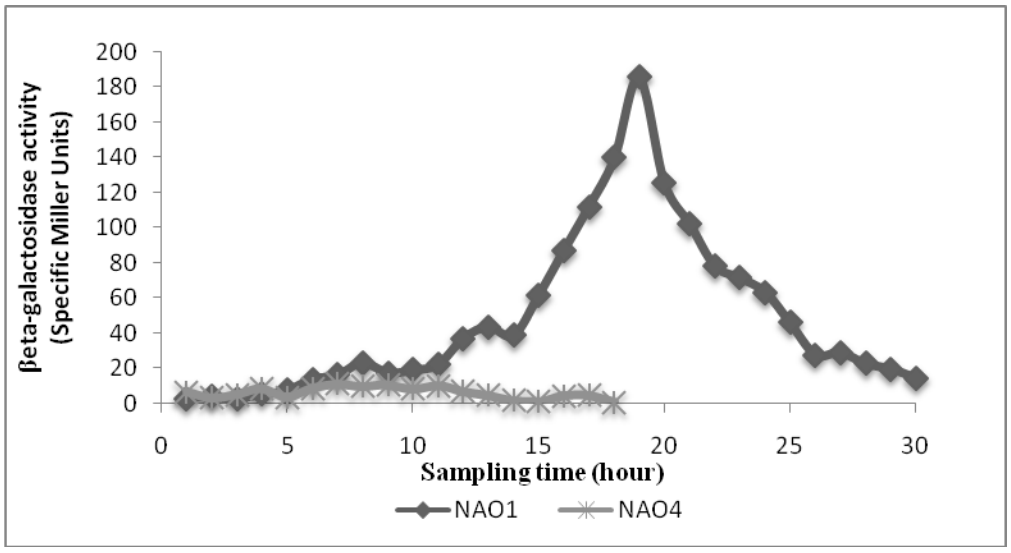


Figure 3.18: β-Galactosidase activities of NAO4 (Δ*spo0H*::*spc* *ywfH*::*lacZ*::*erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (*) NAO4 (Δ*spo0H*::*spc* *ywfH*::*lacZ*::*erm*) and (♦) NAO1 (*ywfH*::*lacZ*::*erm*).

As shown in Figure 3.15, 3.16, 3.17, 3.18, the expression of *ywfH* was impaired completely in case of the deletion of *comA*, *comP*, *comQ* and *spo0H* genes. ComP and ComX(ComQ) were required for signal transduction system that activate ComA and also σ^H required for *phr* gene expression during transition state initiating signal transduction cascade for ComA~P-dependent gene expression as quorum response. A former study also indicated that inactivation of *spo0H* gene in the strain PY79 resulted in the loss of bacilysin biosynthetic ability (Karataş *et al.*, 2003). It can be suggested that the expression of *ywfH* gene regulation might be ComA-dependent. Therefore, ComA, ComP, ComQ and σ^H can regulate biosynthesis of bacilysin by effecting the expression of *ywfH* gene.

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

Spo0A is a classical two domain response regulator that is required for initiation of sporulation in *Bacillus subtilis*. After activation, Spo0A can act as both a transcription repressor and a transcription activator (Seredick and Spiegelman, 2007). On the other hand, additional genes involved in production of mature CSF is probably also controlled by *spo0A* and *abrB*. These genes have much greater effects

on accumulation of extracellular CSF than transcription of *phrC* (Lazazzera *et al.*, 1999).

AbrB represses many stationary phase processes in *Bacillus subtilis*. Especially genes involved in sporulation (e.g. *spo0E, spoVG*), degradative enzyme synthesis (e.g. *aprE*), amino acid utilization (e.g. *dpp*), and antibiotic production (e.g. *tycA*) . The presence of AbrB during exponential growth prevents premature expression of these genes.

Expression of *abrB* declines as a result of transcriptional repression by the activated response regulator Spo0A towards the end of exponential growth. Moreover, in the absence of Spo0A, AbrB becomes overproduced, and the expression of many stationary phase genes, among which found competence genes, is inhibited (Hamoen *et al.*, 2003).

To monitor the effects of *spo0A* and *abrB* null mutations on *ywfH* expression, *spo0A* and *abrB* genes were inactivated in NAO1. For this purpose, firstly, NAO1 competent cells were used for the transformation of chromosomal DNAs of BAL373 (*trpC2 pheA1* Δ*abrB*::*cat*) and JMS175 (Δ*spo0A*::*spc*) mutant derivatives. The selected mutants with regard to specific antibiotic resistance were named as NAO5 (Δ*abrB::cat ywfH::lacZ::erm*), NAO3 (Δ*spo0A*::*spc ywfH::lacZ::erm*) and NAO7 (Δ*spo0A::spc* Δ*abrB::cat ywfH::lacZ::erm*). The expression of *ywfH*::*lacZ* fusion was analyzed in *spo0A-abrB* double mutant genetic background for detection of the effect of absence of both repressor proteins that repress different genes in sequential stages. *spo0A-abrB* double mutant strain NAO7 (Δ*spo0A::spc* Δ*abrB::cat ywfH::lacZ::erm*) was constructed by transforming NAO5 (Δ*abrB::cat ywfH::lacZ::erm*) competent cells with the chromosomal DNA of JMS175 (Δ*spo0A*::*spc*). Transformants were selected on agar plates containing antibiotics Cat (20 μg/ml) and Spc (100μg/ml). In addition step, this double mutant strain (NAO7) was performed on DSM (Appendix A) agar plate in order to detect sporulation ability.

The resulting strains NAO5, NAO3, NAO7, and NAO1, as control, were cultured in PA medium and the specific activities of the samples were measured. Growth profile of mutants was also followed (Fig. 3.19, 3.20, 3.21, 3.22).

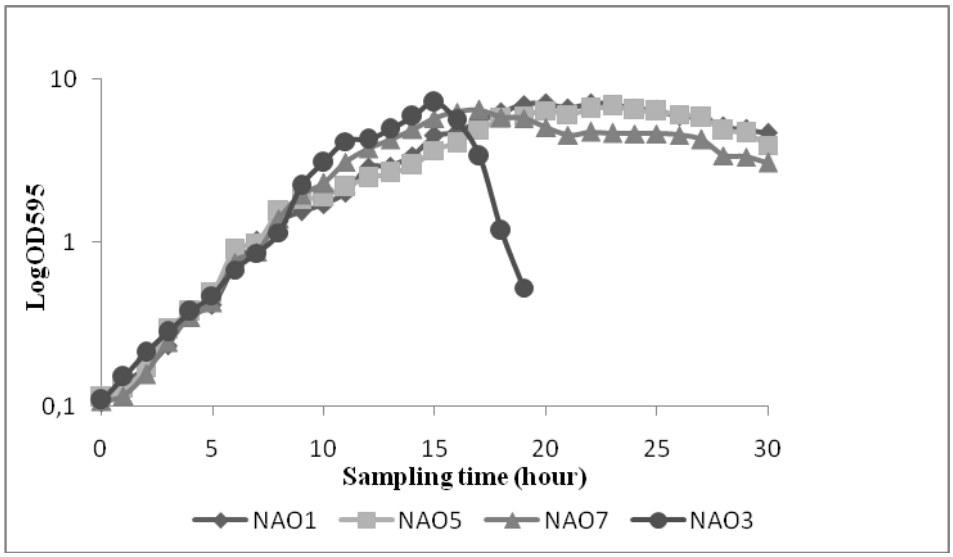


Figure 3.19: Growth profile of NAO5 (Δ*abrB::cat ywfH::lacZ::erm*), NAO3 (Δ*spo0A*::*spc ywfH::lacZ::erm*), NAO7 (Δ*spo0A::spc* Δ*abrB::cat ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (▲) NAO7 (Δ*spo0A::spc* Δ*abrB::cat ywfH::lacZ::erm*); (●)NAO3 (Δ*spo0A*::*spc ywfH::lacZ::erm*); (■) NAO5 (Δ*abrB::cat ywfH::lacZ::erm*); (♦) NAO1 (*ywfH*::*lacZ::erm*).

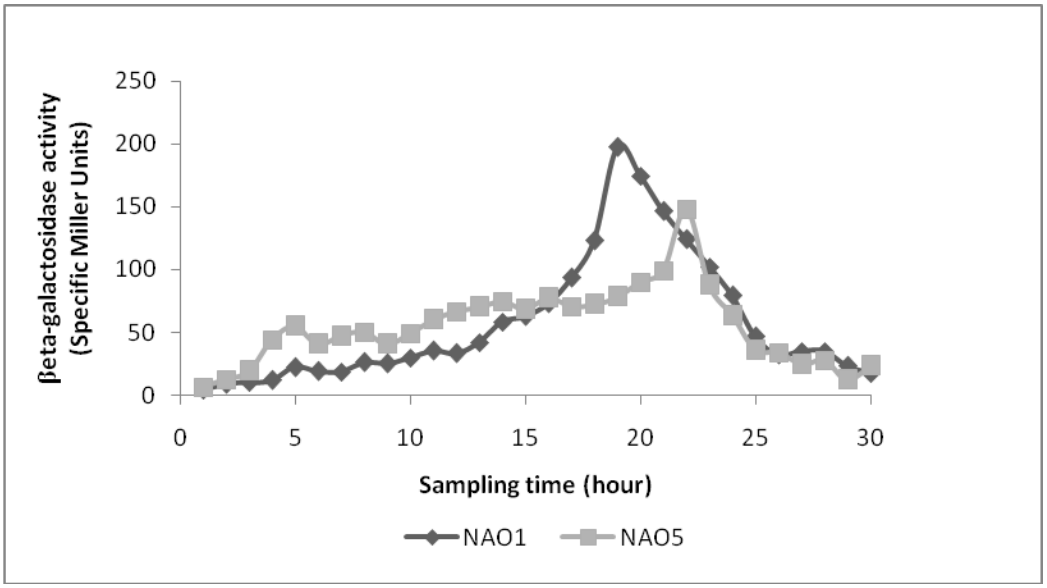


Figure 3.20: β-Galactosidase activities of NAO5 (Δ*abrB::cat ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (■)NAO5 (Δ*abrB::cat ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

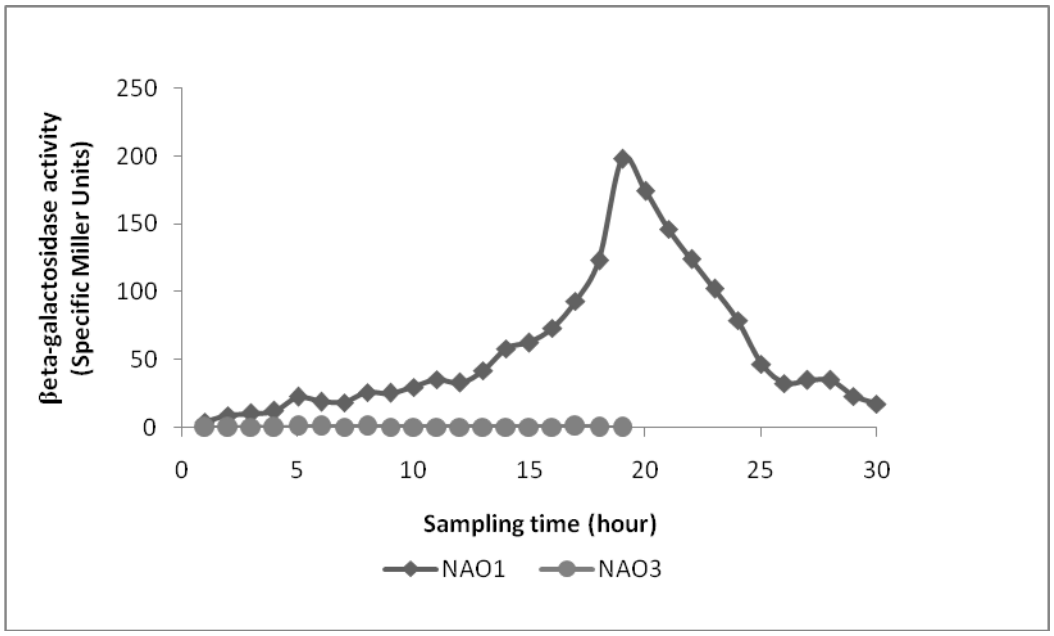


Figure 3.21: β-Galactosidase activities of NAO3 (Δ*spo0A*::*spc ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (●)NAO3 (Δ*spo0A*::*spc ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

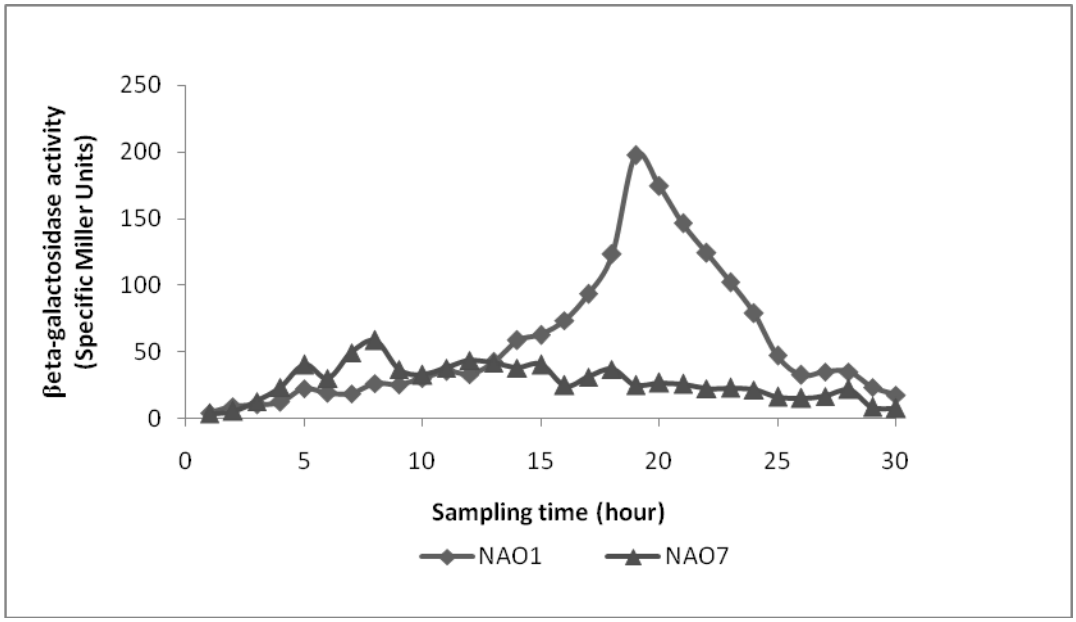


Figure 3.22: β-Galactosidase activities of NAO7 (Δ*spo0A::spc* Δ*abrB::cat ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (▲) NAO7 (Δ*spo0A::spc* Δ*abrB::cat ywfH::lacZ::erm*) and (♦) NAO1 (*ywfH*::*lacZ::erm*).

As seen in *abrB* null mutation, in stationary phase, the expression of *ywfH*::*lacZ* was not severely repressed, there was only a decrease from 197 to 148 Spesific Miller

Units (Figure 3.20). As shown in Fig. 3.21 the expression of *ywfH*::*lacZ* was fully eliminated by deletion of *spo0A* gene. Former studies also showed that *spo0A*, *spo0B* and *spo0H* null mutants were unable to synthesize bacilysin. Also *spo0A* defected mutants of *Bacillus subtilis* were deficient in the synthesis of many antimicrobials and in resistance to a number of antibiotics (Yazgan *et al.*, 2001; Strauch, 1993; Trowsdale *et al.*, 1978). Therefore, this present study pointed that *spo0A* gene was dramatically necessary for *ywfH* gene expression. On the other hand, in case of double mutant, it was seen that disappeared *ywfH* expression was partially relieved by the null mutation of *abrB* which was observed in Figure 3.22. Additionally, the presence of AbrB during exponential growth prevents premature expression of genes in antibiotic production (Hamoen *et al.*, 2003). Consistently, the expression of *ywfH* gene increased during exponential phase in the absence of AbrB. Indicated that *ywfH* expression is repressed by *abrB* during exponential phase (Figure 3.22). This present study also showed that both AbrB and Spo0A were required for the induction as well as maximum *ywfH* expression occurs at the onset of stationary phase.

3.4.6 Deletion of *codY* Gene and Its Effect on the Expression of *ywfH* in *Bacillus subtilis*

CodY, a GTP-binding protein that was first identified as a repressor of the Bacillus subtilis dipeptide permease (dppABCDE) operon. CodY regulates more than 100 genes. The products of these genes are generally involved in the adaptation of bacterium to poor growth conditions. The repressor function of CodY is activated by interaction with two different effectors, GTP and isoleucine (Roggiani and Dubnau, 1993). Under slow-growth conditions like carbon limitation or as stationary phase approaches, the GTP level decreases because of ppGpp production or a reduction in GTP synthesis. In this case, the genes that are under CodY repression become actively transcribed because CodY loses its repressor activity (Serror and Sonenshein, 1996; Molle *et al.*, 2003; Shivers and Sonenshein, 2004; Ratnayake-Lecamwasam *et al.*, 2001).

The effect on competence can be attributed to the binding of CodY to the regulatory regions of two key early competence genes, *srfAA* and *comK*. CodY binding site in the *srfA* promoter region overlaps with the presumed RNA polymerase binding site but not with the ComA binding sites. Furthermore, *srfA* expression is proposed to be

repressed by CodY in the presence of casamino acids whether or not ComA is active. (Roggiani and Dubnau, 1993).

In order to monitor the effect of *codY-null* mutation on the expression of *ywfH* gene, *codY* was deleted in NAO1 (*ywfH*::*lacZ::erm*) by transforming the competent cells of *trpC2 unkU::spc* Δ*codY* mutant strain of PY79 (constructed by transforming the competent cells of *Bacillus subtilis* PY79 with chromosomal DNA of TMH307) with chromosomal DNA of the NAO1 strain with the selection for Erm (1μg/ml) and Spc (100μg/ml). The transformants were then screened on X-gal (40 μg/ml) IPTG, Erm (1μg/ml) and Spc (100μg/ml) containing agar plates for the confirmation of *lacZ* activity. One of the blue transformants was selected and designated as NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*).

Then codY-abrB double mutant strain was constructed by using NAO6 as competent cell. NAO6 was transformed with chromosomal DNA of Δ*abrB*::*cat* mutant strain derivative of *B. subtilis.* The transformants were screened for double antibiotic resistance to Spc (100 μg/ml) and Cat (5 μg/ml). The selected mutant was named as NAO8 (Δ*abrB::cat ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*).

In order to also examine the nutritional regulation system, in the absence of *codY* gene, casaminoacid (casein) addition was applied. So casein was added into growth medium of NAO6 with 0.1% final concentration. The resulting strain NAO6, NAO8, NAO6 with casein and NAO1 as control were cultured in PA medium and the specific activities of the samples were measured. Growth profile of mutants was followed (Fig. 3.23, 3.24, 3.25, 3.26).

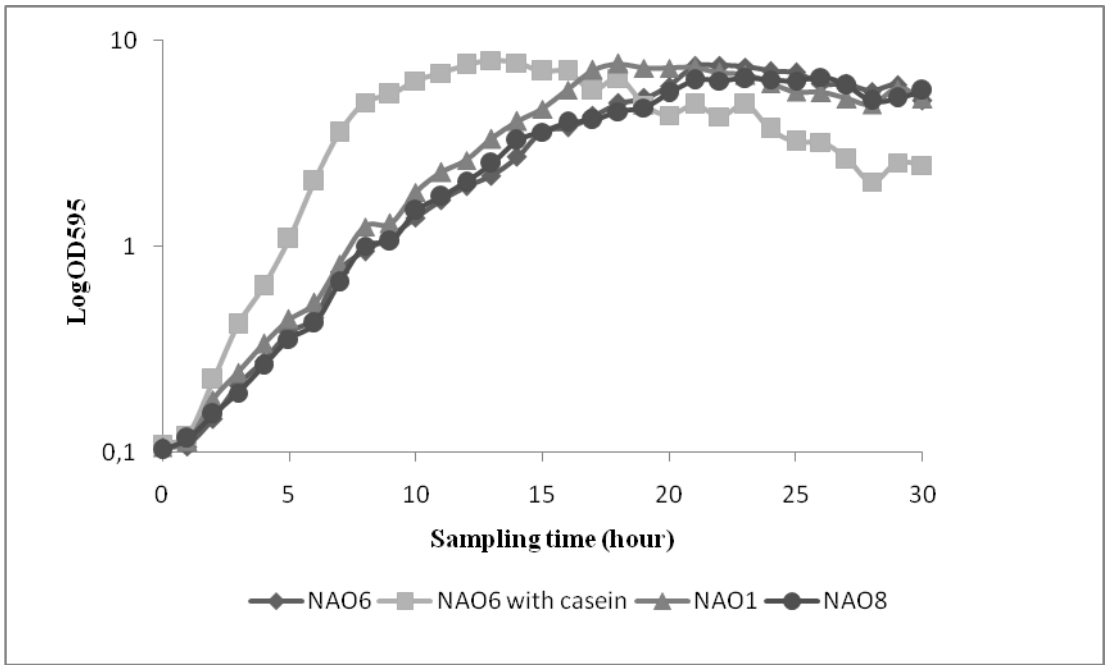


Figure 3.23: Growth profile of NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*), NAO8 (Δ*abrB::cat ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) , NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) with casein and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (▲) NAO1 (*ywfH*::*lacZ::erm*); (●) NAO8 (Δ*abrB::cat ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*); (■)NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) with casein; (♦)NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*).

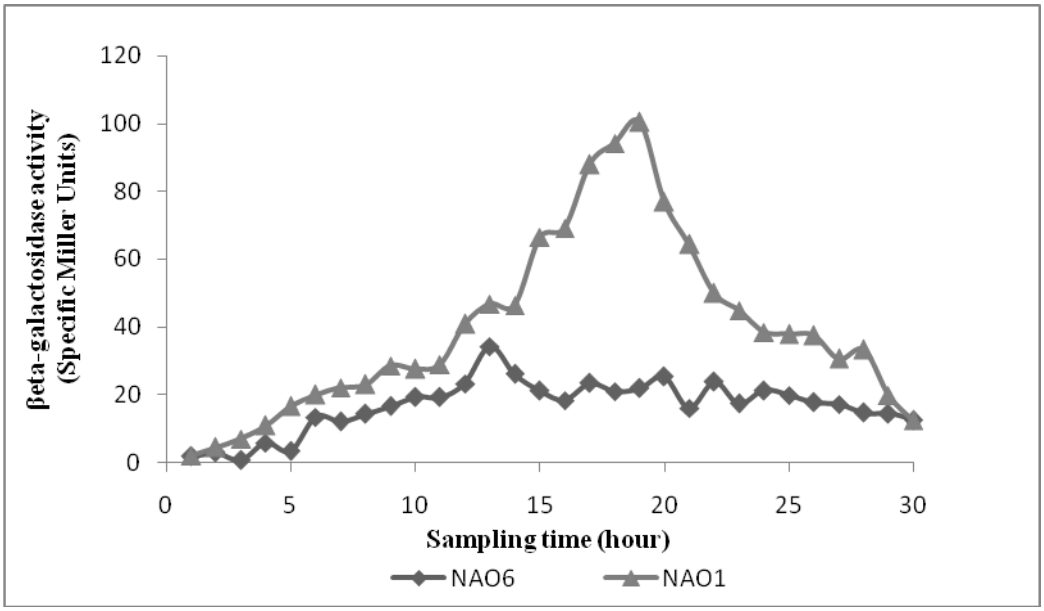


Figure 3.24: β-Galactosidase activities of NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (▲)NAO1 (*ywfH*::*lacZ::erm*) and (♦)NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*).

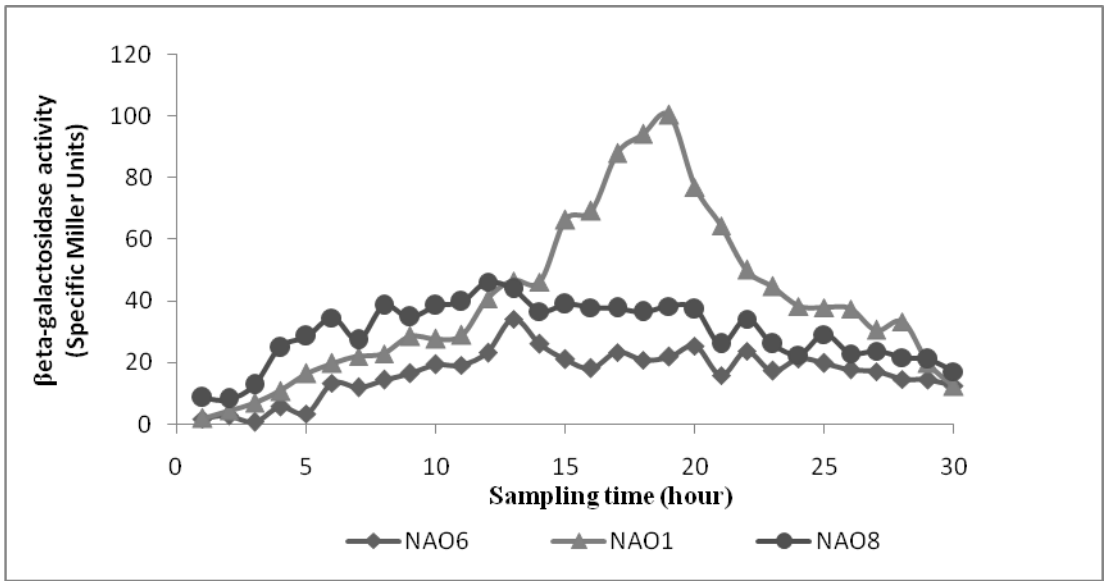


Figure 3.25: β-Galactosidase activities of NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*), NAO8 (Δ*abrB::cat ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (▲) NAO1 (*ywfH*::*lacZ::erm*), (●) NAO8 (Δ*abrB::cat ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) (♦)NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*).

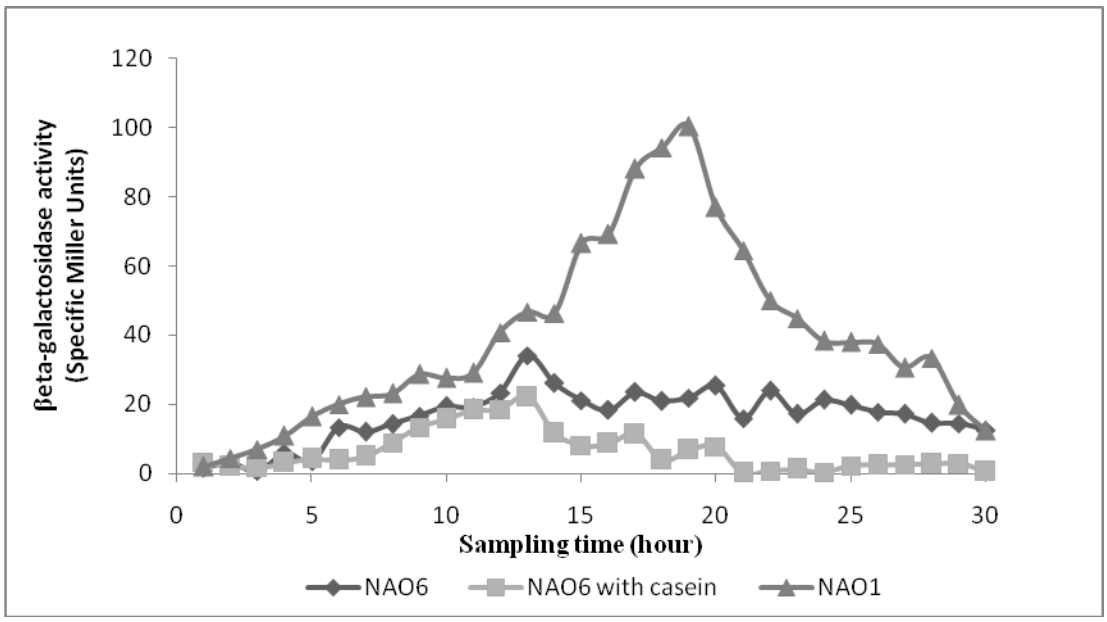


Figure 3.26: β-Galactosidase activities of NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) with casein, NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. Specific Miller Units was calculated with formula as denoted in Section 2.3. The symbols used for the strains are; (▲) NAO1 (*ywfH*::*lacZ::erm*), (■) NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*) with casein (♦) NAO6 (*ywfH::lacZ::erm trpC2 unkU*::*spc* Δ*codY*).

As seen in Figure 3.24 in *codY*-disrupted strain, *ywfH* expression severely decreased during stationary phase but did not become completely eliminated. But, this finding

was inconsistent with results of Inaoka *et al*. (2003) who reported that disruption of *codY* showed a positive effect on *ywfH* transcription. As shown in figure, the expression of *ywfH* gene in Δ*abrB* Δ*codY* double mutant (NAO8) was more than in Δ*codY* mutant (NAO6). Because β-Galactosidase activity of NAO8 might have pointted that a further *abrB* gene null mutation resulted in a relief *on* repression profile for *ywfH* gene expression in case of *codY* deleted mutant. Therefore, absence of AbrB, which is a repressor for the expression of genes in antibiotic production during exponential phase, has induced *ywfH* gene expression especially until the onset of stationary phase (Figure 3.25). Considering the fact that CodY is a very strong element of nutritional regulation circuit, as shown in Figure 3.26, addition of casein into medium of NAO6 showed that casein has a suppressive effect on β-Galactosidase activity of NAO6 during stationary phase as expected. Consequently, it could have been said that *ywfH* gene is probably under control of CodY, effect of which is complemented by AbrB protein.

3.4.7. Deletion of *degU* Gene and Its Effect on the Expression of *ywfH* Gene in *Bacillus subtilis*

The DegS/DegU two-component system provides *B. subtilis* with a developmental switch between different adaptive processes. The two-component regulatory system DegS/DegU plays an important role in the regulation of secreting a large number of degradative enzymes during the transition from exponential to stationary growth in *Bacillus subtilis.* Phosphorylation of DegU is required for the production of degradative enzymes, but not for competence development (Hamoen *et al*., 2003; Msadek et al., 1995). On the other hand, *degU* mutation decreases the expression of *srfA*, an essential competence regulatory gene (Dubnau, 1991; Dahl *et al.*, 1992).

In order to examine the effect of *degU* null mutation, *degU* gene was deleted in the transcriptional *ywfH*::*lacZ* fusion strain NAO1. For this purpose, NAO1 was used as competent cell, then was transformed with the chromosomal DNA of Δ*degU*::*kan* mutant derivative of *B. subtilis.* The transformants were selected with regard to specific antibiotic resistance for Kan (10 μg/ml). Then selected mutant was named as NAO16 (Δ*deg U::kan ywfH::lacZ::erm*).

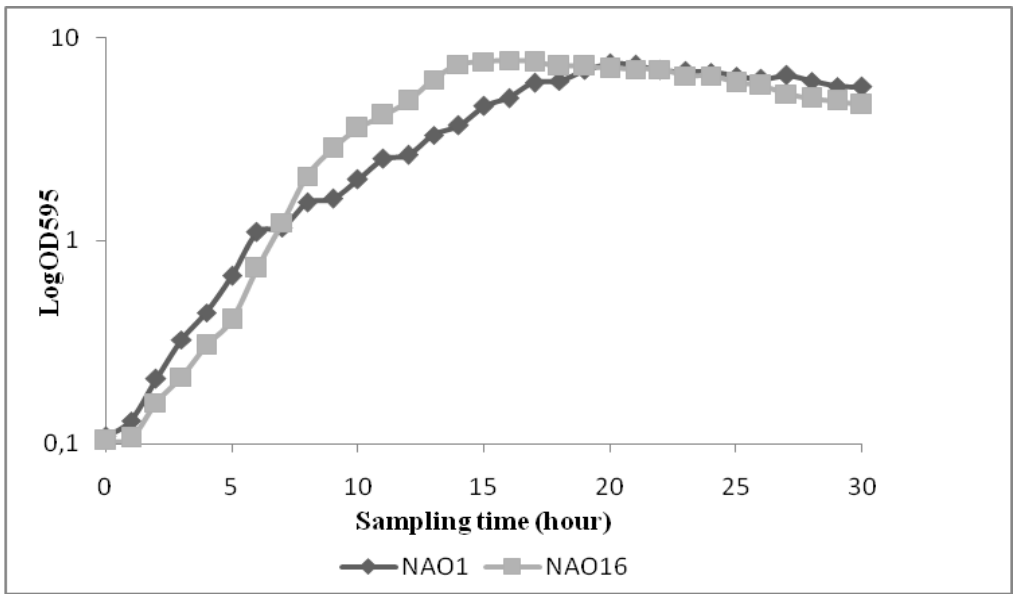


Figure 3.27: Growth profile of NAO16 (Δ*deg U::kan ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (■) NAO16 (Δ*deg U::kanywfH::lacZ::erm*); (♦)NAO1 (*ywfH*::*lacZ::erm*).

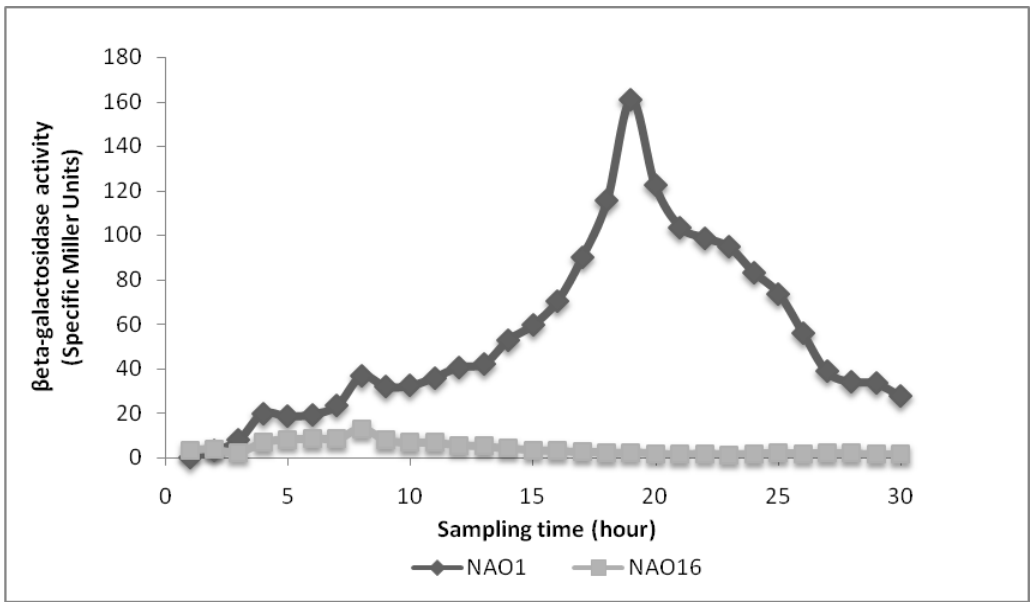


Figure 3.28: β-Galactosidase activities of NAO16 (Δ*deg U::kan ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (■) NAO16 (Δ*deg U::kanywfH::lacZ::erm*); (♦)NAO1 (*ywfH*::*lacZ::erm*).

As seen in Fig 3.28, *ywfH* expression was almost completely blocked when *degU* was deleted. DegU is an effective positive regulator of *srf* operon, so, it was proposed that the expression of *ywfH* gene has required not only *degU* gene but also *srfA* gene.

3.4.8. Deletion of *sigmaB* Gene and Its Effect on the Expression of *ywfH* Gene in *Bacillus subtilis*

Transcription factor, σ^B was the first alternate sigma factor to be discovered in bacteria which is active during the stationary growth phase but its function is obscure. Transcription of the *sigB* gene begins in the early stationary phase when metabolic stress conditions or environmental signals are encountered (Benson and Haldenwang, 1992; Boylan *et al.*, 1992).

In order to monitor the effect of *sigB* null mutation, *sigB* gene was deleted in the transcriptional *ywfH*::*lacZ* fusion strain NAO1. For this purpose, NAO1 was used as competent cell, then was transformed with thw chromosomal DNA of Δ*ML6*::*cat* (*sigB*::cat) mutant derivative of *B. subtilis.* The transformants were selected with regard to specific antibiotic resistance for Cat (5µg/ml). Later on, the selected mutant was named as NAO17 (Δ*ML6::cat ywfH::lacZ::erm*)

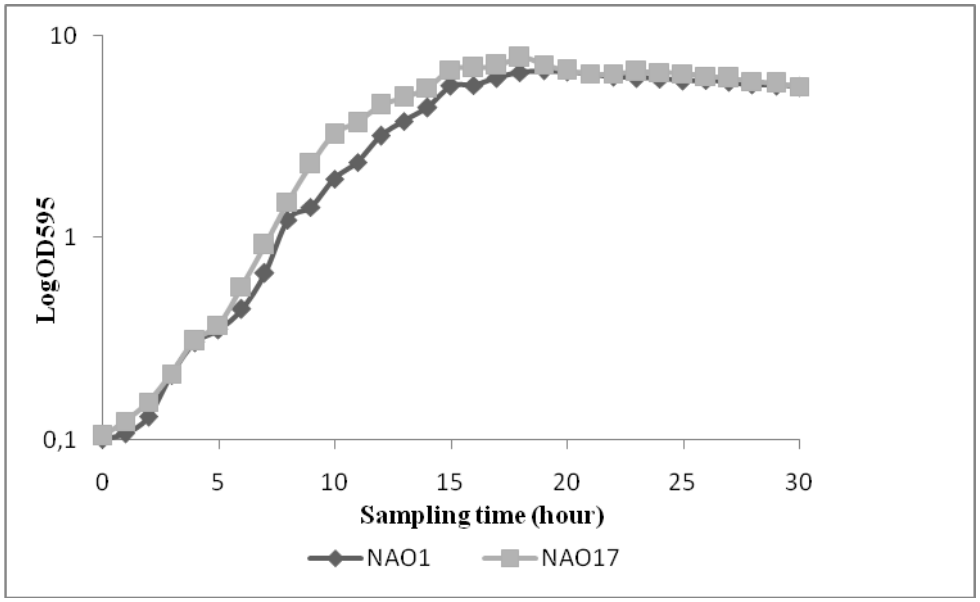


Figure 3.29: Growth profile of NAO17 (Δ*ML6::cat ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (■) NAO17 (Δ*ML6::cat ywfH::lacZ::erm*); (♦)NAO1 (*ywfH*::*lacZ::erm*).

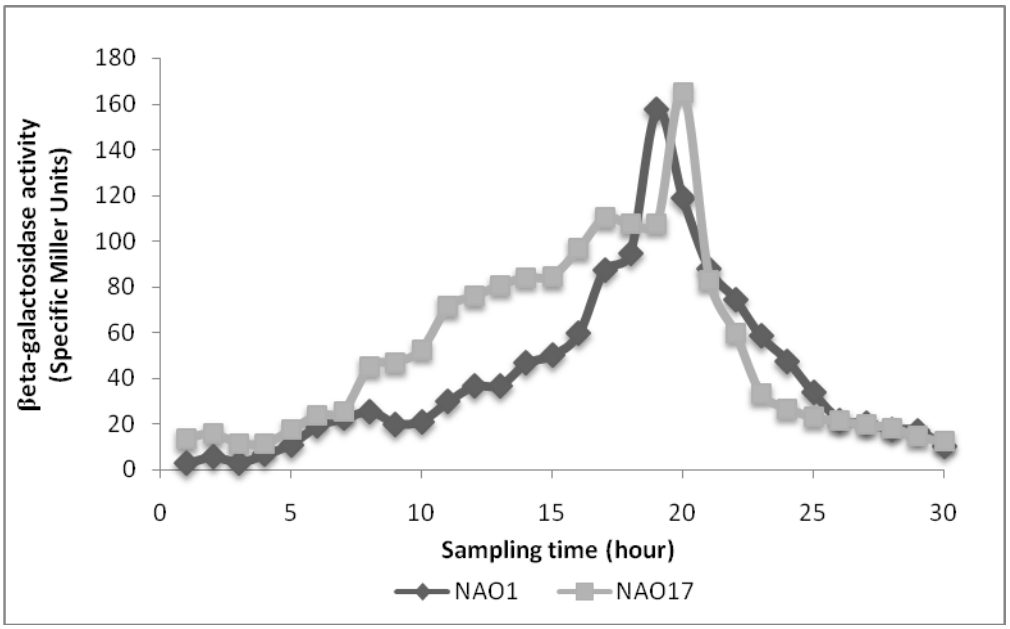


Figure 3.30: β-Galactosidase activities of NAO17 (Δ*ML6::cat ywfH::lacZ::erm*) and NAO1 (*ywfH*::*lacZ*::*erm*) strains grown in PA medium. The symbols used for the strains are; (■)NAO17 (Δ*ML6::cat ywfH::lacZ::erm*); (♦)NAO1 (*ywfH*::*lacZ::erm*).

As observed in Fig. 3.30, *sigB* deletion mutation effected *ywfH* gene expression positively. Nevertheless, the increase was not very considerable. On the other hand, σ^B was required in response to stress conditions. With these facts, function of SigmaB was not considerable for *ywfH* expression.

4. CONCLUSION

In this study, *ywfH::lacZ* fusion mutant, NAO1 was constructed and the expression of *ywfH::lacZ* was observed by the help of β-galactosidase assay in PA medium. The results showed that the expression of *ywfH* increased slowly during the exponential phase and reached to maximal level upon entry into stationary phase in PA medium.

In order to monitor the effects of global regulatory genes on the expression of *ywfH* gene, these genes were deleted on the *ywfH::lacZ* fusion mutant, NAO1 by the transformation and *ywfH*-directed β-galactosidase activities were subsequently analyzed.

The disruption of *srfA* gene completely abolished *ywfH::lacZ* expression, suggesting that the expression of *ywfH* gene depends on an intact surfactin operon. *ywfH::lacZ* expression in the *oppA* blocked mutant was severely abolished, indicating that product *of oppA* gene regulates *ywfH* gene expression as a part of quorum sensing mechnanism.

The deletion of *phrC, phrF* and *phrK* genes impaired *ywfH-lacZ* expression in NAO 12, NAO13 and NAO 14 respectively. PhrC, PhrF and PhrK pheromone peptides are required for the expression of *ywfH* gene by enhancing the full activation of transcriptional factor ComA~P.

In the result of *comA*, *comP*, *comQ* and *spo0H* genes deletion, *ywfH::lacZ* expression was entirely impaired, indicating that products of those genes has regulating effects on the expression of *ywfH* gene. ComP and ComX(ComQ) are required for phosphorylation and activation of ComA. On the other hand, the product of *spo0H*, σ^H is required for increased level of *phr* gene during transition state for inducing the activation of ComA. With these facts, It is sensible to propose that the transcription of *ywfH* gene is ComA~P-dependent.

The deletion of *spo0A* gene resulted in the fully elimination of *ywfH::lacZ* expression and this defect was relieved by a null mutation in *abrB* during exponential

growth which also was partially relieved during the stationary phase in *spo0A-abrB* double mutant. Otherwise, in *abrB* single mutant, the expression of *ywfH* gene increased with the absence of AbrB during exponential phase but not during stationary phase compared to NAO1. This present study generally showed that AbrB and Spo0A were required for the induction as well as the maximum *ywfH* expression occurs at the onset of stationary phase. *ywfH::lacZ* expression decreased in *codY*-deleted mutant during exponential phase, and moreover this deletion strongly caused a decrease in *ywfH::lacZ* expression during the stationary phase. With the deletion of both *codY* and *abrB* genes, the expression of *ywfH* gene was relieved during exponential and stationary phase compared to *codY*-deleted single mutant. In order to identify the nutritional regulation in the absence of *codY* gene, casein was added as supplementery nutrient source in PA medium. The addition of casein into medium showed that casein had a suppressive effect on β-Galactosidase activity in *ywfH::lacZ* strain during stationary phase. Under the light of these results, it was proposed that CodY repressor which plays a key role in the initation of sporulation and genetic competence also plays a negative role in the expression of *ywfH* gene and this effect might be assisted by AbrB repressor protein.

As a result of *degU* gene deletion in NAO1 strain, the expression of *ywfH* was almost fully blocked. Therefore, we suggested that DegU positively regulates the expression of *ywfH* gene. Finally, *sigB* deletion mutation did not make a considerable difference in *ywfH* expression.

REFERENCES

Abraham, L., Sonenshein A., L., 2005. CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria, *Current Opinion in Microbiology*, **8**, 203–207.

Auchtung, J. M., Lee, C. A., and Grossman A. D., 2006. Modulation of the ComA dependent quorum response in *Bacillus subtilis* by multiple Rap proteins and Phr peptides. *J Bacteriol*, **188-14**, 5273-5285.

Basalp, A., Özcengiz, G., and Alaeddinoglu, N. G., 1992. Changes in patterns of alkaline serine protease and bacilysin formation caused by common effectors of sporulation in *Bacillus subtilis* 168. *Curr Microbiol.*, **24**, 129-135.

Benson, A., K., Haldenwang, M., G., 1992. *Bacillus Subtilis* σ^B is Regulated by a Binding Protein (Rsbw) That Blocks its Association with Core RNA Polymerase, *Proc. Natl. Acad. Sci. USA,* **Vol. 90**, Pp. 2330-2334

Boylan, S. A., Redfield, A. R., and Price, C. W. , 1992. Transcription Factor σ^B of Bacillus subtilis Controls a Large, Stationary-Phase Regulon, *Journal of Bacteriology*, P. 3957-3963.

Chmara, H., Woynarowska, B., Borowski, E., 1981. Epoxypeptide antibiotic tetaine mimics peptides in transport to bacteria. *J Antibiot,* **34**, 1608–1612.

Cosby, W. M., Vollenbroich, D., Lee, O. H., and Zuber, P., 1998. Altered srf Expression in *Bacillus subtilis* Resulting from Changes in Culture pH Is Dependent on the Spo0K Oligopeptide Permease and the ComQX System of Extracellular Control, *Journal of Bacteriology*, **Vol. 180**, 1438-1445.

Dahl, S., Msadek, T., Kunst, F., and Rapoport, G., 1992. The Phosphorylation State of the DegU Response Regulator Act as a Molecular Switch Allowing Either Degradative Enzyme Synthesis or Expression of Genetic Competence in *Bacillus subtilis*, *J. Biol. Chem.*, **Vol. 267**, 14509-14514.

Dubnau, D., 1991. Genetic competence in *Bacillus subtilis, Microbiol.Mol. Biol. Rev.*, **55,** 3, 395-424.

Duitman, E., H., **Wyczawski, D., Boven, L. G., Venema, G., Kuipers, O. P. and Hamoen, L. W.**, 2007. Novel Methods for Genetic Transformation of Natural *Bacillus subtilis* Isolates Used To Study the Regulation of the Mycosubtilin and Surfactin Synthetase, *Appl Environ Microbiol.*, **73**, 3490–3496.

Earl, A., M., Losick, R., and Kolter, R., 2008. Ecology and genomics of *Bacillus subtilis*, *Trends Microbiol.*, **Vol. 16**, 6, 269-275.

Fujiya, M., Musch, M.W., Nakagawa, Y., Hu, S., Alverdy, J., Kohgo, Y., Schneewind, O., Jabri, B., and Chang, E.,B., 2007, The *Bacillus subtilis* Quorum-Sensing Molecule CSF Contributes to Intestinal Homeostasis via OCTN2, a Host Cell Membrane Transporter. *Cell Host & Microbe*, **1**, 299–308.

Fuqua, C., Greenberg, E.P., 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Curr.Opin.Microbiol.*, **1**, 183-189.

Furbah, R., M., Gocht, M., Zuber, P., Nakano, M., M., 1991. Interaction of AbrB, a transcriptional regulator from *Bacillus subtilis*, with the promoters of the transition state-activated genes *tycA* and *spoVG*, *Mol. Gen. Genet.*, **225**, 347– 354.

Gobbetti, M., De Angelis, M., Di Cagno, R., Minervini, F. , Limitone, A., 2007. Cell–cell communication in food related bacteria, *International Journal of Food Microbiology*, **120**, 34–45.

Glaser, P., Danchin, A., Kunst, F., Zuber, P., and Nakano, M. M., 1995**.** Identification and Isolation of a Gene Required for Nitrate Assimilation and Anaerobic Growth of *Bacillus subtilis*, *Journal Of Bacteriology*, **Vol. 177**, 4, 1112-1115.

Griffith, L. K., and Grossman, A. D., 2008. A Degenerate Tripartite DNA-Binding Site Required for Activation of ComA-Dependent Quorum Response Gene Expression in *Bacillus subtilis*, *J. Mol. Biol.*, **381**, 261–275.

Guespin-Michel, J., F., 1971. Phenotypic Reversion in Some Early Blocked Sporulation Mutants of *Bacillus subtilis*: Isolation and Phenotype Identification of Partial Revertants, *Journal of Bacteriology*, **Vol. 108**, No. 1, 241-247.

Hahn, J., and Dubnau, D., 1991. Growth Stage Signal Transduction and the Requirements for srfA Induction in Development of Competence, *Journal of Bacteriology*, **Vol. 173**, No. 22, 7275-7282.

Hamoen, L. W., Kausche, D., Marahiel, M. A., van Sinderen, D., Venema, G., Serror, P., 2003. The *Bacillus subtilis* transition state regulator AbrB binds to the 335 promoter region of *comK*, *FEMS Microbiology Letters*, **218**, 299-304.

Hamoen, L. W., Venema, G., Kuipers, O. P., 2003. Controlling competence in *Bacillus subtilis*: shared use of regulators, *Microbiology*, **149**, 9–17.

Harwood, C., R., 1996. Sequencing and functional analysis of the genome of *Bacillus subtilis* strain 168. *FEBS Letters*, **389**, 84-87.

Harwood, C. R., Cutting S. M., 1990. Molecular Biological Methods for *Bacillus*, *A Wiley- Interscience Publication*, pp. 1-3.

Hilton, M., D., Alaeddinoglu, N. G., and Demain, A. L., 1988. Synthesis of bacilysin by *Bacillus subtilis* branches from the prephenate of the aromatic amino acid pathway, *J Bacteriol.*, **170**, 482-484.

Hong, H. A., Duc, L. H., and Cutting, S. M., 2005. The use of bacterial spore formers as probiotics, *FEMS Microbiol. Rev*., **29**, 813–835.

Inaoka, T., Takahashi, K., Ohnishi-Kameyama, M., Yoshida, M., and Ochi, K., 2003. Guanine nucleotides guanosine 5'- diphosphate 3'-diphosphate and GTP co-operatively regulate the production of an antibiotic bacilysin in *Bacillus subtilis*, *J Biol Chem*, **278**, 2169–2176.

Ireton, K., Rudner, D. Z., Siranosian, K. J., and Grossman, A. D., 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor, *Genes Dev.,* **7**, 283-294.

Kenig, M., Abraham, E., P., 1976**.** Antimicrobial activities and antagonists of bacilysin and anticapsin, *J Gen Microbiol*, **94**, 37–45**.**

Kenig, M., Vandamme, E., Abraham, E. P., 1976. The mode of action of bacilysin and anticapsin and biochemical properties of bacilysin-resistant mutants, *J Gen Microbiol*, **94**, 46–54.

Kleerebezem, M., Quadri, L. E., Kuipers, O. P., de Vos, W. M., 1997. Quorum sensing by peptide pheromones and two-component signal transduction systems in Gram-positive bacteria, *Mol Microbiol.*, **24**, 895-904.

Kobayashi, K., Ehrlich, S., D., Albertinid, A., Amatid, G., Andersene, K., K., Arnaudf, M., Asaig, K., Ashikagah, S., Aymerichi, S., Bessieresj, P., Bolandk, F., Brignelll, S., C., Bronm, S., Bunain, K., Chapuisb, J., Christianseno, L., C., Danchinp, A., De´ barbouille´ , M., Dervynb, E., Deuerlingq, E., Devinee, K., Devinee, S., K., Dreesenp, O., Errington J., Fillingeri, S., Fosterk, S., J., Fujitas, Y., Galizzid, A., Gardanf, R., Eschevinsm, C., Fukushimat, T., Hagau, K., Harwoodl, C., R., Heckerv, M., Hosoyaw, D., Hullop, M., F., Kakeshitan, H., Karamatax, D., Kasaharaa, Y., Kawamurah, F., Kogah, K., Koskiy, P., Kuwanaz, R., Imamuraw, D., Ishimaruw, M., Ishikawat, S., Ishios, I., Le Coqi, D., Massonaa, A., Maue, C., Meimam, R., Melladobb, R., P., Moirk, A., Moriyaa, S., Nagakawas, E., Nanamiyah, H., Nakaia, S., Nygaardo, P., Oguracc, M., Ohananq, T., O'Reillye, M., O'Rourkek, M., Pragail, Z., Pooleyx, H., M., Rapoportf, G., Rawlinsr, J., P., Rivasbb, L., A., Rivoltax, C., Sadaieu, A., Sadaieg, Y., Sarvasy, M., Satow, T., Saxildo, H., H., Scanlane, E., Schumannq, W., Seegersaa, J., F., M., L., Sekiguchit, J., Sekowskap, A., Se´ roraa, S., J., Simondd, M., Stragierdd, P., Studerx, R., Takamatsuz, H., Tanakacc, T., Takeuchiw, M., Thomaidesr, H., B., Vagnerb, V., Dijlm, J., M., Watabez, K., Wipatl, A., Yamamotot, H., Yamamotos, M., Yamamotos, Y., Yamanen, K., Yataee, K., Yoshidas, K., Yoshikawau, H., Zuberv, U., Ogasawaraa, N., 2003. Essential *Bacillus subtilis* genes, *Proc Natl. Acad. Sci. USA*, **100**, 4678–4683.

Kobayashi, K., Ogasawara, N., 2002. Genome biology of a model bacterium, *Bacillus subtilis*. *International Congress Series*, **1246**, 15–25.

Kunst, F., Ogasawara, N, Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessiéres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S, Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.-K., Codani, J.-J., Connerton, I.F., Cummings, N.J., Daniel, R.A., Denizot, F., Devine, K.M., Düsterhöft, A., Ehrlich, S.D., Emmerson, P.T., Entian, K.D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, A., Ghim, S.-Y., Glaser, P., Goffeau, A., Golightly,E.J., Grandi, G., Guiseppi, G., Guy, B.J., Haga, K., Haiech, J., Harwood, C.R., Hénaut, A., Hilbert, H., Holsappe, S., Hosono, S., Hullo, M.-F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, B., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.-M., Levine, A., Liu, H., Masuda, S., Mauel, C., Médigue, C., Medina, N., Mellado, R.P., Mizuno, M., Moest, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.-H., Parro, V., Pohl, T.M., Portetelle, D., Porwollik, S., Prescott, A.M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S.J., Serror, P., Shin, B.-S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A.,Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H.-F., Zumstein, E., Yoshikawa, H., and Danchin, A., 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*, *Nature*, **390**, 249-256.

Lazazzera, B. A., 2001. The intracellular function of extracellular signaling peptides, *Peptides*, **22**, 1519–1527.

Lazazzera, B. A., J. M. Solomon, and A. D. Grossman. 1997. An exported peptide functions intracellularly to contribute to cell density signaling in *B. subtilis*, *Cell*, **89**, 917-925.

Lazazzera, B. A., Kurtser, I. G., McQuade, Y. S., Grossman, A. D., 1999. An autoregulatory circuit affecting peptide signaling in *Bacillus subtilis*, *J. Bacteriol.*, **181**, 5193– 5200.

Lazazzera, B., A., 2000. Quorum sensing and starvation: signals for entry into stationary phase, *Current Opinion in Microbiology*, **3**, 177–182

Lazazzera, B., Palmer, T., Quisel, J. & Grossman, A. D., 1999. Cell density control of gene expression and development in *Bacillus subtilis*. In Cell–Cell Signaling in Bacteria (Dunny, G. M. & Winans, S. C., eds), *ASM Press*, pp. 27–46.

Lazdunski, M. A., Ventre, I., Bleves, S., 2007, Cell–Cell Communıcatıon: Quorum Sensıng And Regulatory Circuits In *Pseudomonas aeruginosa, Pseudomonas*, 279–310.

Magnuson, R., Solomon, J., Grossman, A., D., 1994. Biochemical and genetic characterization of a competence pheromone from *B. subtilis*, *Cell*, **77**, 207–16.

McQuade, R. S., Comella, N., Grossman, A. D., 2001. Control of a Family of Phosphatase Regulatory Genes (*phr*) by the Alternate Sigma Factor Sigma-H of *Bacillus subtilis*, *J. Bacteriol.*, **183**, 4905-4909

Miller, M. B., Bassler, B. L., 2001. Quorum sensing in bacteria, *Annu. Rev. Microbiol*, **55**, 165–99.

Molle, V., Nakaura, Y., Shivers, R., P., Yamaguchi, H., Losick, R., Fujita, Y., Sonenshein, A., L., 2003. Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis, *J Bacteriol*, **185**, 1911-1922.

Mootz, H. D., and Marahiel, M. A., 1997. The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains, *J Bacteriol*, **179**, p 6843-50.

Msadek, T., Kunst, F. & Rapoport, G., 1995. A signal transduction network in *Bacillus subtilis* includes the DegS/DegU and ComP/ComA two-component systems, *In Two-Component Signal Transduction*, pp. 447–471.

Nakano, M. M., Magnuson, R., Myers, A., Curry, J., Grossman, A., D., Zuber, P., 1991. *srfA* is an operon required for surfactin production, competence development, and efficient sporulation in *Bacillus subtilis*. *J. Bacteriol.*, **173**, 1770– 1778.

Nicholson, W. L., Setlow, P., 1990. Sporulation, germination and outgrowth. In C.R. Harwood & S.M.Cutting (Editors), Molecular Biological Methods for *Bacillus*, Chichester, England: *John Wiley & Sons,* pp.391- 429.

Özcengiz, G., Alaeddinoglu, N. G., and Demain, A. L., 1990. Regulation of biosynthesis of bacilysin by *Bacillus subtilis*, *J Ind Microbiol.*, **6**, 91-100.

Özcengiz, G., and Alaeddinoglu, N. G., 1991. Bacilysin production by *Bacillus subtilis*: effects of bacilysin, pH and temperature, *Folia Microbiol.*, **36**, 522-526.

Perego, M., 1997. A peptide export-import control circuit modulating bacterial development regulates protein phosphatases of the phosphorelay, *Proc Natl Acad Sci*, 94, 8612-8617.

Perego, M., 1997. A peptide export-import control circuit modulating bacterial development regulates protein phosphatases of the phosphorelay, *Proc. Natl. Acad. Sci.* USA, **94**, 8612–8617.

Perego, M., and Brannigan, J.A., 2001. Pentapeptide regulation of aspartylphosphate phosphatases, *Peptides*, **22**, 1541–1547.

Perego, M., C. F. Higgins, S. R. Pearce, M. P. Gallagher, and J. A. Hoch., 1991. The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation, *Mol. Microbiol.*, **5**, 173–185.

Perego, M., Glaser, P., Hoch, J. A., 1996. Aspartyl-phosphate phosphatases deactivate the response regulator components of the sporulation signal. transduction system, *Mol. Microbiol.*, **19**, 1151–1157.

Perego, M., Hanstein, C. G., Welsh, K. M., Djavakhishvili, T., Glaser, P., and Hoch, J. A., 1994. Multiple protein-aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in *B. subtilis*, *Cell*, **79**, 1047–1055.

Pottathil, M., Jung, A., and Lazazzera, B. A., 2008. CSF, A Species-Specific Extracellular Signaling Peptide for Communication among Strains of *Bacillus subtilis* and *Bacillus mojavensis*, *Journal of Bacteriology*, **Vol. 190**, 4095–4099.

Price, C. W., 2002. The general stress response, In *Bacillus subtilis* and its closest relatives: from genes to cells, *ASM Press*, p. 369–384.

Ramos, H. C., Boursier, L., Moszer., Kunst, F., Danchin, A., and Glaser, P., 1995. Anaerobic transcription activation in *Bacillus subtilis*: identification of distinct FNR-dependent and -independent regulatory mechanisms, *The EMBO Journal*, **14**, 5984-5994.

Ratnayake-Lecamwasam, M., Serror, P., Wong, K. W., and Sonenshein, A. L., 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels, *Genes & Dev.*, **15**, 1093-1103.

Ratnayake-Lecamwasam, M., Serror, P., Wong, K. W., and Sonenshein, A. L., 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels, *Genes & Dev.*, **15**, 1093-1103.

Roggiani, M. & Dubnau, D.,1993. ComA, a phosphorylated response regulator protein of *Bacillus subtilis*, binds to the promoter region of *srfA*, *J Bacteriol*, 175, 3182–3187.

Rudner, D. Z., J. R. LeDeaux, K. Ireton, and A. D. Grossman., 1991. *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence, *J. Bacteriol*., **173**, 1388– 1398.

Ruzheinikov, S. N., Das, S. K., Sedelnikova, S. E., Hartley, A., Foster1, S. J., Horsburgh, M. J., Cox, A. G., McCleod, C. W., Mekhalfia, A., Blackburn, G. M., Rice, D. W., Baker, P. J., 2001. The Structure of a Novel Quorum-sensing Protein, *Bacillus subtilis* LuxS, *J. Mol. Biol.*, **313**, 111-122.

Sakajoh, M., Solomon, N. A., and Demain, A. L., 1987. Cell-free synthesis of the dipeptide antibiotic bacilysin, *J Ind Microbiol.*, **2**, 201-208.

Schwarzer, D., Mootz, H. D., Linne, U., and Marahiel, M.A., 2002. Regeneration of misprimed nonribosomal peptide synthetases by type II thioesterases, *PNAS 99*, **22**, 14083–14088.

Seredick, S. D., Spiegelman, G. B., 2007. *Bacillus subtilis* RNA Polymerase Recruits the Transcription Factor Spo0A~P to Stabilize a Closed Complex during Transcription Initiation, *J. Mol. Biol.*, **366**, 19–35.

Serror, P., and Sonenshein, A., L., 1996. CodY Is Required for Nutritional Repression of *Bacillus subtilis* Genetic Competence. *Journal of Bacteriology*, **Vol. 178**, 5910-5915.

Shivers, R. P., and Sonenshein, A. L., 2004. Activation of the *Bacillus subtilis* global regulator CodY by direct interaction with branched-chain amino acids, *Mol. Microbiol.*, **53**, 599-611.

Shivers, R., P., Sonenshein, A., L., 2004. Activation of the *Bacillus subtilis* global regulator CodY by direct interaction with branched-chain amino acids. *Mol. Microbiol.*, 53, 2, 599-611.

Solomon, J., M., Lazazzera, B., A., and Grossman, A., D., 1996. Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in *Bacillus subtilis*, *Genes Dev.*, **10**, 2014-2024.

Solomon, J., M., Magnuson, R., Srivastava, A., Grossman A.D., 1995. Convergent sensing pathways mediate response to two extracellular competence factors in *Bacillus subtilis, Genes Dev.*, **9**,547– 558.

Solomon, J., Su, L., Shyn, S., Grossman, A., D., 2003, Isolation and Characterization of Mutants of the *Bacillus subtilis* Oligopeptide Permease with Altered Specificity of Oligopeptide Transport, *Journal of Bacteriology*, p. 6425–6433.

Stein, T., 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology*, **56**, 845-857.

Steinborn, G., and Hofemeister, J., 1998/2000. Genes for the biosynthesis of anticapsin and bacilysin, their isolation and use, PCT/DE99/ 02179, WO 00/03009.

Steinborn, G., Hajirezaei, MR., Hofemeister, J., 2005. *bac* genes for recombinant bacilysin and anticapsin production in *Bacillus* host strains, *Arch Microbiol*, **183**, 71–79.

Stephens, C., 1998. Bacterial sporulation: A question of commitment?, *Current Biology*, **8**, 45-48.

Stock, J., B., Ninfa, A., J., and Stock, A., M., 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.*, **53**, 450-490.

Strauch, M. A., 1993. Regulation of *Bacillus subtilis* gene expression during the transition from exponential growth to stationary phase, *Prog. Nucleic Acid Res. Mol. Biol.*, **46**, 121-153.

Strauch, M., A., 1993. Regulation of *Bacillus subtilis* gene expression during the transition from exponential growth to stationary phase, *Prog. Nucleic Acid Res. Mol. Biol.*, **46**, 121-153.

Strauch, M., A., 1993. AbrB, a transition state regulator. In: *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology and molecular genetics, *American Society for Microbiology*, p. 757–764.

Strauch, M., A., and Hoch, J., A., 1993. Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression, *Mol. Microbiol.*, **7**, 337-342.

Sturme, M. H. C., Kleerebezem, M., Nakayama, J., Akkermans, A. D. L., Vaughan1, E. E., de Vos, W. M., 2002. Cell to cell communication by autoinducing peptides in gram-positive bacteria, *Antonie van Leeuwenhoek*, **81**, 233–243.

Tabata, K., Ikeda, H., Hashimoto, S., 2005, *ywfE* in *Bacillus subtilis* Codes for a Novel Enzyme, L-Amino Acid Ligase, *Journal of Bacteriology*, **187**, 5195-5202.

Taga, M. E., Bassler, B. L., 2003. Chemical communication among bacteria, *PNAS Early Edition.*

Tanaka, T., Kawata, M., and Mukai, K., 1991. Altered Phosphorylation of Bacillus subtilis DegU Caused by Single Amino Acid Changes in DegS, *Journal of Bacteriology*, **Vol. 173**, 5507-5515.

T**rowsdale, J., Chen, S. M. H. and Hoch, J. A.,** 1978. Genetic analysis of phenotype revertants of *spo0A* mutants in *Bacillus subtilis*: a new cluster of ribosomal genes. In: Spores VII (Chambliss, G. and J.C. Vary, J.C., Eds.), *American Society for Microbiology*, p. 131-135.

Turgay, K., Hahn, J., Burghoorn, J., Dubnau, D., 1998. Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *EMBOJ.*, **17**, 6730–38.

Turgay, K., Hamoen, L., W., Venema, G., Dubnau, D., 1997. Biochemical characterization of a molecular switch involving the heat shock protein ClpC, which controls the activity of ComK, the competence transcription factor of *Bacillus subtilis*, *Genes Dev.*, **11**, 119–28.

van Sinderen, D., A. Luttinger, L. Kong, D. Dubnau, G. Venema, L.Hamoen, 1995**.** *comK* encodes the competence transcription factor, the key regulatory protein for competence development in *Bacillus subtilis*, *Mol. Microbiol.*, **15**, 455– 462.

vanSinderen, D., Withoff, S., Boels, H., and Venema, G., 1990. Isolation and characterization of comL, a transcription unit involved in competence development in *Bacillus subtilis*, *Mol. Gen. Genet.*, **224**, 396-404.

von Bodman, S. B., Willey, J. M., and Diggle, S. P., 2008. Cell-Cell Communication in Bacteria: United We Stand. *Journal of Bacteriology*, **Vol. 190**, 4377–4391.

Walker, J. E., and Abraham, E. P., 1970. The structure of bacilysin and other products of *Bacillus subtilis*, *Biochem. J.*, **118**, 563-570.

Walton, R., B., and Rickes, E., L., 1962. Reversal of the antibiotic bacillin by N-acetylglucosamine, *J Bacteriol*., **84**, 1148-1151.

Weinrauch, Y., Penchev, Y., Dubnau, R., 1990. A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems, *Genes Dev.*, **4**, 860– 872.

Williams, P., 2007. *Bacillus subtilis*: A Shocking Message from a Probiotic, *Cell Host & Microbe*, **Vol. 1**, 248-24.

Yazgan, A., Özcengiz, G., and Marahiel, M. H., 2001. *Tn 10* insertional mutations of *Bacillus subtilis* that block the biosynthesis of bacilysin, *Biochim Biophys Acta.*, **1518**, 87-94.

Yazgan, A., Özcengiz, G., Özcengiz, E., Kılınc, K., Marahiel, M. A., and Alaeddinoğlu, N. G., 2001. Bacilysin biosynthesis by a partially-purified enzyme fraction from *Bacillus subtilis*, *Enzyme Microb Technol*., **9**, 400-406.

Yazgan-Karatas, A., Y., Cetin, S., and Özcengiz, G., 2003. The effects of insertional mutations in *comQ, comp, srfA, spo0H, spo0A* and *abrB* genes on bacilysin biosynthesis in *Bacillus subtilis*, *Biochim Biophys Acta.*, **1626**, 51-56.

APPENDIX

APPENDIX A : Composition and Preperation of Culture Media
APPENDIX B : Composition and Preparation of Buffers and Solutions
APPENDIX C : Chemicals and Enzymes
APPENDIX D : Markers
APPENDIX E : *ywfH* DNA Sequence

APPENDIX A

Luria Bertani (LB) Agar Medium (1000 ml)

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

Distilled H_2O was added up to 1000ml and then autoclaved for 4 minutes.

2xYT Medium (1000ml)

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g
Agar	15 g

(Added before autoclaving for solid 2xYT medium)

HS medium (30 ml)

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

Completed up to 30 ml with sterile distilled H_2O and store at cold (+4°C) for a week.

LS Medium (20 ml)

10X-S-Base	2 ml
Glucose	200 µl
Tryptophan	200 µl
Phenylalanine	30 µl
Casaminoacid	100 µl
Yeast Extract	200 µl
Spermine (50mM)	200 µl
MgCl2 (1M) (filter steriled)	50 µl

Freshly prepared and completed up to 20 ml with sterile distilled H2O.

Perry and Abraham (PA) Medium (pH 7.4)

KH2PO4	1 g/L
KCl	0.2 g/L
$MgSO_4.7H_2O$*	0.5 g/L
Glutamate.Na.H_2O	4 g/L
Sucrose*	10 g/L
Ferric citrate**	0.15 g/L
Trace elements**	1 ml
$CoCl_2.6H_2O$	0.0001 g/L
Ammonium molybdate	0.0001 g/L
$MnCl_2.4H_2O$	0.001 g/L
$ZnSO_4.7H_2O$	0.0001 g/L
$CuSO_4.5H_2O$	0.00001 g/L

*Autoclave separately

**Filter sterilization

DSM Medium (Difco's Sporulation Medium)

Nutrient Broth	8 g
KCl (10 % w/v)	10 ml
$MgSO_4.7H_2O$ (1.2 %)	10 ml
NaOH (1 M)	0.5 ml

Autoclaved for 30 min. and cooled own to 50 ˚C.

$Ca(NO_3)_4$ (1 M)	1 ml
$MnCl_2$ (0.01 M)	1 ml
$FeSO_4$ (1 mM)	1 ml (resuspended before use)

1.5 % Agar was added if needed.

APPENDIX B

P1 Buffer (pH 8)

Tris-base	6.06 gr
EDTA.2H2O	3.72 gr

Dissolve Tris-base and EDTA with 800 ml dH_2O. Adjust pH to 8 with HCl. Adjust volume to 1 L dH_2O. Add 100 mg RNaseA per liter of P1.

P2 Buffer

NaOH	8 gr
SDS solution (20%)	50 ml

Dissolve NaOH in 950 ml dH_2O. Add 50 ml SDS solution.

P3 Buffer (pH 5.5)

Potassium acetate	294.5 gr

Dissolve in 500 ml dH_2O. Adjust pH

TE Buffer (pH 7)

Tris base	10 mM
EDTA	1 mM

Adjusted pH 7 with HCl

TAE Buffer (50X)

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

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

Low Melting Agarose Gel (1%)

Agarose 0.5 g

TAE buffer (1X) 50 ml

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

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

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

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

NaCl2 8.5 g

Dissolve in 1000 ml distilled water and autoclave.

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

Tris (hydromethyl)aminomethane 3.03 g

Dissolve in 1000ml distilled water and adjust pH to 7.4 with HCl (1 M)

Z Buffer (500 ml, pH 7.0)

Na2HPO4.7H2O	(60mM)	5.33 g
NaH2PO4	(40 mM)	3.12 g
KCl2	(10 mM)	0.373 g
MgSO4.7H2O	(1 mM)	0.123 g

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

β-mercaptoethanol final concentration: 270 µl / 100 ml (add to Z buffer on immediately before using)

Lysozyme final concentration: 2.5 mg/ml

ONPG final concentration: 4.0 mg/ml

APPPENDIX C

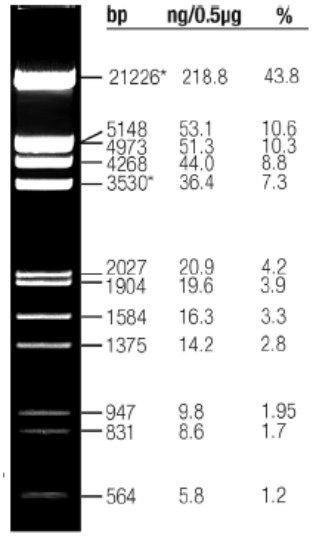
Chemicals	**Suppliers**
Agar Bacteriological	AppliChem
Agarose	Prona
Ammonium persulfate	Carlo Erba
Arginine	Merck
$CaCl_2.2H_2O$	Merck
Casamino acid	AppliChem
Cloramphenicol	Sigma
$CoCl_2.6H_2O$	Carlo Erba
$CuSO_4.5H_2O$	Carlo Erba
CTAB	Sigma
EDTA	Sigma
Ethanol	Botafarma
Ethidium bromide	Sigma
Glucose	Riedel deHaen
Glycerol	Merck
HCl	Carlo Erba
Histidine	Merck
IPTG	Sigma
KH_2PO_4	J.T.Baker
K_2HPO_4	J.T.Baker
KCl	Carlo Erba
Mercaptoethanol	Merck
$MgCl_2.6H_2O$	Carlo Erba
$MgSO_4.7H_2O$	Carlo Erba
$MnCl_2.4H_2O$	Carlo Erba
Na_2CO_3	Riedel deHaen
Na_3citrate.$2H_2O$	Riedel deHaen
NaCl	Carlo Erba
NaOH	Carla Erba
2-Nitrophyl β-D-galacto pyranoside (ONPG)	Sigma
Nutrient Broth	Merck

Chemicals	**Suppliers**
PEG 8000	Merck
Phenol-chloroform-isoamylalcohol	Fluka
Phenylalanin	Merck
SDS	Merck
Spermine	Fluka
Sucrose	Merck
Tris-base	Merck
Triton-X100	Sigma
Trypton	Acumedia
Tryptophan	Merck
X-Gal	Sigma
Yeast Extract	Acumedia

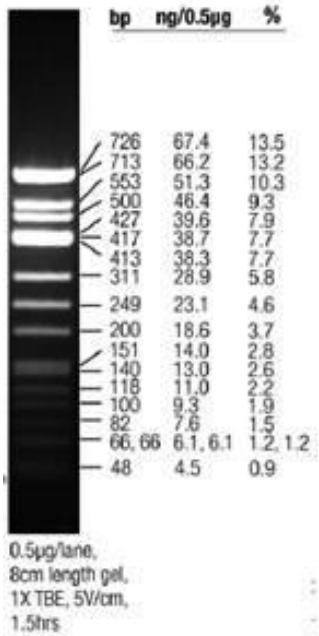
Enzymes	
*Bam*HI	Fermentas
*Hin*dIII	Fermentas
Lysozyme	AppliChem
Proteinase K	Sigma
RNAse A	Sigma
Taq DNA Polymerase	Fermentas
T4 DNA Ligase	Roche

APPENDIX D

Marker	**Supplier**
Lambda DNA/*Eco*RI+ *Hind*III Marker (Marker 3)	MBI Fermentas



ΦX174 DNA/HinfI Marker (Marker 10)	MBI Fermentas



APPENDIX E

***ywfH* DNA Sequence**

TTGTCAAAACGAACCGCATTTGTTATGGGAGCCAGTCAAGGGATCGGGA
AAGCAATCGCTCTGAAATTAGCAGACCAGCACTTTTCCCTCGTCATTAAT
TCGCGAAATTTGGATAATATTGAATCTGTCAAAGAAGACATTTTGGCCAA
GCATCCTGAGGCGAGCGTCATTGTCCTTGCGGGCGATATGTCTGACCAGC
ATACGAGAGCGGGCATTTTTCAGAAAATCGAATCTCAATGCGGACGGCTT
GATGTTCTGATTAATAATATCCCGGGCGGCGCGCCTGACACATTTGATAA
CTGCAATATAGAGGATATGACGGCCACGTTCACTCAAAAGACCGTTGCCT
ATATTGACGCAATCAAGCGTGCTTCCTCACTGATGAAACAAAACGAGTTT
GGCAGAATCATCAACATTGTCGGAAATCTGTGGAAAGAACCCGGCGCCA
ACATGTTCACAAACAGTATGATGAATGCCGCTTTAATTAACGCCAGTAAA
AATATTTCCATCCAGCTTGCTCCTCACAACATTACTGTCAACTGTCTGAAT
CCTGGTTTTATCGCTACAGACCGTTATCATCAATTTGTGGAAAATGTGAT
GAAAAAGAACAGCATATCCAAACAGAAAGCAGAAGAACAGATTGCTTCC
GGGATTCCGATGAAACGGGTCGGATCAGCCGAAGAAACCGCAGCGCTCG
CCGCATTTCTTGCCTCTGAGGAAGCCTCCTACATCACGGGACAGCAAATT
TCCGCTGACGGCGGCAGCATGAAAAGCATATAA

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