ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

GENOME-WIDE ANALYSIS OF *yvfI* GENE in *Bacillus subtilis*

Ph.D. THESIS

Öykü İRİGÜL SÖNMEZ

Advanced Technologies Department

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

*Bacillus subtilis'*DE *yvfI* GENİNİN GENOM ÖLÇEĞİNDE ANALİZİ

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TEMMUZ 2012

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Date of Submission : 20 February 2012
Date of Defense : 2 July 2012

To my mother,

FOREWORD

Foremost, I would like express my most sincere gratitude to my supervisor Assoc. Prof. Ayten Yazgan Karataş. Her wide knowledge and her logical way of thinking have been of great value for me. Her understanding, encouraging, stimulating advices and personal guidance have provided a strong basis for this thesis. I also want to express my appreciation to my thesis committee for their guidance in this academic path.

I also want to thank Prof. Dr. Oscar Kuipers and his group at the Groningen University in the Netherlands. It was a pleasure for me to conduct a crucial part of this thesis under his guidance and with the help of their technical support.

It is a pleasure to thank those who made this thesis possible. I want to express my deep appreciation to Türkan Ebru Köroğlu. She has completed me not only scientifically but also personally. Once again I want to emphasize that she was the strongest promoter that provided me with a gain of function. A whole bunch of thanks also go to my lab mates who have contributed to this project. It is maybe impossible to acknowledge each and every (past and present) member of our bacteriology lab but I want them to know that they all be remembered with great appreciation and deep love. I am indebted to many of my colleagues from Istanbul Technical University and from other universities whom I had the chance to work with.

I would like to express my special gratitude to my family starting with my dear mother Sema İrigül, who would always be my biggest inspiration. No illnesses, events of life or obstacles would and could let us apart from each other spiritually. I also want to thank my father Hasan Raşit İrigül and my brother Kemal Özgün İrigül for always being there for me.

Finally, my husband İbrahim Can Sönmez has made available his support in a number of ways. He provided a cinnamon scented life for us and tried his best to support me at hard times. Love made us one and I hope our life would be filled with more success stories.

FEBRUARY 2012 Öykü İRİGÜL SÖNMEZ

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ABBREVIATIONS

bp : Base pair
Cm : Chloramphenicol
dH_2O : Distilled water
DNA : Deoxyribonucleic acid
DSM : Difco's Sporulation Medium
Erm : Erythromycin
EtBr : Ethidium bromide
IPTG : Isopropyl-b D- thiogalactopyranoside
kb : Kilobase
LB broth : Luria Bertani broth
lacZ : Structural gene for β-galactosidase
Ln : Lyncomycin
MLS : Macrolide-Lincosamide-Streptogramine
OD : Optical density
ONPG : *O*-nitrophenyl-*β*-galactoside
PA : Perry and Abraham Medium
PCR : Polymerase Chain Reaction
RNA : Ribonucleic acid
RT-qPCR : Reverse transcriptase quantitative PCR
Spc : Spectinomycin
QS : Quorum Sensing
TAE : Tris acetate EDTA
Tris : Hydroxymethyl aminomethane

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GENOME-WIDE ANALYSIS OF *yvfI* GENE in *Bacillus subtilis*

SUMMARY

Bacillus subtilis is a highly adaptive organism that can change its physiological state in response to received environmental signals and different growth conditions. Its genome is completely sequenced and its 4,2 Mbp pair long genome is known to encode for approximately 4,100 genes. About one third of total genes have specific defined functions and this numbers increases with each new study that targets genes with unknown functions. Explored patterns on *B. subtilis* genome uncovered the ability of *B. subtilis* to use highly diverse types of carbon sources and to produce secondary metabolites, including antibiotics and industrially important enzymes. Moreover, *B. subtilis* is also known to encode for prophages that signifies a possible role for bacteriophage infection in the transfer of genes during bacterial evolution. *B. subtilis* also evolved diverse types of cellular processes which involve two-component signal transduction systems that sense environmental signals and develops an adaptive response, which can in turn act as a transcriptional regulator to change gene expression profile. These cellular processes include sporulation, biofilm formation, chemotaxis, virulence, antibiotic production and resistance.

Among the different types of transcriptional regulator proteins, GntR family transcriptional repressor consists of about 2000 different proteins that have different action sites and also that function in different organisms. These proteins act as regulators on many different types of regulons. In *Bacillus subtilis*, the protein encoded from a novel gene, *yvfI* which was recently found to be essential for bacilysin biosynthis, consists of a FadR C-terminal ligand binding (FCD) domain which is very common among GntR family of proteins. Furthermore, 44 amino acid extended YvfI protein was identified to display an incomplete but considerable match to the wHTH domain of GntR family type proteins. Moreover, very recent study has shown that the *yvfV-yvfW-yvbY* (*lutABC)* operon which is required for lactate utilization is under the negative control of YvfI in *Bacillus subtilis.* The present research aimed at identification of genes that are regulated by YvfI transcriptional factor and therefore a genome wide comparative transcriptome analysis of wild type control strain PY79 that produces YvfI trancriptional factor and *yvfI* disrupted TEK1 mutant, *yvfI::T10::spc* during logarithmic phase (~OD_{600} 0,7) and stationary phase (~ OD_{600} 7) were performed. Subsequently, in order to confirm previously evaluated microarray data, quantitative analysis of expression of many genes that were identified to be induced or repressed during stationary phase were realized by using reverse transcription quantitative-PCR (RT-qPCR). Finally, YvfI affected genes were grouped according to their functional properties. As a result, this work enabled the identification of many pathways that are regulated by YvfI protein during stationary growth phase. These pathways include degradative enzyme production, antibiotic production and resistance, carbohydrate utilization and transport, nitrogen metabolism, membrane transport, fatty acid and lipid metabolism, protein synthesis and translocation, cell-wall synthesis, energy production and

membrane bioenergetics, mobile genetic element, phage infection, sporulation, delay of sporulation and cannibalism, biofilm formation. In a harmony with YvfI regulation during stationary phase, YvfI was also found to inhibit carbohydrate metabolism and participate in regulation of genes involved in lipid metabolism during exponential growth phase. Furthermore, YvfI was found to induce resistance against bacitracin and repress pyrimidin biosynthesis during that phase.

Furthermore, comparative studies were performed in order to define expressional differences between IPTG induced (*yvfI* gene overexpressed) "*B. subtilis* PY79 *amyE*::P_{spac}::*yvfI*, *yvfI::Tn10"* mutant strain and non-induced (*yvfI* gene not overexpressed) "*B. subtilis* PY79 *amyE*::P_{spac}::*yvfI*, *yvfI::Tn10"* mutant strain and this part of study clarified that YvfI is involved in the regulation of nitrogen metabolism, amino acids. catabolism and transport. Moreover, YvfI regulator protein probably induces indirectly the conjugal transfer of "ICEBs1" locus while it is also involved in the expression of prophage-like elements when overexpression achieved.

Moreover, identification and localization of promoter region of *yvfI* gene by 5'-RACE-PCR analysis was also achieved. As a result, *yvfI* transcription was shown to be controlled from a σ^A-type promoter. Furthermore, a consensus sequence that shows a perfect match to Spo0A binding motif, three putative AbrB binding and one CodY binding sites were identified on the sequence that indicate that this gene is most probably under the control of global regulatory system operated by Spo0A, CodY and AbrB.

Promising diverse regulatory behavior of YvfI displays a harmony with the fact that YvfI belongs to GntR family regulatory proteins that exert regulatory activity by applying repressions and inductions on many different types of regulons. Therefore, this study introduced YvfI as a critical regulator protein that helps to the adaptation of *B. subtilis* cells to different stimuli.

Bacillus subtilis'DE *yvfI* GENİNİN GENOM ÖLÇEĞİNDE ANALİZİ

ÖZET

Bacillus subtilis, çevresinden aldığı sinyallere ve büyüme koşullarına göre fizyolojik durumunu değiştirebilen, yüksek adaptasyon yeteneğine sahip bir organizmadır. Bu değişimler organizmanın morfolojisi ve fenotipine keskin bir şekilde yansırlar. Büyüme hızındaki dalgalanmalar, oksijen direnci, sıcaklık, pH ve besin kaynaklarının ulaşılabilirliği, *B. subtilis*'in fizyolojik durumunu farklılaştırmaya yönelten başlıca sebeplerdir. Örneğin, logaritmik büyüme fazı sonlarına doğru sınırlı besin kaynağı sinyalini alan organizmada, hareketlilik ve kemotaksis sistemleri uyarılarak, hayatta kalma stratejileri gelişmektedir. Besin kaynağındaki kısıtlılık devam ettiğinde ise, organizmanın metabolizmasında antibakteriyel, antifungal ve antimetabolik özellikleri olan antibiyotiklerin üretimi, diğer organizmalarla rekabet ortamında galip gelebilmek için hızlanmaktadır. Alternatif kaynaklardan besin tüketimi için üretilen proteazların yanında, yabancı DNA'nın hücre içine taşınımını sağlayan kompetans ve son olarak sporlanma da değişen çevresel şartlara adaptasyon için *B. subtilis* tarafından verilen cevaplardır.

B. subtilis genomunun tamamen dizilenmesiyle birlikte, bu organizma patojen olmaması, kolay idare edilebilmesi, yüksek büyüme ve bölünme hızına sahip olması, kolay transforme edilebilmesi, adaptasyona uygun metabolizması, hidrolitik enzimler, polipeptit antibiyotikler ve çeşitli biyokimyasallar üretebilmesi sayesinde çok araştırılan bir model organizma olarak kabul edilmiştir. Genom dizilemesi sonucu 4,2 Mbp uzunluğundaki genomun yaklaşık olarak 4,100 geni kodladığı ortaya çıkmıştır. Gen setlerinin haritalanması ile total genomun üçte birinin kendine özgü tanımlanmış fonksiyonu olduğu ortaya çıkarken, bu sayı bilinmeyen fonksiyonlara sahip genler üzerindeki yeni araştırmalar sayesinde artmaktadır.

B. subtilis genomunun yapısının keşfi ile *B. subtilis*'in antibiyotik ve sanayi açısından önemli enzimler gibi ikincil metabolitleri üretebildiği ve çeşitli karbon kaynaklarını yüksek oranda kullanılabildiği ortaya çıkmıştır. Ayrıca, *B. subtilis*' in genomunun 10 civarında profaj ve profaj benzerlerini kodladığı ve böylece bakteriyel evrim sırasındaki genlerin transferindeki bakteriyofaj enfeksiyonunda rol alma ihtimali ortaya çıkmıştır. Bunların yanında, büyük gen ailelerinin varlığı gen düplikasyonlarından ortaya çıkmıştır ve en büyük gen ailesi 77 aday ATP-bağlayıcı taşıyıcı proteinden oluşmaktadır.

Proteinlerde tek bileşenli sistemler, sinyal algılayıcı ve fonksiyonel cevap verici bölgelere sahiptirler. Prokaryotlarda sinyal iletimi bu sistemler aracılığıyla gerçekleşirken, fonksiyonel çıktı bölgesi büyük oranda sarmal-dönüş-sarmal (helix-turn-helix) bölgesinden oluşmaktadır. Tek bileşenli sistemler ve HTH transkripsiyonel düzenleyiciler, DNA bağlanma bölgelerindeki dizilerin benzerliklerine göre gruplanmışlardır. Transkripsiyonel düzenleyici proteinlerin değişik türleri arasında, farklı aksiyon bölgeleri ve aynı zamanda farklı organizmalarda işleyen yaklaşık 2000 farklı proteinden oluşan GntR ailesi

bulunmaktadır ve bu aile yaygın olarak kanatlı sarmal-dönüş-sarmal motifi ile karakterize edilmişlerdir. GntR ailesin mensup 4 büyük altgrup kategorize edilmiştir. Bu gruplardan ilki olan FadR en kalabalık alt grup olarak göze çarpmaktadır. FadR-benzeri proteinlerin fonksiyonlarındaki çeşitlilik oldukça yoğundur. Bu proteinler, aminoasit matabolizmasının regülasyonuyla ve aspartat, pirüvat, glycolat, galaktonat, laktat, malonat ya da glukonat gibi metabolik yol izleriyle ilişkilidir.

Bunların yanı sıra, yeni tanımlanan bir gen olan, *B. subtilis yvfI* geninin GntR ailesinde çok yaygın bulunan FadR C-ucu ligand bağlayıcı (FCD) bölgesini ihtiva ettiği gösterilmiştir. Ayrıca bu genin *B. subtilis*'de basilisin üretiminde gerekli olduğu da gösterilmiştir. Ayrıca YvfI proteinin genişletilmiş 44 amino asit daha içeren versiyonunun da GntR ailesi benzeri proteinlerinin kanatlı sarmal-dönüş-sarmal bölgesiyle tam anlamıyla olmasa da önemli bir derecede benzeştiği gösterilmiştir. Ayrıca, çok yeni bir çalışma göstermiştir ki, *yvfV-yvfW-yvbY (lutABC)* operonu, *yvfI*'nın baskın kontrolü altındadır.

Bu sunulan çalışmada, YvfI transkripsiyonel faktörü tarafından etkilenmesi mümkün genleri tanımlayabilmek için YvfI kodlayamayan TEK1 mutantı, *yvfI::T10::spc,* ve YvfI transkripsiyonel faktörü üreten kontrol suşu PY79 arasında, logaritmik faz sırasında (~OD_{600} 0,7) ve durağan fazda (~OD_{600} 7) detaylı karşılaştırmalı transkriptom analizi gerçekleştirilmiştir. Çalışmanın bu bölümünde daha önce değerlendirilen, micoarray yöntemi ile elde edilen verileri doğrulamak için durağan faz sırasında baskılanmış ya da indüklenmiş genlerin kantitatif analizleri RT-qPCR tekniği kullanılarak gerçekleştirilmiştir. Çalışmanın bu kısmının sonucunda, YvfI tarafından düzenlenen genler fonksiyonel özelliklerine göre gruplanmışlardır. Sonuç olarak bu çalışma ile büyümenin durağan evresinde YvfI proteini tarafından düzenlenmiş bir çok yol tanımlanmasının keşfi sağlanmıştır. Bu yollar; indirgenmiş enzim üretimi, antibiyotik üretimi ve direnci, karbonhidrat kullanımı ve iletimi, nitrojen metabolizması, zar iletimi, yağ asidi ve lipit metabolizması, protein sentezi ve yer değiştirmesi, hücre duvarı sentezi, enerji üretimi ve zar biyoenerjisi, mobil genetik elementi, bakteriyofaj enfeksiyonu, sprolanma, sprolanma gecikimi, yamyamlık ve biofilm oluşumudur.

Durağan faz sırasında YvfI düzenlemesi ile uyum içerisinde olarak, eksponensiyel fazda YvfI proteinin karbonhidrat metabolizmasını engellediği ve lipit meatbolizmasında görevli genleri düzenlemede rol aldığı bu çalışmada gösterilmiştir.

Bunların yanı sıra, karşılaştırmalı transkriptom çalışmaları IPTG ile indüklenen (*yvfI* olağandan fazla düzeyde ifade edilmiş) *B. subtilis* PY79 *amyE*::P_{spac}::*yvfI, yvfI::Tn10* mutant suşu ve indüklenmeyen (*yvfI* olağan düzeyde ifade edilmiş) *B. subtilis* PY79 *amyE*::P_{spac}::*yvfI, yvfI::Tn10* mutant suşu arasında yürütülmüştür. Bu çalışma YvfI'nın nitrojen metabolizmasında görev aldığını ve amino asitlerin katabolizması ve taşınmalarında düzenleyici etki gösterdiğini ortaya koymuştur. Ayrıca, YvfI düzenleyici proteinin olağandan fazla düzeyde ifade edilmiş olmasının "ICEBs1" lokusunun konjugal transferini dolaylı olarak indüklediği ve profaj benzeri elementlerin ekspresyonuna sebep olduğu ortaya konmuştur.

Ayrıca, bu araştırma 5'-RACE-PCR analizi ile *yvfI* geninin promotör bölgesinin tanımlanması ve yerinin saptanmasını amaçlamaştır. Sonuç olarak *yvfI* transkripsiyonunun σ^A-tipi promotör tarafından kontrol edildiği gösterilmiştir. Bunun dışında, yvfI gen dizisi ve promotör bölgesi üzerinde Spo0A proteini bağlanma motifiyle mükemmel bir benzerlik gösteren konsensüs motifi ile üç AbrB bağlanma

motifi ve bir CodY motifi gösterilmiştir. Böylece, bu genin quorum-sensing yol izi üzerinden, global düzenleyici proteinler tarafından düzenlendiği belirlenmiştir.

Sonuç olarak, YvfI'nın umut verici muhtelif düzenleyici davranışları YvfI'nın, bir çok değişik tipte regulona baskı ve indüksiyon uygulayarak düzenleyici aktivite gösteren GntR ailesi düzenleyici proteinlerine ait olduğu önerisi ile uyum göstermektedir. Bu yüzden bu çalışma *B. subtilis*'in farklı uyaranlara uyumlu tepkiler vermesine yardımcı olmada, YvfI'yı kritik bir düzenleyici protein olarak konumlandırmaktadır.

1. INTRODUCTION

1.1 *Bacillus subtilis*

Members of the *Bacillus* genus are Gram positive organisms that are omnipresent in the nature; therefore can be found to be facing a diverse variety of environmental conditions and habitats. These ubiquitous organisms are well known to be rod-shaped, aerobic or facultative, endospore-forming bacteria (Sonenshein et al., 1993; Harwood et al. 1990). The most extensively studied member of the Bacillus genus is *Bacillus subtilis* which was identified and named after by Ferdinand Cohn in 1872. Among the many characteristics that are attributed to *Bacillus subtilis*, being a chemoorganotroph, producing endospores, altering its physiology to develop adaptive responses and secreting many types of secondary metabolites are the most distinguishing features.

As being a chemoorganotroph, *Bacillus subtilis* displays the ability to oxidize a wide variety of organic compounds. Due to this characteristic, *B.subtilis* can grow on a simple salt medium which would offer glucose or other sugars as carbon and energy source, inorganic nitrogen and adequate supply of oxygen (Trun and Trempy, 2004; Harwood et al. 1990). Furthermore, *B. subtilis* displays a mesophilic characteristic which allows it to grow as normal-sized colonies within a day in case it is incubated at 37^{o}C (Harwood et al., 1990). Additionally, *B. subtilis* can survive under anaerobic conditions in such a case that nitrate is supplemented as the terminal electron acceptor to the environment (Earl et al., 2008, Harwood, 1996, Glaser et al., 1995, Cruzramos et al., 1995, Stein et al., 2005). During its growth, certain features of its metabolism might drive the organism into autolysis, which arises from the production of the peptidoglycan degrading enzymes that choose its own cell membrane structure as the target and resulting in autolysis caused by the increasing osmotic pressure across the cell membrane (Trun and Trempy, 2004).

Moreover, *Bacillus subtilis* is highly adaptive organism that smoothly responds to changes in the environment and subsequently, different growth conditions create big affects on its phenotype and morphology. The ultimate causes of these changes can

be listed as fluctuations on the growth rate, oxygen tension, temperature, pH or nutrient availability (Sonenshein et al., 1993). For instance, when exposed to limited nutrient condition at the end of the exponential phase, *B. subtilis* develops a motility and chemotaxis system that leads to a promotion of its survival strategies. In case this limitation does not cease to exist, then its metabolism initiates antibiotic production which stands for over than two dozen of ribosomal and non-ribosomal antibiotics that have antibacterial, antifungal and antimetabolic features to eliminate competition and secretion of enzymes such as proteases to consume nutrients from alternative resources. Furthermore, genetic competence development in order to uptake exogenous DNA and sporulation are also other responses that might be exerted by *Bacillus subtilis*, when nutritional limitation remains effective (Sonenshein et al., 1993; Hamoen et al., 2003).

Thus, especially after the sequencing of its genome is completed *B. subtilis* became a highly investigated model organism thanks to its non-pathogenicity, easy handling, high growth rate, easily transformability, adaptive metabolism, ability to produce hydrolytic enzymes (e.g. alkaline proteases, amylases), polypeptide antibiotics (e.g. bacitracin), biochemicals (e.g. nucleosides) and insecticides (e.g. endotoxins) (Kunst et al., 1997, Harwood, 1996; Sonenshein et al.,1993; Hamoen et al., 2003). Hence, it is not only a highly rated model organism but it also displays commercially important health-beneficial properties; such that its spore form helps to prevent gastrointestinal disorders by acting as a probiotic and it is confirmed that it can be used as an alternative to many antibiotics thanks to its characteristic of being a novel prophylactic, therapeutic and growth promoting agent (Hong et al., 2004, Williams, 2007, Fujiya et al., 2007).

1.2 Genetic View of *Bacillus subtilis*

Bacillus subtilis stands as one of the most articulately studied member of *Bacillus* species and it is a model organism for low GC Gram positive bacteria (Kobayashi et al., 2003). G+C content of the *B.subtilis* genome is reported to be about %43 while A+T regions can be found on specific regions of the chromosome (Kunst et al., 1997). Studies on its genomic sequence was completed and published in Nature in November 1997 by Kunst and his friends. 4,214,810 base pair long genome of *Bacillus subtilis* is now known to consist of ≈4,100 genes (Kunst et al., 1997,

Kobayashi and Ogasawara, 2002). Following the first studies, a large set of genes were mapped, revealing out that about one third of total genes had specific defined functions and proteins encoded from another 1000 were classified as having unknown functions. Another prediction was set for about a quarter of the *Bacillus subtilis* genome to encode fueling and biosynthetic proteins (Moszer, 1998; Harwood et al., 1990). Additionally, *B. subtilis* genotype has further supported its being a highly adaptive organism, such that, large sets of genes were proposed to be responsible for environmental stress adaptations (Harwood et al., 1990). Explored patterns on *B. subtilis* genome further strengthened the capacity of *B. subtilis* to use highly diverse types of carbon sources and its ability to produce secondary metabolites, including antibiotics and industrially important enzymes. Furthermore, it was reported to encode for about ten prophages or prophage remnants that signifies a possible role for bacteriophage infection in the transfer of genes during bacterial evolution. Furthermore, the presence of large gene families has rised as the results of gene duplications and the largest family contains 77 candidate ATP-binding transport proteins (Hoch and Losick, 1997).

Formerly, when the sequence was first published, only about 58% of the genes had assigned functions. However, this number has incresead up to 63% in the current databases (Kobayashi and Ogasawara, 2002). In a more recent study, published by Kobayashi and his colleagues in 2003, they have performed gene inactivation studies in order to estimate the minimal gene set required to maintain survival. In comparison to the results obtained in previous studies, which pointed out 192 genes to be essential for survival in given optimum conditions, another 79 genes were predicted to be essential as a result of this current study. Nearly half of the essential genes were categorized to fuction in information processing. Remaining genes were proposed to function in cell envelope synthesis, morphology and division specification and scarcely in cell energetics. On the other hand, 4% of essential genes were revealed out to display unknown functions (Kobayashi et al., 2003). Moreover, 4215 kb genome further consists of a set of 86 tRNA genes, 30 rRNA genes and three small stable RNA genes (Kobayashi and Ogasawara, 2002). Additionally, the polarity of *B. subtilis* genome comes from the residence of 75% of genes in the leading strand and clustering of of highly expressed genes near the origin of replication (Rocha et al., 2000).

1.3 *Bacillus subtilis* Antibiotics

Bacillus antibiotics that have been recognized for more than 50 years exhibit a highly diverse range of antimicrobial activity: while bacitracin, pumulin, laterosporin, gramicidin and tyrocidin exerts their effect towards Gram-positive bacteria; colistin and polymyxin are anti-Gram-negative antibiotics; and a broad spectrum antibiotic, difficidin is known to be produced as secondary metabolites (Stein et al., 2005; Trun and Trempy, 2004). Furthermore, antifungal agents are widely synthesized by *B. subtilis* strains as small antibiotic peptides, which cover less than 2000 Da in molecular weight and these peptide agents include iturin, surfactin, fengymycin, bacilysin, bacillomycin, mycosubtilin, B29I and mycobacillin (Majumdar and Bose, 1960; Sengupta et al., 1971; Tsuge et al., 2001; Stein, 2005; Peypoux et al., 1999; Carrillo et al., 2003; Peypoux et al., 1980, Peypoux et al., 1986; Li et al., 2009).

Number of antibiotics that are known to be produced by several hundred wild-type *B. subtilis* strains have reached to about two dozen and these antibiotics display a variety of structure. Genomic region that has been occupied by the genes that encode for these antibiotics is estimated to be about 350 kb which stands for approximately 4-5% of the genome of *B. subtilis* (Stein, 2005). The function that is attributed to these antibiotics is beyond to be solely anti-microbial agents, but it is now known that some types of antibiotics have role in setting the morphology and physiology and promote the survival strategies of *B. subtilis* in diverse niches. Peptide antibiotics represent the largest and the most diverse class and they are synthesized either through ribosomal machinery followed by post-translational modifications and proteolytic processing (recognized as lantibiotics and lantibiotic-like peptides) or through non-ribosomal machinery by large megaenzymes, the non-ribosomal peptide synthetases (NRPSs). These two different biosynthetic pathways allow the incorporation of unusual (non-proteinaceous) constituents to these peptide antibiotics that exert a highly rigid, mainly hydrophobic with unusual components like non-essential and D-amino acids (Katz and Demain, 1977; Stein, 2005; Li et al., 2009; Moszer, 1998).

1.3.1 Bacilysin

Bacilysin is a simple and small sized (125kDa) dipeptide antibiotic strain which is produced and excreted by *B. subtilis* Marburg 168 strain against a wide range of bacteria and fungi especially *Candida albicans* (Steinborn et al., 2005). *B. amyloliquefaciens, B. licheniformis* and *B. pumilus* are among the species that are known to produce bacilycin which is considered to be a species trait of *Bacillus subtilis* (Loeffler et al., 1986). Bacilysin [L-alanine-(2.3-epoxycyclohexanone-4)-L-alanine] (Figure 1.1) is composed of L-alanine residue at N-terminus and non proteinogenic L-anticapsin residue at C terminus (Walker and Abraham, 1970).

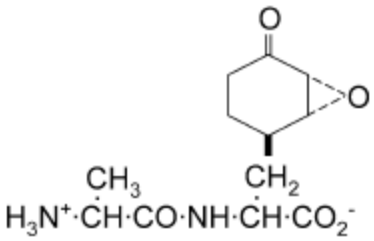


Figure 1.1 : The structure of bacilysin.

Antibiotic moiety of this dipeptide is displayed by its L- anticapsin residue (Kenig et al., 1976). The synthesis of anticapsin is initiated from prephenate of the aromatic acid pathway which is the primary precursor of bacilysin (Hilton et al., 1988). Prior to get subjected to peptidase, bacilysin is carried by a distinct peptide permease system into the cytosol where L-anticapsin residue is released and this free intracellular anticapsin residue acts as an inhibitor of glucosamine synthetase which in turn determines bacterial peptidoglycan or fungal mannoprotein biosynthesis and then ultimately leading to cell protoplasting and lysis (Kenig et al., 1976; Perry and Abraham 1979). On the other hand, antibiotic moiety of anticapsin is antagonized by glucosamine or N-acetylglucosamine (Walton and Rickes 1962; Kenig and Abraham, 1976).

Bacilysin production of *B. subtilis* 168 was found to be induced in specific circumstances including growth in a synthetic medium and to be inhibited by a number of growth conditions, such as certain nutrient supplements like glucose or casaminoacids and environmental factors such as pH and temperature (Özcengiz et al. 1990; Özcengiz and Alaeddinoglu 1991a, b; Basalp et al. 1992). Regulation of bacilysin biosynthesis has been widely studied revealing out that its production is

regulated on different levels both positively and negatively. Positive regulation is known to drived by guanonsine 5'-diphosphate 3'-diphosphate (ppGpp) (Inaoka et al., 2003) and also by quorum-sensing mechanism via peptide pheromone PhrC (Yazgan et al., 2003). ppGpp plays a crucial role in the transcription process of the biosynthetic *ywfBCDEFG* operon and that the transcription of these genes is dependent upon the level of intracellular GTP concentration of which is sensed by CodY-mediated repression system (Inaoka et al., 2003; Yazgan et al., 2003). Furthermore, negative regulation of CodY is more effectively sensed in the presence of excessive glucose or casamioacids (Ratnayake-Lecamwasam et al.,2001; Shivers and Sonenshein 2004). On the other hand, CodY also displays an important role in case of the regulation of *spo0A* and additionally, it also represses *srfA* operon. Therefore, when there is a codY null mutation, an increase in bacilysin production in wild-type cells becomes observable and repression on *oppA*, *srfA* and *spo0A* disappears. Moreover, recent studies have suggested that srfA operon deletion in the bacilysin producing wild type cells caused the bacilysin-negative phenotype (Mascher et. al., 2004 and Yazgan et al., 2003). Transposon mutagenesis studies have revealed out that bacilysin biosynthesis is directly linked to and under control of quorum sensing global regulation of ComQ/ComX, PhrC (CSF), ComP/ComA in a spo0K-dependent manner (Yazgan et al., 2001). Besided, the relationship between the sporulation and bacilysin biosynthesis has revealed out that changes in bacilysin formation pattern was caused by common effectors of sporulation (Basalp et al., 1992).

Biosynthetic core function on bacilysin production depends on a polycistronic operon *ywfBCDE* renamed as *bacABCDE* and the adjacent ywfGH genes (Figure 1.2) (Steinborn and Hofemeister 1998/2000; Inaoka et al., 2003).

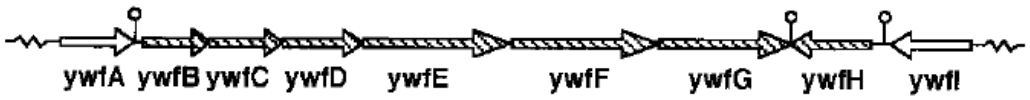


Figure 1.2: Organization of the bacilysin gene cluster *ywfABCDEFG* and *ywfH* gene of *Bacillus subtilis* 168 (Inoaka et al., 2003).

The unusual amino acid anticapsin is generated as a branching off from prephenate of the aromatic amino acid pathway through the action of BacA which is an atypical prephenate decarboxylase and BacB which is an aminotransferase that catalyzes an allylic isomerization (Hilton et al., 1988, Mahlstedt and Walsh 2010). BacA and

BacB products are found to be regioisomers of H(2)HPP (dihydro-4-hydroxyphenylpyruvate) and then BacB is chosen as the substrate by YwfH, a short chain reductase that ultimately causes to promotion of cyclohexenol-containing tetrahydro-4-hydroxyphenylpyruvate H(4)HPP product. H(4)HPP gets subjected to transamination by YwfG which induces therefore formation of tetrahydrotyrosine (H(4)Tyr). This four enzyme (BacA, BacB, YwfH, and YwfG) pathway leads to the H(4)Tyr production and ultimately to anticapsin formation through a unknown reaction (Figure 1.3) (Mahlstedt and Walsh 2010).

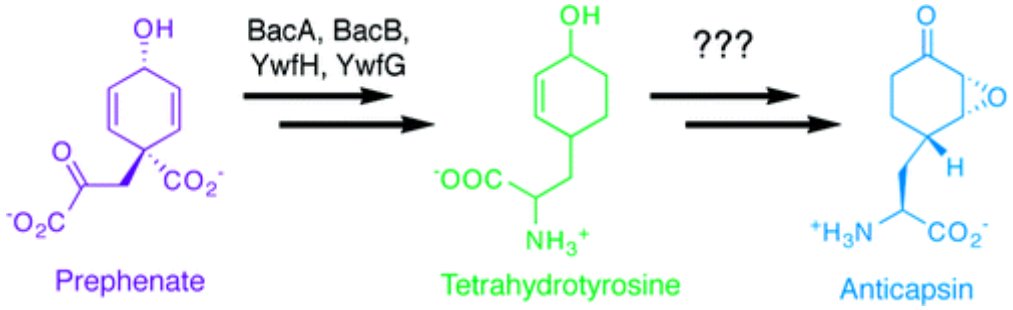


Figure 1.3: Four enzyme (BacA, BacB, YwfH, and YwfG) pathway leading to the H(4)Tyr production and ultimately to L-anticapsin formation.

BacD gene is responsible for the ligation of L-alanine and L-anticapsin residues and it encodes for a L-amino acid ligase belonging to ATP-dependent carboxylate-amine/thiol ligase superfamily and this study revealed this ligase as the branching point of bacilycin biosynthesis from nonribosomal peptide synthetase (NRPS) system (Stein, 2005). Furthermore, studies on the in vitro enzymatic synthesis of bacilysin proposed that formation of bacilysin was not carried out by multiple-carrier thiotemplate mechanism because adenylation and thiolation were observed only for L-alanine, but not for L-anticapsin (Marahiel, 1997; von Döhren et al., 1999). On the contrary, it should be noted that formation of tetrahydrotyrosine (H(4)Tyr) from above mentioned four enzyme complex was pointed out to play building role in cyanobacterial nonribosomal peptides such as micropeptins and aeruginopeptins (Mahlstedt and Walsh 2010). Furthermore, BacE is responsible for self-protection from bacilysin by encoding an efflux protein for bacilysin excretion (Steinborn et al., 2005).

1.4 GntR-Type Regulatory Proteins

When mentioning about proteins, a two-component signal transduction system contains input and response domains in two different proteins that communicate via a His-Asp phosphotransfer. On the other hand, one component system refers to the proteins that have signal sensing domain and a functional response domain (Ulrich et al. 2005). In prokaryotes, signal transduction is acquired through these systems where sensor input domain is usually small-molecule binding domain and functional output domain is predominantly helix-turn-helix domain (Ulrich et al. 2005). It should be noted that although helix-turn-helix domain is the most common output domain, several other transcriptional regulator groups have been identified that consist of zinc-fingers, leucine-zipper, homeodomain, and β-sheet DNA-binding domains (Harrison, 1991; Pabo and Sauer, 1992). Still, the most extensively characterized and widely experimented group stands as the HTH group (Haydon and Guest, 1991; Henikoff et al., 1988; Brennan and Matthews, 1989; Weickert and Adhya, 1992; Gallegos et al., 1993; Nguyen and Saier, 1995) in which the the second α-helix is often called as the the "recognition" helix and it is addressed to the DNA major groove. Generally, HTH proteins bind to their target as dimers, therefore they possess the ability to bind 2-fold symmetric DNA sequences. Accordingly, this group is now counted useful for estimating general axioms that direct protein-DNA interactions (Pabo and Sauer, 1992; Wintjens and Rooman, 1996).

One component systems and hence HTH transcriptional regulators are subdivided into families according to sequence similarities, generally in the DNA binding effector domain (Haydon and Guest, 1991; Ulrich et al., 2005; Henikoff et al., 1988; Weickert and Adhya, 1992; Gallegos et al., 1993; Nguyen and Saier, 1995; Rosinski and Atchey, 1999). These families are usually classified after the best-characterized member and these families include LysR (Henikoff et al. 1988), TetR (Ramos et al. 2005), IclR (Nasser et al. 1994), GntR (Haydon and Guest 1991), and AraC (Gallegos et al. 1997) superfamilies. Sequence comparisons and phylogenetic, structural, and functional studies have resulted in exploration of a common consensus sequence that is recognized by each family and wherefrom it offered a way to label new members based on their families (Karmirantzou and Hamodrakas, 2001).

Sequence similarities are not common between DNA binding or oligomerization domains therefore these sites are not usually taken into consideration when deciding for which member belongs to which family. On the other hand, they display a crucial part in the regulating process. Most importantly, it is often these domains that provide correct alignment of HTH motif and DNA binding ability of transcriptional regulatory protein (Rigali et al., 2002). Therefore a recent study conducted by Rigali and friends in 2002, has focused on exploration of the link that exists between regions involved in the regulating process by studying the HTH GntR family of bacterial regulators.

Regulators from GntR family transcriptional repressor consist of a conserved N-terminal DNA binding domain (D-b domain) followed by C-terminal domain involved in the effector binding and/or oligomerization (E-b/O domain) and additionally they are best characterized by a winged helix-turn-helix motif which acts as the DNA recognition domain (Haydon and Guest 1991; Rosinski and Atchley 1999; van Aalten et al. 2000; Lee et al. 2000; Rigali et al. 2002; Aravind and Anantharaman 2003). First description addressing this family was given by Haydon and Guest in 1991 and it was named as GntR, based on the repressor of the gluconate operon in *Bacillus subtilis* (Fujita et al., 1986; Reizer et al., 1991). These proteins act as regulators by applying regulations on many different types of regulons. GntR family of transcriptional regulators consists of about 2000 different proteins that have different action sites and also that function in different organisms including archeal organisms (Rigali et al., 2002).

Study by Rigali and friends in 2002 has led to defining family signatures and therefore many subfamilies. Initially, a phylogenetic tree was created by using full-length multiple alignment of GntR-resembling members (Figure 1.4). As a result, it became obvious that C-terminal heterogeneity has divided the families into four major classes based on effector binding and/or oligomerization (E-b/O domain) types. Two-dimensional structural predictions has supported four major types prediction (Figure 1.5, *a*–*d*) including distinct varying members in each subfamily and this subdivision was accepted to be highly efficient considering the fact that only 7% of tested proteins had escaped from this subdivision. It is also suggested by this study that members that belong to a defined subfamily were commonly created by duplication events.

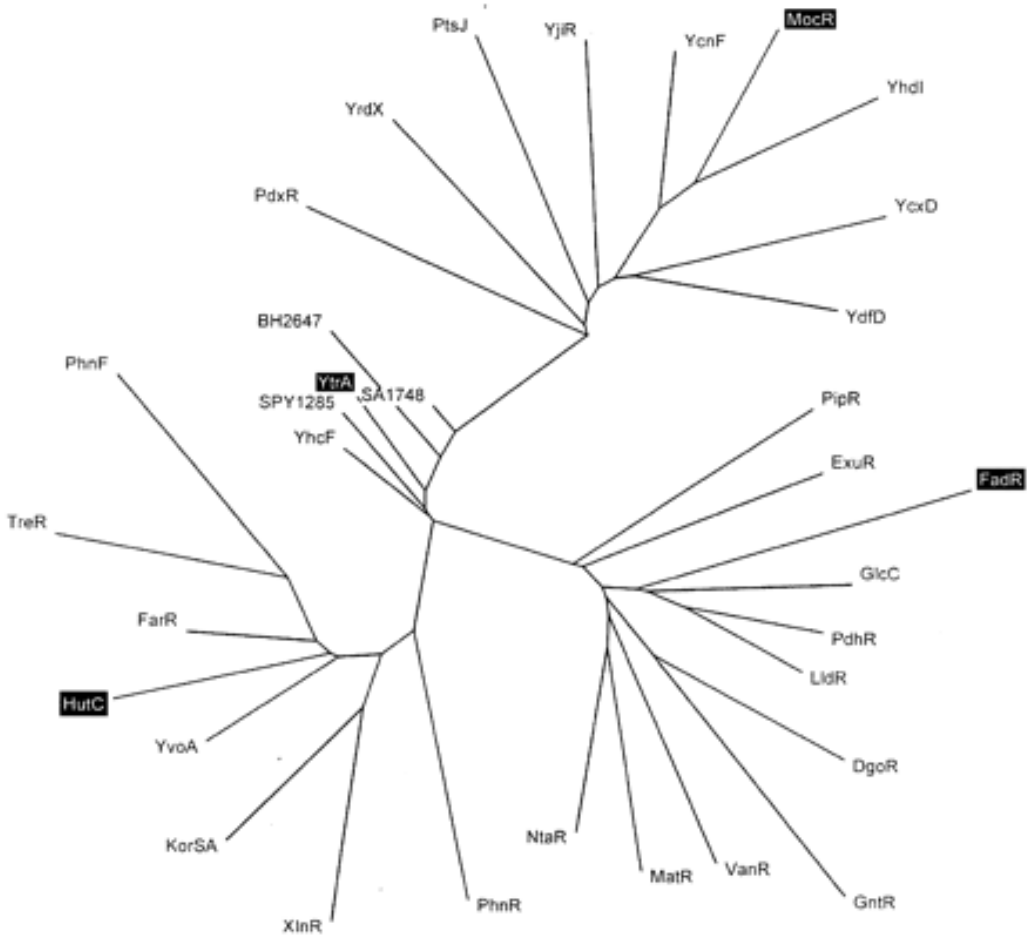
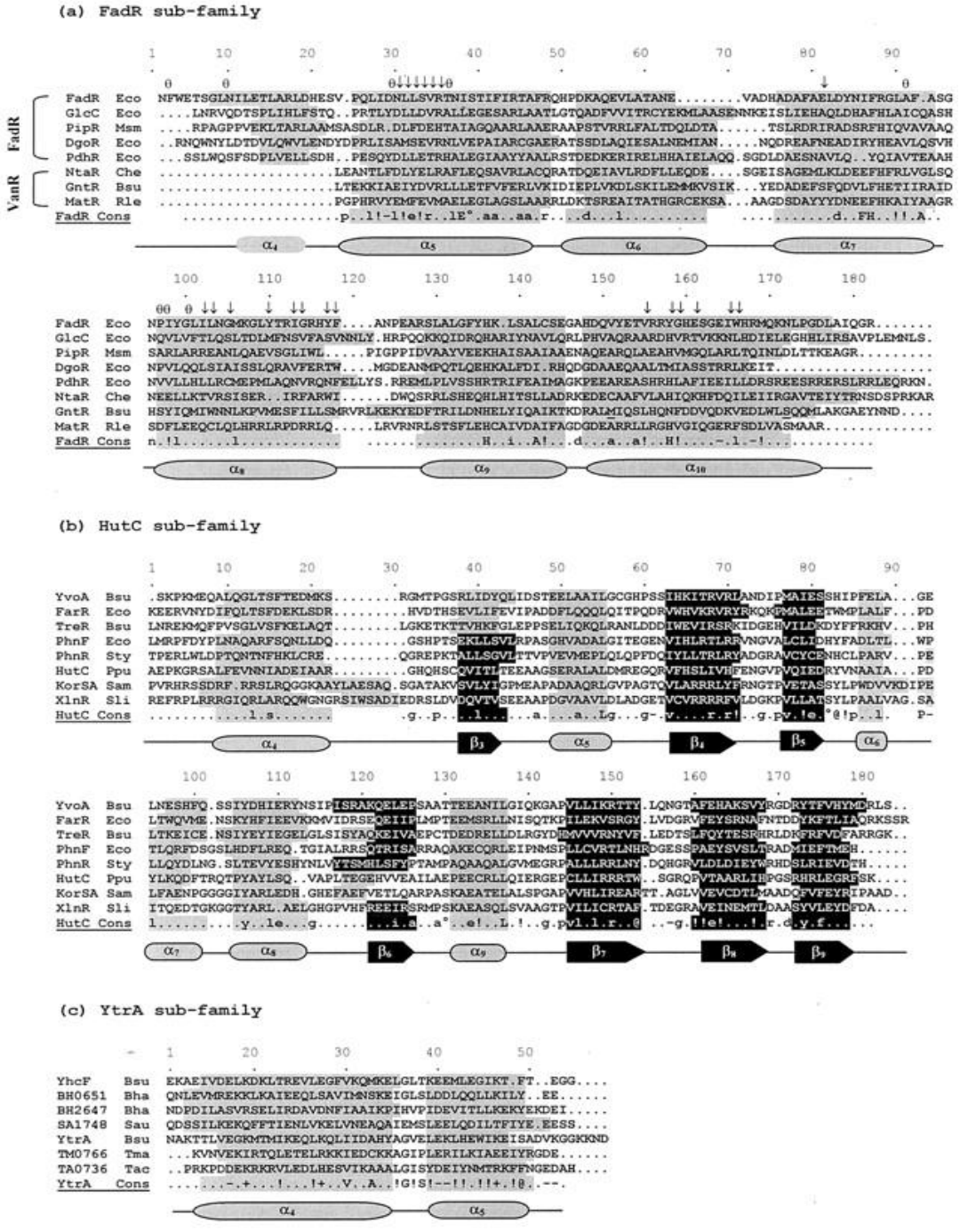


Figure 1.4. Phylogenetic tree created by using full-length multiple alignment of GntR-like members. GntR-resembling transcriptional regulators were categorized into four major subfamilies based on the observed C-terminal structural topologies.

The first GntR subfamily, FadR, is the most crowded subfamily as it covers 40% of GntR-like regulatory proteins. Members of this subfamily consist of a six or seven α-helices containing C-terminal domain depending on subgroups (Figure 1.5*a*). VanR-like regulators differ from FadR protein subgroup based on the loss of the first α-helix (α_4). The crystal structure of the C-terminal domain of FadR has been enlightened (van Aalten et al., 2000; van Aalten et al., 2001) and its three-dimensional structure was decided to be useful to stand for a scaffold to conduct studies on the entire subfamily (Rigali et al., 2002). Considering functional properties of the FadR-like proteins, it is remarkable that these proteins are associated to the regulation of amino acids metabolism or to various metabolic pathways such as aspartate (AnsR), pyruvate (PdhR), glycolate (GlcC), galactonate (DgoR), lactate (LldR), malonate (MatR), or gluconate (GntR). For instance,

in *Escherichia coli* FadR protein from GntR repressor family acts regulating fatty acid biosynthetic and degradation genes (DiRusso et al., 1999). Corynebacterium glutamicum protein LldR, which is responsible for the regulation of L-Lactate and sugar utilization operon was identified very recently to belong to the FadR subfamily of GntR family regulators (Georgi et al., 2008).



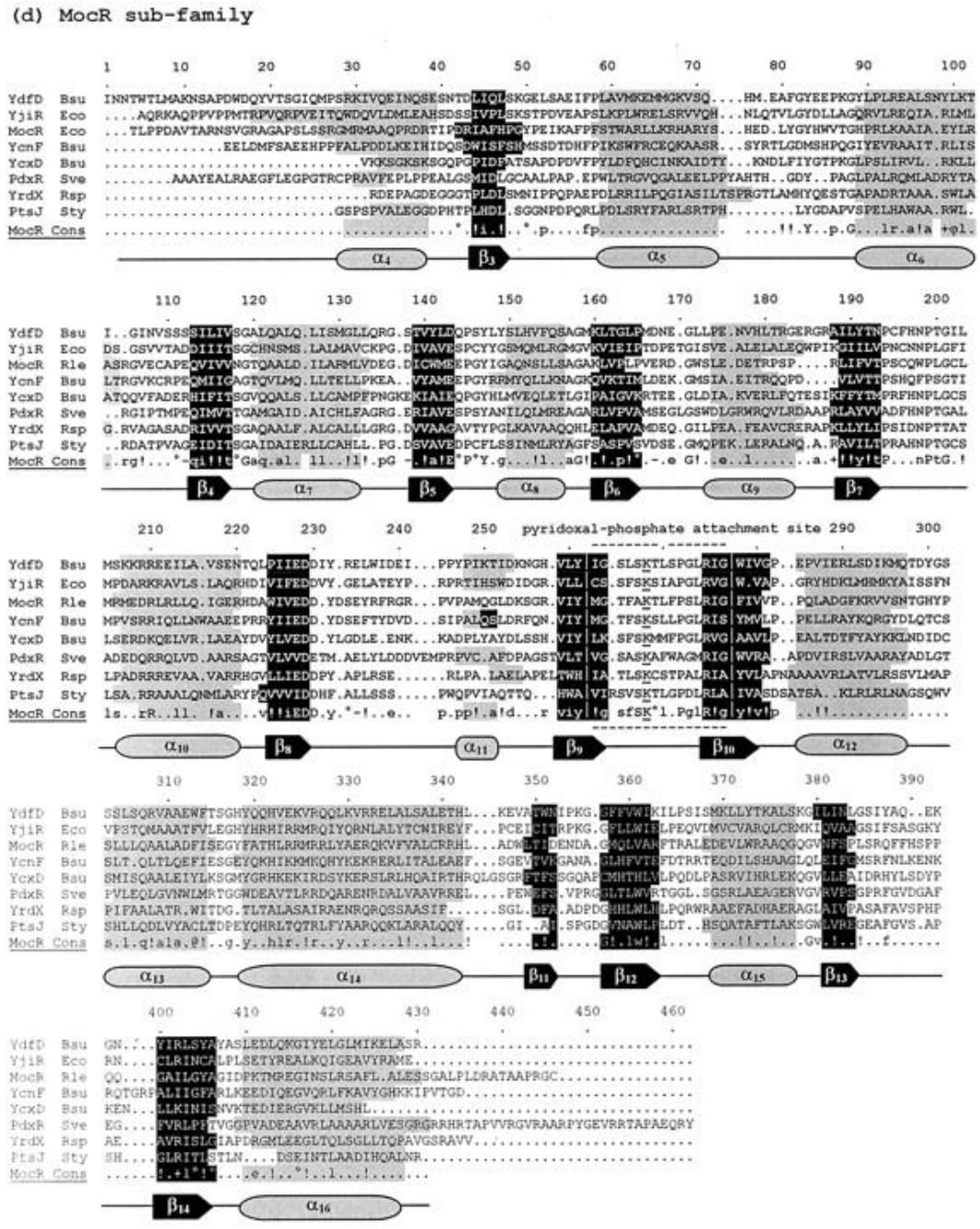


Figure 1.5. Sequence alignments of the C-terminal domains of proteins belonging to the GntR family on the basis of their structure. Symbols for conserved amino acid properties were given as follows: !, conserved hydrophobic residues (ILVAMFYW); @, aromatic residues (FYW); −, negatively charged residues (ED); +, positively charged residues (RKH); ○, small residues (GSATPN). ↓ and θ indicate residues implicated in effector binding and dimerization of the FadR protein (van Aalten et al., 2000; van Aalten et al., 2001). Also in panel a, the underlined residue represents the single amino acids mutations on what affect gluconate binding ability in GntR (Yoshida et al., 1995). In panel d, the underlined residue corresponds to the amino acid lysine that established the covalent link with the cofactor in aminotransferases. Spaces in consensus sequences refers to insertions within the alignment.

Second subfamily, HutC, differs from all-helical FadR C-terminal domains, because the C-terminal domain of HutC-like proteins contains both α-helical and β-sheet structures (Figure 1.5b). The subfamily represents 31% of GntR-like regulators and its 170 amino acid long C-terminal domain resembles highly to FadR subgroup of FadR subfamily in length (Rigali et al., 2002; van Aalten et al., 2000). At several positions, including β_3, α_7, and β_6, structural elements were observed to be changed in the means of conservation and this alteration in E-b/O topology was accorded to the structural adaptations as responses to high diversity of biological processes regulated by HutC-like members (Rigali et al., 2002). This subfamily was named after the HutC regulator from *Pseudomonas putida*, which was previously suggested to display an inhibitory effect on the expression of histidine utilization genes and to be repressed by the binding of uroconate (Allison and Phillips 1990). YvoA protein that belongs to GntR/HutC family of bacterial transcriptional regulators regulates many biological pathways from antibiotic production to profileration and growtn not only in *Bacillus subtilis* but also in other organisms like *Streptomyces griseus* or *Streptomyces coelicolor* (Rigali et al., 2006; Resch et al., 2010; Seo et al. 2002). Furthermore, many other proteins like TraR that functions in oligomerization were found to belong to the GntR/HutC subfamily repressor (Kataoka et al., 2008). Among other studied members of the HutC family FarR from Escherichia coli was reported to regulate citric acid cycle pathway and reacts against long-chain fatty acids (Quail et al. 1994). Contrarily, Sampaio and friends suggested that FarR regulates genes coding for 2-O-α-mannosyl-D-glycerate transport and metabolism system. On the other hand, FarR-dependent regulation in C. glutamicum has put out a support to FarR function described by Quail et al. İn 1994 (Haenssler et al., 2007). TreR from *Bacillus subtilis* regulates trehalose operon and this protein is inhibited by trehalose-6-phosphate (Schock and Dahl 1996), furthermore proteins like KorSA, KorA are repressors of the genes involved in conjugative plasmid transfer in Streptomyces species (Kendall and Cohen 1988; Hagege et al. 1993).

In case of third subfamily, called MocR, E-b/O domain is extensively distinct from the first two types because of average length of C-terminal domain of about 350 amino acids and because of its homology to the class I of aminotransferase proteins (Sung et al., 1991) (Figure 1.5d). These proteins catalyze a transamination reaction which is important for amino acid metabolism as well as in linkages to carbohydrate

and fatty acid metabolism. During transamination, an amino group of an amino acid is transferred to a α-keto acid in a reversible manner by these regulatory proteins which are usually found as dimers during action and pyridoxal 5'-phosphate (PLP) works as a cofactor of their regulating activity (Ko et al., 1999). One example of these regulatory proteins is PdxR in *Streptomyces venezuelae*, which is involved in the regulation of pyridoxal phosphate synthesis (Magarvey et al., 2001). Another example can be given from *Rhodobacter capsulatus* in which the GntR-Like regulator TauR which shows the highest similarity to the MocR subfamily, named after *S. meliloti* MocR, was found to activate expression of taurine utilization genes (Wiethaus et al., 2008; Rossbach et al., 1994). In contrast to FadR, HutC, and YtrA subfamilies, which exert their regulatory effects on identified pathways by binding to inverted repeats that are found on the promoter regions of their target genes, MocR-like proteins bind to direct repeats on the promoter regions (Rigali et al., 2002). For instance, MocR-like protein GabR from *B. subtilis* acts as both an autorepressor and an inducer of genes involved in the utilization of γ-aminobutyrate (GABA) as the sole nitrogen source (Belitsky, 2004; Belitsky and Sonenshein, 2002).

The fourth subfamily, YtrA was shown to be moderately smaller than the other three subfamilies based on the fact that it possesses a short C-terminal domain consisting of only two α-helices (Figure 1.5c). The subfamily represents 6% of GntR-like regulators and most of this limited number of proteins regulates some of the operons involved in ATP-binding cassette (ABC) transport systems (Rigali et al., 2002).

In case the DNA-binding (D-b) domain topology was investigated through structural predictions, all of the secondary structure elements in the whole GntR family was found to be rather well conserved in similar relative positions. They were found to consist of three α-helices and two (sometimes three) β-sheets. Their permutation was proposed to be: $\alpha_1\alpha_2\alpha_3\beta_1\beta_2$ (Figure 1.6).

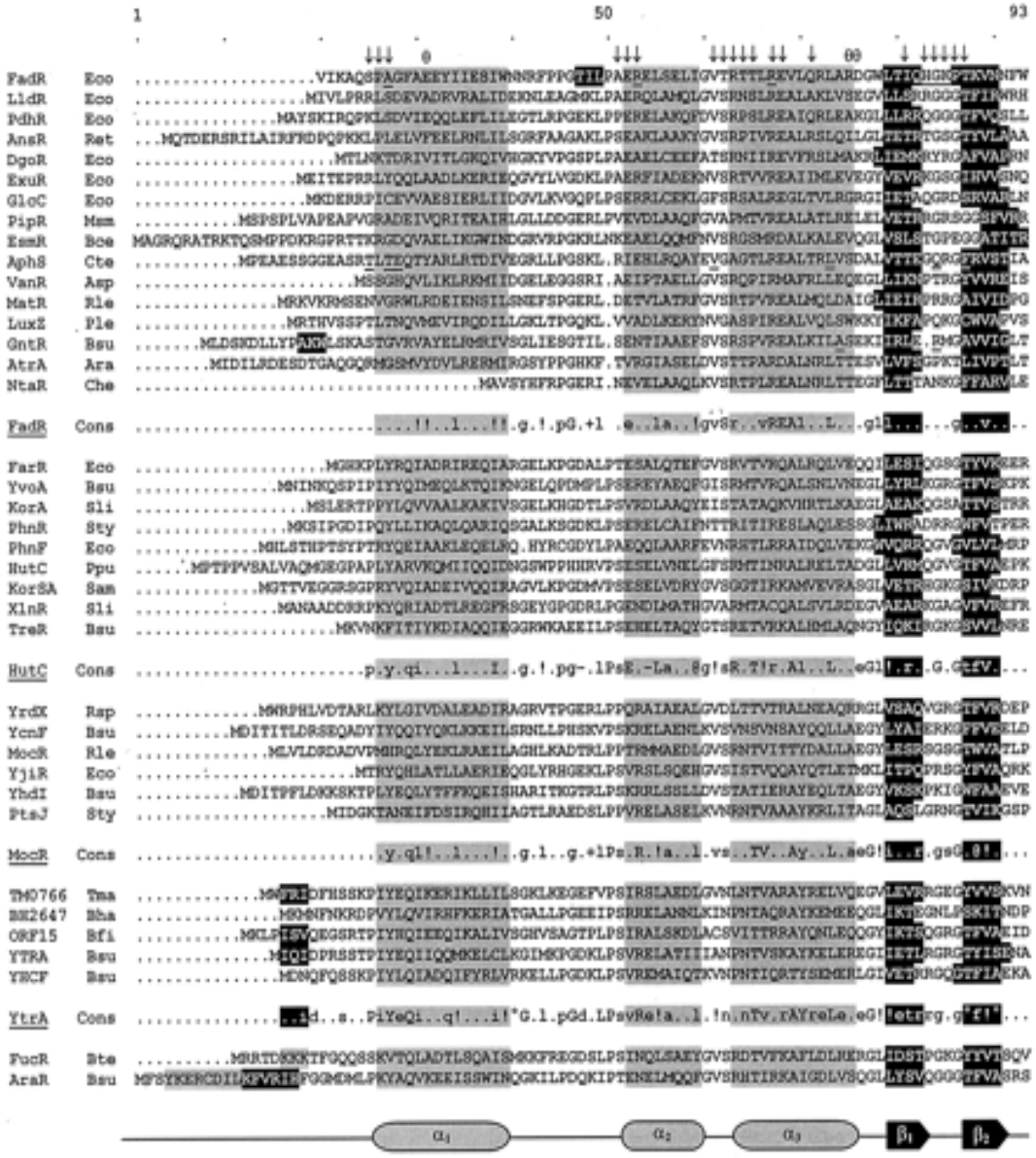


Figure 1.6. Structure-based sequence alignment of the N-terminal DNA-binding domain of proteins of the GntR family. Symbols for conserved amino acid properties are as follows: !, conserved hydrophobic residues (ILVAMFYW); @, aromatic residues (FYW); −, negatively charged residues (ED); +, positively charged residues (RKH); ○, small residues (GSATPN). ↓ and θ indicate, in FadR, residues refers to DNA binding and dimerization (van Aalten et al., 2000; van Aalten et al., 2001). The mutation on underlined amino acid residues is known to alter the DNA binding ability of AphS (Ortuno-Olea and Duran-Vargas, 2000), FadR (Raman et al., 1997), and GntR (Yoshida et al., 1993). Spaces in consensus sequences refer to insertions within the alignment.

When D-b domains of GntR type regulator proteins were compared, similarities between amino acid alignments of these domains were found to be constricted to 25%. Therefore, it was concluded that common DNA-binding domain ancestor of

GntR family is represented by structural topology rather than by amino acids alignment. On the other hand, one quarter percentage was proven to elevate to 40 and 60% when subfamilies are analyzed separately. Therefore, although subfamilies were categorized by the C-terminal domains, C-terminal similarity was reflected on the DNA-binding domain and on the HTH motif itself. Figure 1.6 further displays the fact that similarities between MocR and YtrA subfamilies were distinctively higher. The fusion between the D-b domain and the E-b/O domain was proposed to realized separately for the FadR, HutC, and MocR/YtrA subfamilies hence explaining that these subfamilies were definitely not emerged from each other. Still, figure 1.4, strengthens the observation of high level similarity between the D-b domains belonging to the MocR and YtrA subfamilies because phylogenetic tree also displayed higher similarity such that these two subfamily was designated to emerge from a common branch and this fact emphasize a conserved amino acids composition in their N-terminal domain (Rigali et al., 2002). It should be noted that some unusual secondary structure element alignments was observed in the N-terminal structural consensus ($\alpha_1\alpha_2\alpha_3\beta_1\beta_2$). These anomalities include NtaR from *Chelatobacter heintzii* and EmoR from the EDTA-degrading bacterium, BNC1 lacking their first α-helix (α_1), WhiH from *Streptomyces aureofaciens* or PdxR from *S. venezuelae* containing one extra helix upstream of α_1, YtrA regulators displaying an additional β-sheet upstream of α_1 (Rigali et al., 2002).

In the means of operator sites analysis it was supposed in the same study by Rigali et al. that highly conserved DNA-binding motifs may bind similar operator sequences. When C-terminal inverted repeat operator site sequences of previously studied GntR-like proteins were aligned, bound sites has given a palindromic 5′-(N)yGT(N)xAC(N)y-3′ sequence. (y) stands for divergence on the number of nucleotides whereas (N) stands for the nature of nucleotide. Based on the observed palindromic sequence, it was concluded that the center of the sequence was highly conserved, while the adjacent sequence diverged and this model was also observed by Weickert and Adhya in the study that focused on LacI/GalR family. Hence the central region of the operator was accepted to be the signal-attracting region, whereas the peripheral zones worked as the operator discriminator in these regulatory proteins.

1.5 YvfI, a GntR-Type Regulatory Protein, Involved in Bacilysin Production

In addition to global regulatory genes, bacilysin biosynthesis in *Bacillus subtilis* was also reported to be regulated by a novel gene, *yvfI* (Koroglu et al., 2008). In this study, three mutants that display bacilysin negative phenotype were isolated using transposon mutagenesis studies (Figure 1.7).

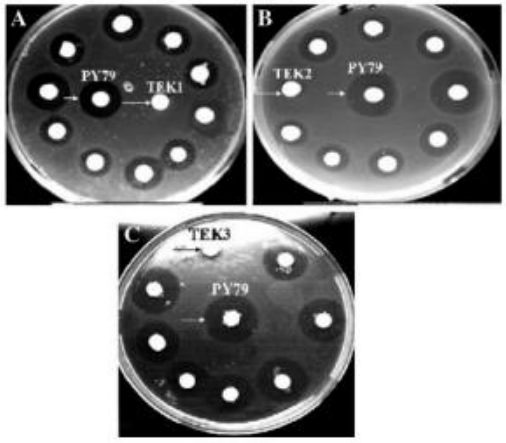


Figure 1.7 : mini-Tn10 insertional mutagenesis derivatives of wild type PY79 strain of *Bacillus subtilis* and three mutants TEK1, TEK2 and TEK3 assayed for their antimicrobial activity by paper disc-agar diffusion assay.

Following the confirmation that antimicrobial activity came directly from lack of bacilysin, DNAs from TEK1, TEK2 and TEK3 were subjected to southern blot analysis and by using mini-Tn10 (ori-spc) probe three independent mutant isolates were shown to carry the same insertional mutation on the same locus. Tn10 insertions and their flanking regions were cloned as further step and as a result of sequencing analysis, insertion in all three mutants were found to be in the *yvfI* gene which displays the genomic organization given in figure 1.8.

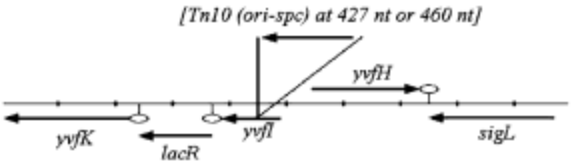


Figure 1.8 : Genomic organization of the *yvfI* gene and peripheral chromosomal regions (Kunst et al., 1997). Also shown is the 427 or 460 nucleotide long transposon insertion in the *yvfI* gene (Köroğlu et al., 2008).

Hence, transposon mutagenesis was applied in order to point out mutations on which genes resulted in bacilysin-negative phenotype and as a result, determined mutation on *yvfI* gene has shown that *yvfI* was essential for dipeptide antibiotic bacilysin

biosynthesis in *Bacillus subtilis* (Köroğlu et al., 2008). Furthermore, A conserved Domain Database (CDD) (Marchler-Bauer et al. 2005) search was applied for YvfI protein and this study pointed out FadR C-terminal ligand binding (FCD) of YvfI protein which is located at the C-terminal of protein and very common among GNTR family of proteins. FCD domain-harboring proteins were previously published by van Aalten and colleagues (2000). 44 amino acid extended proposed YvfI protein (Kunst et al., 1997) was also examined to reveal an incomplete but considerable match to the GntR wHTH domain. Therefore, it was demonstrated that YvfI sequence shows great homology with its orthologs from different species. Studies on YvfI sequence suggested that this protein displays high homology with its orthologs from different species including FadR (Marchler-Bauer et al., 2005). FadR-like proteins function in the regulation of many pathways involved in amino acids metabolism, L-lactate and sugar utilization, fatty acid transport and degradation as well as in many metabolic pathways including those for aspartate, pyruvate, glycolate, galactonate metabolism (Rigali et al., 2002).

Moreover, very recent study has shown that the *yvfV-yvfW-yvb*Y (*lutABC)* operon is under the control of *yvfI,* consequently renamed as *lutR* and was further accepted to be a part of GNTR regulatory proteins that consists of a HTH recognition domain (Chai et al., 2009). Furthermore, it was speculated by Chai an coworkers that the *yvfV-yvfW-yvbY* operon specifies a pathway for the utilization of L-lactate and therefore this operon was renamed as LutABC. Furthermore, homologous operons were demonstrated in different bacterial genomes, suggesting that LutABC operon stands for a widely conserved genomic organisation for L-Lactate utilisation (Figure 1.9b). However, a 15 kb loop was observed that separates lutABC operon from the lutR, which encodes for GntR-type repressor, *lutP*, which putatively encodes for a lactate permease *sigL*, and *yvfG* genes and the *epsA-to-O* operon so that *B. subtilis* differs from *B. licheniformis* on that matter (Figure 1.9a) (Chai et al., 2009; Veith et al., 2004). Hence these observations all together have led to the conclusion that function of the *lutABC* operon is related to not all but to one or more members of the cluster. Keeping in mind that *lutP* putatively encodes for a lactate permease, it was suggested by Chai and coworkers that *lutABC* operon is involved in lactate metabolism.

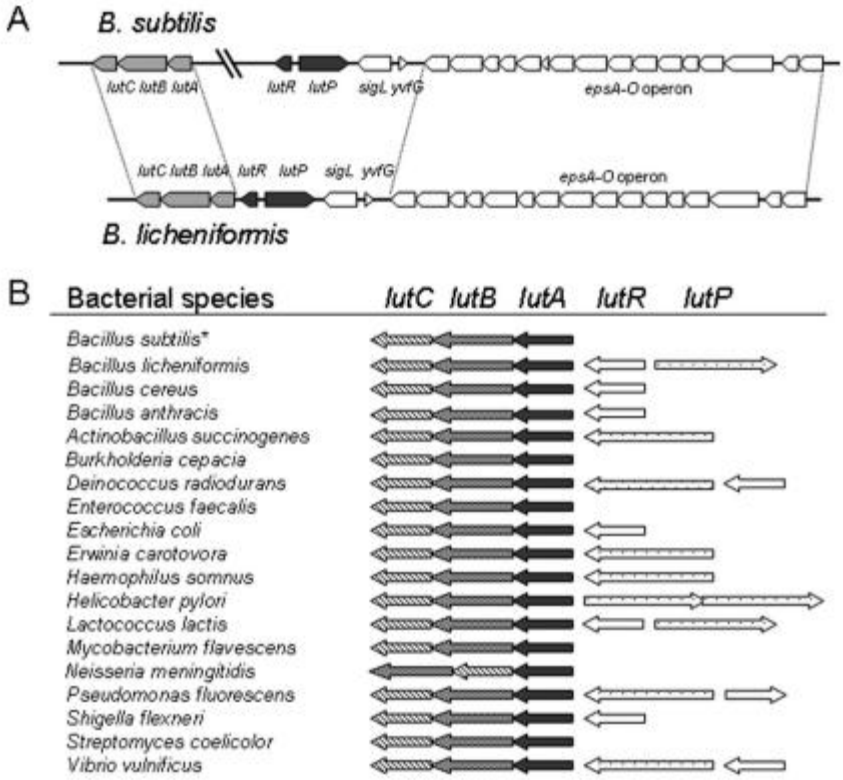


Figure 1.9 : Genomic organisation the *lutABC* (formerly *yvfV-yvfW-yvbY*) operon. (A) An alignment of genomic organisation of lutABC and *epsA-to-O* operons of *B. subtilis* and B. licheniformis. (//) represents 15 kb loop between lutR gene and lutABC operon. (B) Homologous operons from different bacterial genomes. (*) represents 15 kb loop between *lutR* gene and *lutABC* operon. (Chai et al., 2009)

Because *lutA-lacZ* fusion strain showed an increased activity when strain was subjected to *lutR* deletion, it was hypothesized that lutABC operon was subjected to repression by LutR (Chai et al., 2009). In this study, it was demonstrated that *lutABC* operon is not solely under the control of LutR but also SinR (Figure 1.10). Moreover it was concluded that LutR and SinR act in cooperation on the repression of LutABC operon. SinR is known to repress 18 genes that belong *epsA-to-O* and *yqxM-sipW-tasA* operons and these operons are responsible for extracellular matrix that hold the long chains cells known as biofilm (Branda et al., 2005; O'Toole and Kaplan, 2000; Stoodley et al., 2002; Sutherland, 2001; Ren et al., 2004; Kearns et al., 2005; Chu et al., 2006; Branda et al., 2006).

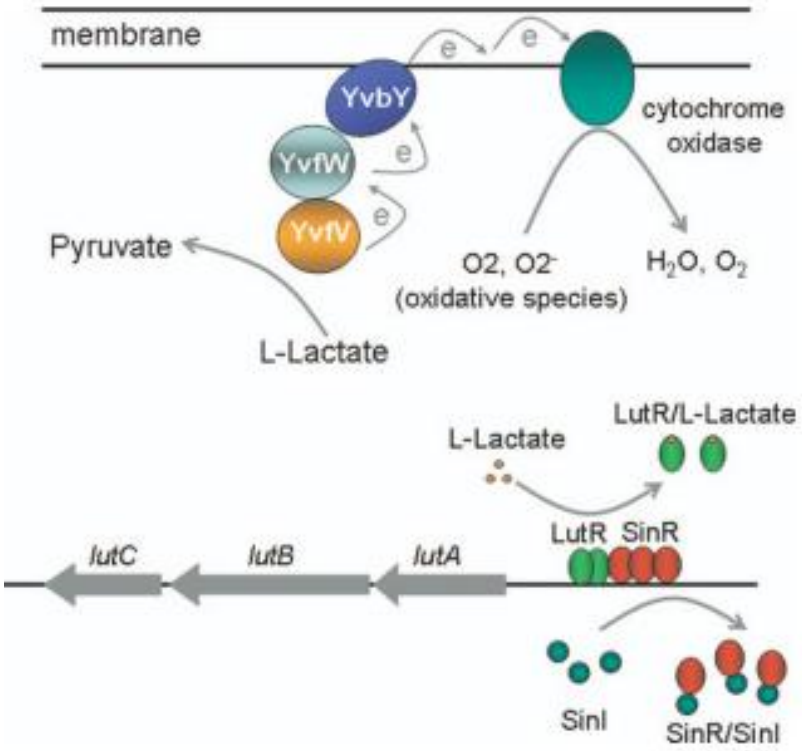


Figure 1.10 : Proposed model for LutABC mediated utilization of L-lactate and LutR and SinR drived regulation of the operon during which the LutR and SinR repressors are hypothesized to act in cooperation on the repression of LutABC operon by binding to DNA. L-lactate is proposed to interact with LutR, thereby derepressing the operon, which demands both LutR and SinR to be repressed.

1.6 Aim of the Study

Prior to the studies reporting the involvement of YvfI on the regulation of *lutABC* operon, YvfI in *B. subtilis* has first been identified as a novel gene required for the biosynthesis of the dipeptide antibiotic bacilysin. Furthermore, extended YvfI protein was accepted to display a partial but important match to the wHTH domain which is signifant for GntR family regulatory proteins. This protein also found to show high homology with its orthologs from different species including FadR and proteins that belong to this subfamily function in the regulation of many pathways involved in amino acids metabolism, L-lactate and sugar utilization, fatty acid transport and degradation as well as in many metabolic pathways including those for aspartate, pyruvate, glycolate, galactonate metabolism. The present research aimed at the understanding the broader regulatory role of YvfI in *B. subtilis.* For this purpose, DNA microarray technology was applied for the detailed comparative transcriptome analysis of wild type control strain PY79 that produces YvfI transcriptional factor

and *yvfI* disrupted TEK1 mutant, *yvfI::T10::spc* during stationary phase (~OD600; 7) where *yvfI* expression reaches a maximum level in cells grown in PA medium and during logarithmic phase of growth (~OD600; 0,7) Furthermore, quantitative reverse transcriptase PCR was also performed for the validation and articulation of data obtained from array analysis. For the genes that did not meet Bayesian probability criteria, lacZ-fusions were further constructed to observe their expression profile changes conducted by YvfI. Furthermore, comparative studies were performed in order to define expressional differences between IPTG induced (*yvfI* gene overexpressed) "*B. subtilis* PY79 *amyE*::P_{spac}::*yvfI, yvfI::Tn10"* mutant strain and non-induced (*yvfI* gene not overexpressed) "*B. subtilis* PY79 *amyE*::P_{spac}::*yvfI, yvfI::Tn10"* mutant strain.

This research also focused on gaining an insight about the regulation of *yvfI* gene expression. For this purpose, transcription initiation site of *yvfI* gene was determined by performing 5' RACE-PCR analysis in this study.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Throughout this study, *Bacillus subtilis* PY79, which is a prototrophic derivative of *Bacillus subtilis* 168 standard strain was used as the wild type strain. Strains and their genotypes that are used in this project are listed in Table 2.1.

Table 0.1 : Bacterial strains and their genotypes used in this project.

Strain	Genotype	Source
Bacillus subtilis PY79	Wild type, BSP cured prototrophic derivative of *B.subtilis* 168	P.Youngman
E.coli Top10F'	[*lac*Iq Tn10(Tet^r)], *mcrA* Δ(*mrr-hsdRMS-mcrBC*), *f80lacZΔM15 ΔlacX74, deoR, recA1, araD139 Δ(ara-leu)7697, galU, galK, rpsL (Strr), endA1, nupG*	M.A.Marahiel
MI105	*spoIIE::lacZ::kat*	Grossman
TEK1	*yvfI::Tn10::spc*	T. E. Köroğlu
TEK7	*yvfI::lacZ::erm*	T. E. Köroğlu
OYK1	*amyE::P$_{spac}$::yvfI*	in this study
OYK2	*rapI::lacZ::erm*	in this study
OYK3	*yvfI::Tn10::spc rapI::lacZ::erm*	in this study
OYK4	*spoIIE::lacZ::kat*	in this study
OYK5	*yvfI::Tn10::spc spoIIE::lacZ::kat*	in this study

2.1.2 Bacterial culture media

Culture media compositions and preparation techniques are given in Appendix C.

2.1.3 Buffers and solutions

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

2.1.4 Chemicals and enzymes

The chemicals and enzymes that were used in this study are given in Appendix E along with their suppliers.

2.1.5 Laboratory equipment

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

2.1.6 Maintenance of bacterial strains

B. subtilis and *E. coli* strains were grown in Luria-Bertani (LB) liquid medium and kept on Luria-Bertani (LB) agar plates at +4 °C. For extended storage, 10 % LB glycerol stock was prepared for each strain and stocks were kept at -80°C.

2.1.7 pGEM®-T easy cloning vector

pGEM®-T Easy Vector System that is supplied by Promega, is used in this study because it is a convenient system for the cloning of PCR products (Figure 2.1). Within the α-peptide coding region of the enzyme β-galactosidase, pGEM®-T Easy Vectors contains promoter regions for T7 and SP6 RNA polymerase flanking the multiple cloning region within which there reside multiple restriction sites. The pGEM®-T Easy Vector multiple cloning region is flanked by restriction enzymes EcoRI, BstZI and NotI digestion sites. Hence, these three enzyme provide the opportunity of three single-enzyme digestions for the release of the cloned insert.

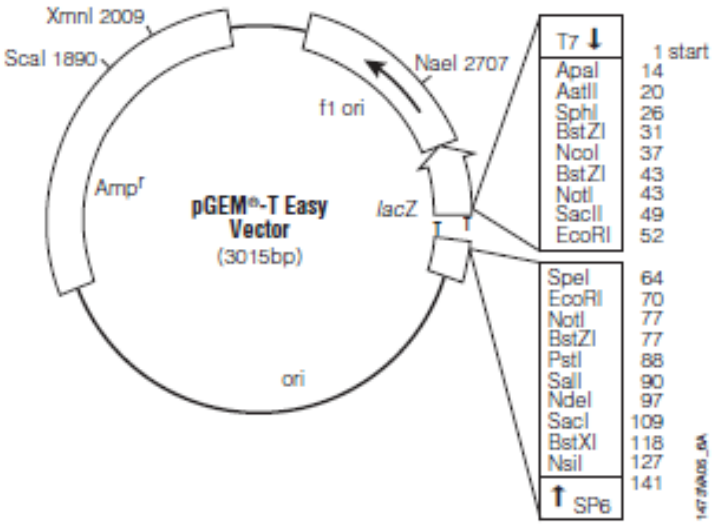


Figure 2.1 : pGEM®-T Easy Vector circle map and sequence reference points.

2.1.8 pDrive cloning vector

pDrive Cloning Vector is provided by QIAGEN in a linearized form with a U overhangs at each 3' and 5' ends which allows direct ligation with A overhangs of PCR products generated by iTaq and other non-proofreading DNA polymerases (Figure 2.2). The vector allows *kan* and *amp* selection and blue/white screening and includes α–peptide portion of *LacZ* gene. This vector contains several restriction endonuclease recognition sites around the cloning site and on either site a T7 and SP6 promoter is located. These promoters allows not only *in vitro* transcription procedure but also sequence analysis with standart sequencing primers. By means of the multiple cloning sites, restriction analysis of the recombinant plasmid can be performed easily. pDrive Cloning Vector also exibits a phage f1 origin which allows production of single stranded DNA. A map of the pDrive Cloning Vector is retrieved from Qiagen official web site.

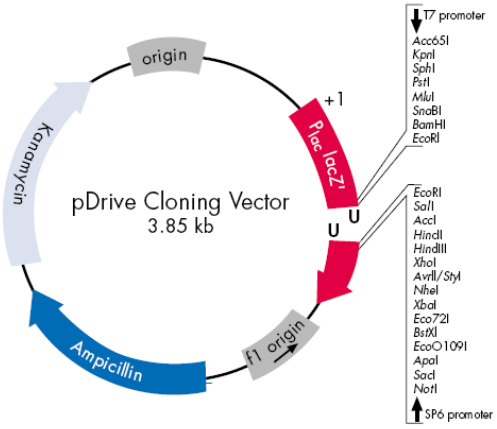


Figure 2.2 : Genomic map of pDrive Cloning Vector.

2.1.9 pMUTIN T3 cloning vector

Insertional mutagenesis is a commonly used method in order to study the characterization of open reading frames found on *Bacillus subtilis*. Therefore, several constructed vectors are used for the systematic gene inactivation and to observe the changes in the phenotype or life cycle of the bacterium (Vagner et al., 1998). For this purpose a special integrational plasmid, called pMUTINT3, that offer many efficient properties is commonly chosen (Figure 2.3). These plasmids are unable to replicate in *B.subtilis* and in order to be able to measure the target gene expression they carry a reporter *lacZ* gene. Moreover, pMUTIN T3 plasmid has an inducible promoter, *Pspac*, developed by Yansura and Henner (1984) in order to control the expression of the genes found downstream of the target gene. The Pspac inducible promoter includes one of the three-lac operators, known as "O1" and RNA polymerase recognition sequences of the SPO1 phage. Besides, the plasmid carries *amp* and *erm* antibiotic resistance genes expressed in *E.coli* and *B. subtilis*, respectively.

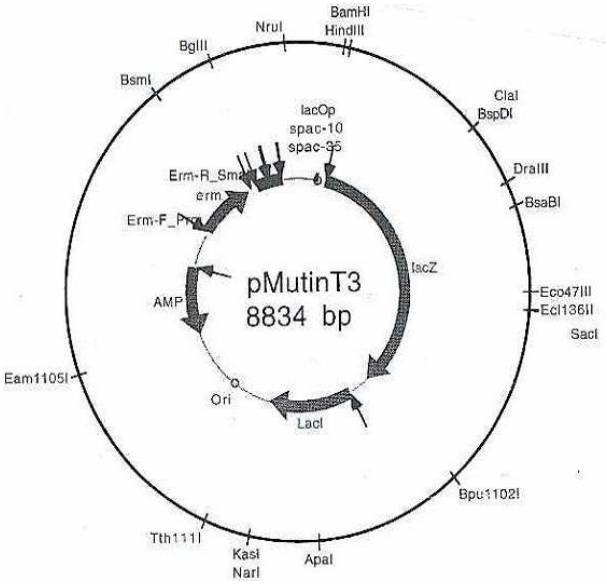


Figure 2.3: Genomic map of pMUTINT3 vector including the functional genes in the structure as well as the restriction map (Vagner et al., 1998).

2.1.10 pDR66 cloning vector

pDR66 was constructed by Ireton et al. from pDH32 (Shimotsu and Henner 1986) and pAG58 (Jaacks et al. 1989) vectors. This multipurpose cloning vector contains the selectable marker amp for growth in *E.coli* and a selectable marker for *B. subtilis* (cat) and an origin of replication for maintenance in *E. coli* (Figure 2.4). Another important trait of pDR66 is its regions of homology to the nonessential amyE locus (amyE front and amyE back). pDR66 displays multiple cloning site with HindIII, XbaI, SalI, and SphI sites downstream of Pspac. Furthermore, intact lacZ is removed from the vector.

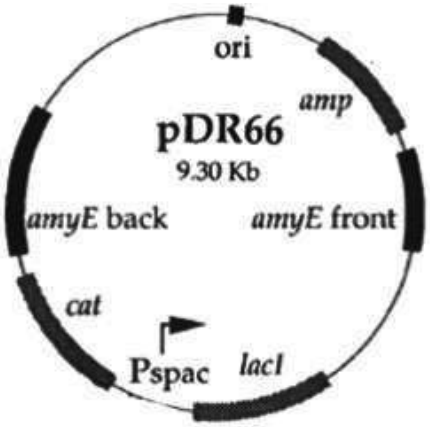


Figure 2.4 : Map of pDR66, used as a cloning vector that was constructed to create a fusion of the desired gene to the LacI-repressed/IPTG-inducible promoter, Pspac.

2.2 Methods

2.2.1 DNA techniques and manipulation

2.2.1.1 Plasmid DNA isolation

Qiagen Plasmid Purification Mini Kit (Qiagen Inc., Valencia, CA) was preferably used for isolation of *E. coli* plasmid DNA. Bacterial cells that were cultured overnight in LB medium at 37^{o}C, were harvested at 13.000 rpm for 5 minutes. After removing supernatant, the pellet was thoroughly resuspended in 300 µL P1 buffer (Appendix B). Then, solution mix was supplemented with 300 µL P2 (Appendix B) buffer and mix was then incubated at room temperature for 5 minutes. Following 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. Following centrifugation at 13.000 rpm for 15 minutes, 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. DNA containing pellet was washed with 1 mL of 70% ethanol. Ethanol was evaporated at 37 $^{\circ}$C for 15 minutes and finally, the pellet was dissolved in 15 µL elution buffer (EB) at 37°C, and stored at -20°C. The isolated DNA was run on 1 % agarose gel.

2.2.1.2 Chromosomal DNA isolation

Chromosomal DNA's of *B. subtilis* strains were 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. Supernatant was discarded and obtained pellet was resuspended in 567 µL of TE buffer (Appendix B). 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 to the cell mixture and homogenized mixture was incubated for 1 hour at 37°C in a water bath. Then, 100 µL of 5M NaCl solution and 80 µL of CTAB/NaCl (Appendix B) (prewarmed to 65^{o}C because of viscosity) solution were added and the sample and it was incubated for 10 minutes in 65°C water bath. Freshly prepared phenol/chloroform/isoamyl alcohol (25:24:1) was then added to the mixture in equal volume 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 supplied. Following gently mixing, 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, ethanol residues were evaporated at 37°C for 1 hour and isolated DNA was dissolved in 10 µL of TE buffer. Obtained chromosomal DNA was stored at 4°C.

2.2.1.3 RNA isolation

Bacillus subtilis cells grown in PA medium were collected and total RNA was immediately isolated using "Qiagen RNeasy Midi Kit". Cells were pelleted by centrifugation at 10000xg for 5 minutes and biomass was stored at -80°C before isolation. Procedure was applied at room temperature and in order to prevent RNA degradation, RNAse free environment was provided. Lysis buffer RLC containing guanidine hydrochloride, was supplemented with 10 µl β-Mercaptoethanol (β-ME), just before use. On column DNase I digestion with RNase free DNase set (Qiagen) was applied in order to avoid any DNA contamination. Isolated RNA was divided into aliquots and stored at -80°C.

2.2.1.4 Polymerase chain reaction (PCR)

The sequences of primers used in PCR reactions are given in Table B.1. PCR components and amounts used are given in Table 2.2.

PCR was performed using *i*-Taq polymerase supplied by iNtRON Biotechnology, Inc. Reaction steps were as follows:

Initial denaturation,	94 ^{o}C for 3'	
Denaturation,	94 ^{o}C for 1'	for 5 cycles
Annealing,	55 ^{o}C for 1'	
Extension	72 ^{o}C for 1'	
Denaturation,	94 ^{o}C for 1'	for 25 cycles
Annealing,	60 ^{o}C for 1'	
Extension,	72 ^{o}C for 1'	
Final extension,	72 ^{o}C for 15'	

Table 2.2 : PCR components and amounts used.

Component	**Amount** (20 µL in total)
10X Buffer (+$MgCl_2$)	2 µL
dNTP	2 µL
Template	1 µL
Reverse primer	0,5 µL
Forward primer	0,5 µL
i-Taq polymerase	0,3 µL
dH_2O	13,7 µL

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.

2.2.1.5 Polymerase chain reaction (PCR) for sequencing

PCR components for sequencing and amounts used are given in Table 2.3. The sequence of M13 reverse primer used for Sequence PCR was as follows: 5'- AGC GGA TAA CAA TTT CAC ACA GGA -3'

Table 2.3 : Components and amounts used for Sequence PCR.

Component	**Amount** (10 µL in total)
5X Sequencing Buffer	2 µL
Big Dye Terminator v3.1	2 µL
Template	1 µL
Primer (10pmol)	1 µL
dH_2O	4 µL

Following PCR for sequence analysis, 25 μL ethanol and 1 μL of sodium acetate solution was mixed with 10 μL of PCR product and the mixture was incubated on ice for 15 minutes. The mixture was then centrifuged at 14000 rpm for 15 minutes. Supernatant is discarded and 250 μL of ethanol was used in order to wash the pellet. The mixture was centrifuged at 14000 rpm for 15 minutes. Supernatant is discarded once again and the pellet was dried off from ethanol residues completely by incubating at 95^{o}C for 3 minutes. 20 μL formamide eas finally added onto the DNA pellet and incubated at 95^{o}C for 3 minutes and then vortexed. After denaturation with formamide, the sample was incubated at -20^{o}C for 2-3 minutes.

In order to perform sequence analysis, Avant 3130 Genetic Analyzer (POP7 polymer with a capillary array length of 50 cm) together with the particular components provided by Applied Biosystems (ABI) were used.

2.2.1.6 Agarose gel electrophoresis

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus and in a gel system composed of ~1% agarose gel (%0,8 for chromosomal DNA visualization, 1% for plasmid DNA and PCR product visualizations) containing 1xTAE buffer (Appendix B) and ethidium bromide of a 0.2 μg/mL final concentration. 6X Loading dye was diluted to 1X in the samples. Electrophoresis was performed at 90-120 Volts for 20-30 minutes. 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.

2.2.1.7 Gel extraction

Desired DNA fragments were extracted and purified from agarose gel by using "QIAquick Gel Extraction Kit" (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The DNA fragment was excised from the gel and protocol was applied to the gel pieces containing DNA fragments. After obtaining DNA, an aliquot was run on 1 % agarose gel to monitor the final DNA concentration and stored at -20°C.

2.2.1.8 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 were mixed in a microfuge tube. Reaction mixture was incubated overnight at 4°C. After ligation was completed, the mixture was used to transform electrocompetent *E. coli* Top10F' cells.

2.2.1.9 Ligation of PCR products into pDrive vector

Ligation of PCR products to pDrive Vector was performed as follows: 5 µL ligation master mix buffer, 1 µL (50 ng/µL) pDrive vector, 3 µL insert DNA (PCR product) and 1 µL dH_2O were mixed in a microfuge tube. Reaction mixture was incubated at 16°C for 1,5 hours and then enzyme denaturation was achieved at 70°C for 10 minutes. After ligation was completed, the mixture was used for transformation of *E.coli* Top10F'.

2.2.1.10 Ligation of pMutinT3 vector

Ligation procedure for cloning into the pMutinT3 vector was carried out as follows: 9,5 µL of PCR products as insert DNA and 0,5 µL of pMutinT3 vector were mixed in a microfuge tube. Reaction mixture was incubated for 5 min at 65°C. Then, the tube was cooled on ice. Then, 2µL 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 reaction mixture. Finally, the mixture was incubated at 16°C for 16 h and the mixture was use to transform electrocompetent *E. coli* Top10F' cells.

2.2.1.11 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, containing 20 μg/mL Tetracyclin. Then incubated at 37 ^{o}C by shaking until the optical density of the culture at 600 nm has reached 0,6. After incubating the cells on ice for 30 minutes, cells were harvested by centrifugation at 5000 rpm for 5 minutes. Following the removal of supernatant, pellet was resuspended in 40 mL ice cold distilled water and centrifuged at 5000 rpm for 15 minutes. Supernatant was discarded and pellet was resuspended once again in 20 mL ice cold distilled water and centrifuged at 5000 rpm for 15 minutes again. After this step, supernatant was discarded for the last time and pellet was resuspended in 1 mL of cold sterile 10% glycerol. Finally, 40 μL aliquots were prepared from the competent cells and these aliquots were frozen immediately in liquid nitrogen and stored at -80 ^{o}C.

For transformation, competent *E. coli* cells were melted on ice shortly. 10 μL of ligation products was placed gently into the competent cell aliquot and was mixed gently. Then, mix was transferred into pre-cooled electroporation tube. The sample was placed onto electroporator and the pulse at 1800V was applied to the cells. 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 pelleted at 5000 rpm for 10 min and supernatant was discarded. Obtained pellet was resuspended in 100 μL 0,85% saline solution (Apendix B). Transformed cells were further plated on selective medium containing appropriate antibiotic (100μg/mL ampicillin). For the blue-white colony screening, cells 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 their transformation process 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. Adequate amount of *B. subtilis* cells were inoculated into 3 mL of HS medium and 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 the optical density of the culture at 600 nm has reached 0,55. Then, 1 mL of competent cells was transferred into 1 µL of chromosomal or plasmid DNA containing eppendorf tubes. 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 antibiotics and incubated at 37°C for 16 h.

2.2.2.3 Selection of transformants by MLS resistance method

Competent *B. subtilis* cells were transformed by the same conditions given in the Section 2.2.2.2 except directly spreading out on LB agar plates containing selective antibiotics. In the MLS resistance method, firstly, 2 µL of 2 mg/mL erythromycin (*erm*) antibiotic was added into 2,5 mL 0,7 % LB agar which was melted and kept at 50°C. Then, immediately, mixture was mixed and quickly poured on a antibiotic-free LB agar plate containing solidified 25 mL of 1,5% LB agar. Following incubation at 37°C for 2 hours, a second overlay was carried out by overlaying this second layer that 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.

2.2.3 Microarray analysis

Total RNA was isolated from *B. subtilis* strains by using the RNeasy Midi RNA isolation kit (Qiagen). Detailed transcriptome analysis was performed at the University of Groningen with the help of their technical support. Superscript cDNA Post Labelling Kit was used to label cDNAs. Fort this purpose, isolated total RNAs were firstly reverse transcribed into cDNAs. 10-20 ug total RNA was annealed using 2 µl random nonamers 1,6 g/l and total end volume was completed to 18 µl. Reaction mix was gently mixed, held at 70°C for 5 minutes and cooled down on room temperature for 10 minutes. Following this step, reverse transcription mix was prepared as 6 µl of 5x Superscript III buffer, 3 µl of 0.1 M DTT, 1.2 µl of 25x AA-dUTP / nucleotide mix and 1.8 µl of Superscript III reverse transcriptase. This mix was added to the 18 µl annealing mix and held at 42°C for 16 hours in order to be

sure that reverse transcription takes place. Later on, the nontranscribed mRNA was degraded using 3 µl of NaOH and holding at 37°C for 15 minutes. Finally 15 µl of 12M HEPES free acid was added to the cDNA solution. Purification of amino allyl-modified cDNA was performed using NucleoSpin® Extract II columns (Biokè, Leiden, the Netherlands) and the exact protocol of the kit. Measurement of cDNA concentration was done using Nanodrop and labeling protocol was made continued if the cDNA concentration is arround 200 ng/ul in total volume of 60 ul. In order to label amino allyl-modified cDNA with CyDye(GE Healthcare, Amersham, The Netherlands), 5 µl of CyDye NHS esters were added to amino allyl modified cDNA and incubated at room temperature, in the dark for 60 to 90 minutes. 15 µl of 4 M Hydroxylamine were added to each Cy5 and Cy3 coupling reaction and incubated at 30°C , in the dark, for 15 minutes. Purification of CyDye labelled cDNA was made using NucleoSpin ExtraxtII columns and the exact protocol of the kit. Measurement of incorporation of Cy dyes in the cDNA was done using Nanodrop and concentration of Cy3 and Cy5 were expected to be at least 0.5 pmol/ul in a total volume of 50 ul. Labelled cDNA's were mixed in equal quantities cDNA of Cy3/Cy5 for hybridization. Samples were dried using the speedvac high temperature. Dried Cy3/Cy5 samples pellet were dissolved in 7 µl of H_2O and incubated at 94°C for 2 minutes. Then, 35 µl of preheated Hybridisation buffer, Ambion Slidehyb #1 buffer (Ambion Europe Ltd) (68°C) was gently mixed and spinned at maximum speed for 1 minute to get rid of precipitates. Then, microarray slides were placed with Lifter Slip on a heat-block at 50°C shown in figure 2.5. The arrays were constructed as described elsewehere (van Hijum et al., 2003a). Furthermore, slide spotting, slide treatment after spotting and also slide quality control were done as before (van Hijum et al., 2005).

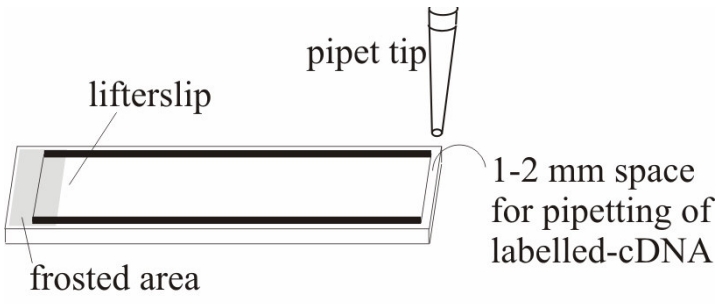


Figure 2.5 : Placement of Lifterslip on microarray slide.

35 µl of the prepared sample was targeted to the end of the slide and the sample was let go between the glass surfaces by capillary force. Then, the microarray slides were placed in the prewarmed Hybridization cassette and they were held in the hybridization oven at 48 °C for more than 16 hours. Following hybridization, slides were washed for the removal of hybridization buffer and unbound cDNAs. Fresh wash buffers I (2 x SSC / 0,5 % SDS), II (1 x SSC / 0,25 % SDS), III (1 x SSC / 0,1 % SDS) were preheted before use and lifterslips were taken out gently in a falcon tube filled with 50 mL wash buffer I until the glass rests on the conical bottom of the tube. Then the washings were continued immediately. Slides were washed in buffer I for 5 minutes, then twice in buffer 2 for 5 minutes and finnally in buffer III for 5 minutes. Finally slides were dried for 2 minutes in a centrifuge at 2000 rpm and scanning of the slides was done using GenePix 4200AL Microarray Scanner (Axon Instruments, CA, USA). Fluorescent signals were quantified using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). Following the acquisition of expression data, expression levels were processed and normalized with Micro-Prep in order to handle microarray data and transforming raw data into processeable Excel data through avoidance of inconsistencies (van Hijum et al., 2003b). Then the expressional difference ratios were further processed using Cyber-T tool that works on t-test variant combined with a Bayesian statistical framework (Baldi and Long, 2001). Parameters used in Cyber-T tool are: 2 as 'minimum non-zero replicates', 'sliding window' of 101 and confidence value of 10. Accepted Cyber-T (Bayes) p value was set to 0,01. Gene lists obtained through the analysis are in supplementary materials. Microarray data has been deposited in Gene Expression Omnibus database (GSE34414).

2.2.4 Quantitative PCR analysis

Total RNA was isolated by using the RNeasy Mini RNA Isolation Kit (Qiagen). Equal amounts (2 µg) of total RNAs were reversely transcribed by using a "Transcriptor cDNA Synthesis Kit" (Roche) with random hexamer primers (60µM) supplied with the kit. The obtained cDNA was used as template for the reverse transcriptase quantitative PCR. Amplification and detection of PCR products were performed with the "SYBR Green Master Mix Kit" (Roche) and Light Cycler 480 (Roche) instrument. As recomended by the constructors, 2 µls of the cDNA synthesis reaction mixture was directly used as template in a 20 µl of reverse transcriptase

PCR mixture with 10 pmols of gene specific primers. Reverse transcriptase PCRs were run at 52^{o}C annealing temperature, as all the primers used in this study were designed to work at this temperature to obtain the comparable data. Melting curve analysis was used to monitor the specificity of the reaction. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expressions (Pfaffl, 2004). The expression levels of the investigated genes were determined relative to the wild type *Bacillus subtilis* PY79 sample. The ratios ($2^{-\Delta\Delta Ct}$) were calculated and log2 transformed. Experiments were performed as replicates and minimum three independent experiments were run. The *sigA*, *veg* and *qcrA* genes were used as internal controls, since the expression of those genes were constant under both control and mutant conditions in both microarray and real-time PCR experiments.

2.2.5 5'-Rapid amplification of cDNA ends (5'-RACE)

Total RNA was isolated from *B. subtilis* cells grown in PA medium to midexponential growth phase and to early stationary growth phase by using the RNeasy Midi RNA isolation kit (Qiagen). SMART^{TM} RACE cDNA Amplification kit (Clontech) was used through which total RNA was reverse transcribed into the 5'-RACE cDNA (Figure 2.6).

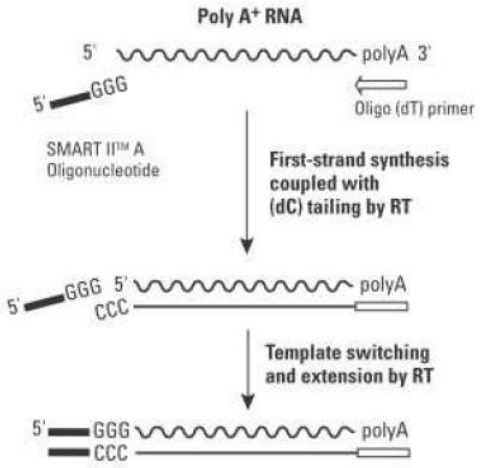


Figure 2.6 : Mechanism of SMART™ cDNA synthesis. (SMART RACE cDNA synthesis kit manual).

In this procedure, PowerScript™ Reverse Transcriptase (RT), a variant of MMLV RT and SMART II™ A Oligonucleotide were used. During the reaction, PowerScript reverse transcriptase enzyme applies terminal transferase activity and reaches the end of the RNA template and adds 3 to 5 generally dC residues to the 3'

end of the first-strand cDNA. SMART II A oligonucleotide, containing a terminal strain of guanine nucleotides, anneals to the dC-rich cDNA tail and therefore, reverse transcriptase utilizes this annealed oligo an extended template. Those converted cDNAs were served as templates for 5'-RACE PCR amplification reactions by using Advantage^{TM} 2 PCR Kit (Clontech) in the presence of UPM and the gene-specific primer (Table B.1). PCR program was initiated at 94 °C for 3 min, followed by 35 cycles of 94°C for 1 min, 52 ºC for 1 min, 72 ºC for 2.5 min and a final extension at 72 ºC for 10 min. One additional nested PCR reaction was performed in order to gain more distinct results by using Nested Universal Primer A (NUP) supplied with kit and a second nested gene-specific primer. Resulting PCR product were cloned in pDrive Cloning Vector (Qiagen) and sequenced.

2.2.6 Beta-galactosidase activity assay

As the initial step, *Bacillus subtilis* cells grown overnight at 37°C in PA medium (Appendix A). Then, 100 mL of PA medium were inoculated to an initial optical density of about 0,1 at 595 nm (OD595). Cultures were incubated at 37°C shaking at 250 rpm for 30 h and sampling was done at each hour.

1 mL of culture samples were 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. After the removal of washing buffer, the pellet was resuspended in 640 µL Z-buffer and 160 µL of lysozyme was added in order to provide cell lysis. Following the incubation at 37°C for 5 min, the samples were taken on ice and 8 µL of 10% Triton-X100 was added. Prior to incubation on ice, mixtures were vortexed for 2 seconds. Following the incubation, β-galactosidase assay was initiated by prewarming the extracts to 30°C. Subsequently, 200 µL of ONPG solution was added and the solution was watched for the progression of the yellow color. At the exact time that yellow color stabilized, the reaction was stopped by the addition of 100 µL 1 M Na_2CO_3 and reaction time was recorded. Finally, the samples were centrifuged at 13000 rpm for 5 min. and their supernatant subjected to the measurement of OD at A_{420} and A_{550}. Calculations for β-galactosidase specific activity were carried out according to the formulation given by Miller in 1972 which was Miller Units=((A_{420} – (1.75 x A_{550}))/(Reaction time (min) x A_{595})) x 1000.

Furthermore a graph that represents both the β-galactosidase activity and log A_{595} were drawn.

2.2.7 Spore count assay

B. subtilis cells were grown in LB medium at 37 ^{o}C overnight and DSM medium was inoculated with the overnight culture to 0,1 OD at 600 nm. Following the incubation at 37^{o}C, a sample was removed from the cultures, diluted serially 10-fold in 0,85% saline solution and 0,1 mL aliquots of dilutions were plated on LB agar plates for total viable cell count. Afterwards these dilutions were heated at 80^{o}C for 30 min and plated once again for total spore forming units count. All the plates were incubated overnight at 37^{o}C and colony forming units were counted and sporulation percentage of the cells were calculated.

3. RESULTS AND DISCUSSION

3.1 Strain Constructions

3.1.1 Construction of *amyE*::P_{spac}::*yvfI* recombinant strain

In order to construct the ectopic integration vector for *yvfI*, *yvfI* gene fragment which stands between 94378 and 95051 bp was amplified by PCR using chromosomal DNA of *B. subtilis* PY79 wild type strain as template (Figure 3.1). On the oligonucleotide *yvfI* F_1 and *yvf* R_1 primers given below, underlined sequences represent restriction sites for *Hind*III and *Pae*I enzymes on forward and reverse primers respectively.

yvfI F_1 : 5' – C C G A A G C T T C G G A G G C C C T A T T A G A T A T G A -3'

yvfI R_1 : 5' – C G G G C A T G C T T A T T G C A C A T T T T C C T C G A A - 3'

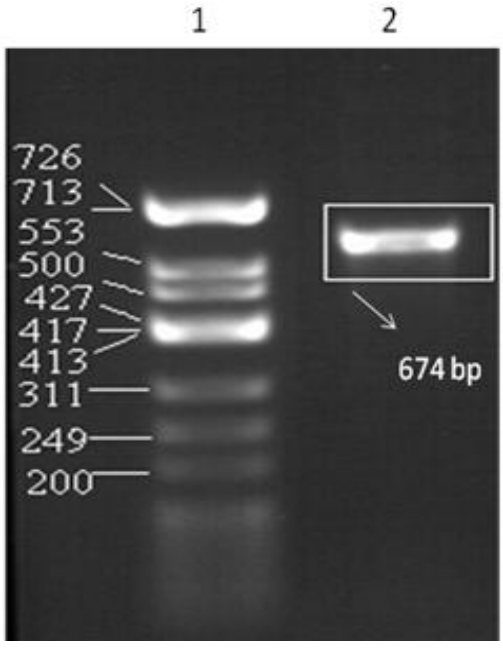


Figure 3.1 : Amplification of 674 base pair long *yvfI* gene fragment. Marker 10 : PhiX174 DNA/*Bsu*RI (lane 1). Amplified *yvfI* gene fragment (lane 2).

Following 674 base pair long *yvfI* internal gene fragment amplification by PCR, direct cloning of *yvfI* to pGEMT was performed and the recombinant vector was used

to transform *E.coli* Top10F' cells. Obtained plasmids were isolated and were digested with *Hind*III and *Pae*l restriction enzymes in order to visualize cloning of the correct fragment.

Digestion of the plasmid DNAs with *Hind*III and *Pae*I has resulted in 692 bp long fragment (containing extra residues from the restriction sites on the primers) and 3,15 kbp PGEMT vector. Hence it was concluded that some of the transformants included 692 bp long *yvfI* gene fragment. The selected clone is shown in figure 3.2. Furtehrmore, transformed plasmid was further subjected to sequence PCR and *yvfI* fragment was confirmed.

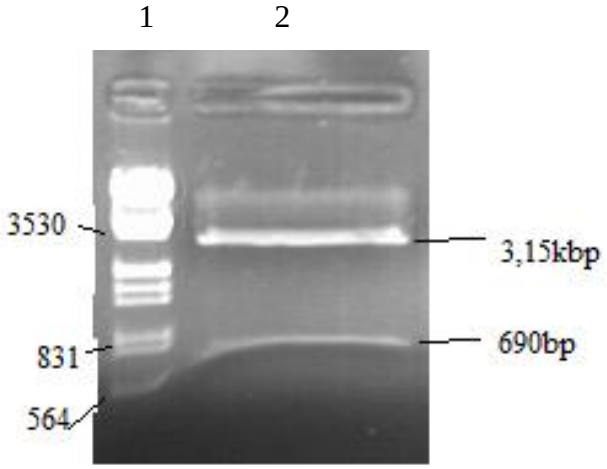
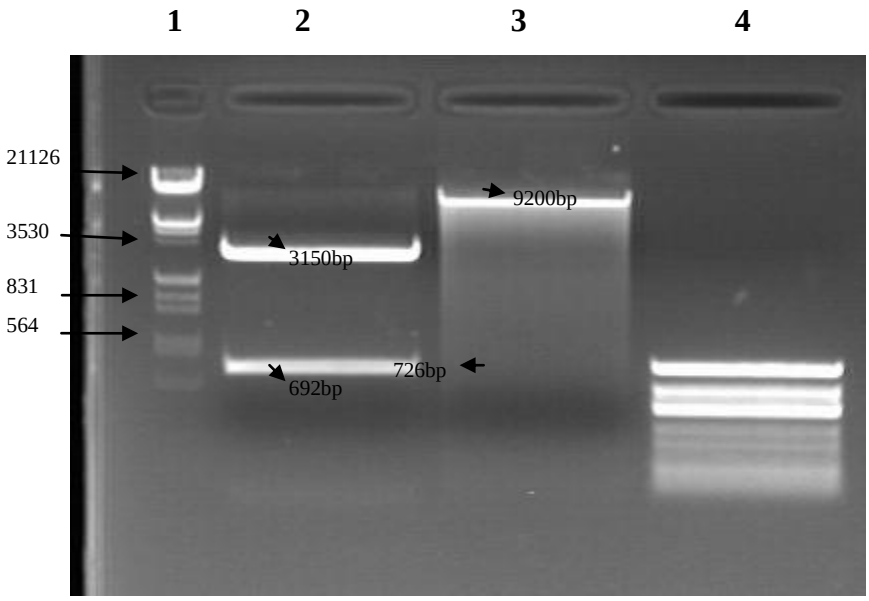


Figure 3.2 : *Pae*I and *Hind*III double digestion of plasmid DNA from selected tranformant. Marker 3: Lambda DNA / *Eco*RI + *Hind*III (lane 1), digested plasmid (lane 2).

Cloned fragment in pGEMT was used for the ligation into the pDR66 vector. In this purpose, pDR66 vector DNA and previously constructed, *yvfI* carrying recombinant vector were digested with HindIII and PaeI in order to create sticky ends that could be hybridized to each other. As a result of this double digestion of the selected transformant, two bands were obtained, one of them was a 692 bp DNA fragment which equal to cloned *yvfI* gene and the other one a 3.15 kbp fragment which represents the lineer form of the pGEMT. As a consequence of the double digestion of pDR66 plasmid, only a 9.2 kbp band which equals to linear form of the pDR66 vector was observed. (figure 3.2).



Fiure 3.3 : Double digestion of selected transformant DNA (lane 2) and pDR66 vectors (lane 3) with *Hind*III and *Pae*I. Marker 10 : PhiX174 DNA / *Bsu*RI (lane 4), Marker 3: Lambda DNA / *Eco*RI + *Hind*III (lane 1).

Following extraction of both gene fragment and linearized pDR66, *yvfI* gene fragment was inserted into pDR66 by using T4 DNA ligase protocol. Following, transformation of pDR66 vector carrying *yvfI* gene into the *E.coli* competent cells, confirmation of the insertion of *yvfI* gene fragment into pDR66 was performed through double digestion of the obtained plasmids with *Hind*III and *Pae*I. As a consequence of restriction analysis, it was proven that *yvfI* gene had been inserted into pDR66 as shown in figure 3.4.

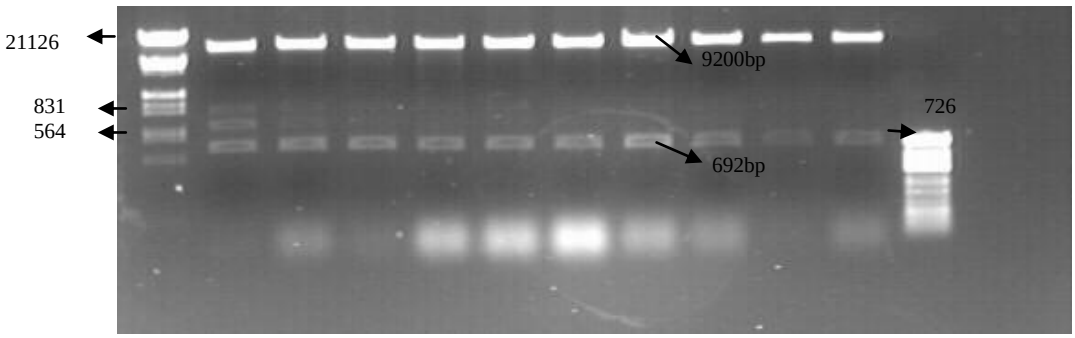


Figure 3.4 : The double digestion of plasmids isolated from *E.coli* Amp^R transformants. Marker 3: Lambda DNA / *Eco*RI + *Hind*III (lane 1) Marker 10 : PhiX174 DNA / *Bsu*RI (lane 12) and *yvfI* gene fragment cleaved from the rest of the pDR66 vector as a consequence of a double digestion with *Hind*III and *Pae*I. (lane 2-11).

In order to transform *B.subtilis* wild type PY79 cells with recombinant pDR66 vector, plasmid DNA of selected clone 7 was used to transform competent cells of *B. subtilis yvfI::Tn10::spc* strains to chloramphenicol resistance. Obtained strains were tested for their ability to metabolize starch on starch media in order to check the disruption of *amyE* locus (Figure 3.5). Consequently, recombinant strain of *amyE*::P_{spac}::*yvfI* was selected for detailed transcriptome analysis.

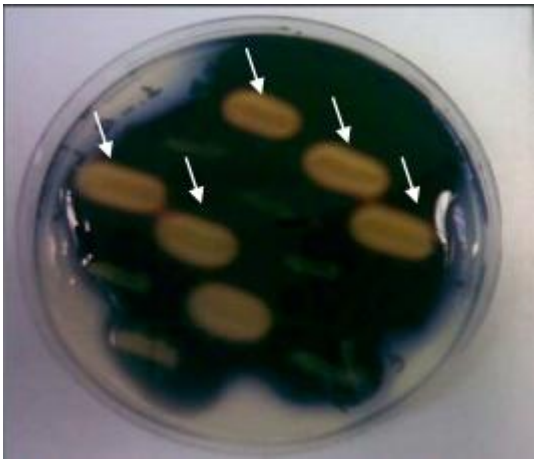


Figure 3.5 : Starch test performed on B.subtilis *amyE*::P_{spac}::*yvfI* Cm^R Spc^R transformants.

3.1.2 Construction of *rapI::lacZ* insertional plasmid

In order to determine the expression profile of *rapI* gene in the absence and presence of *yvfI* gene, mutant *B. subtilis* strains containing *yvfI::lacZ* fusion at *yvfI* locus were constructed using wild type PY79 and TEK1 strains of *B. subtilis*. In order to construct the lacZ fusion vector, 390-bp-long rapI gene fragment was amplified by PCR using chromosomal DNA of *B. subtilis* PY79 wild type strain as template (Figure 3.6). On the oligonucleotide *rapI* F and *rapI* R primers given below, underlined sequences represent restriction sites for *Hind*III and *Bam*HI enzymes on forward and reverse primers respectively.

rapI F : 5' – G C C A A G C T T T T G C G G G G T G T T T T C T T A - 3'

*rapI*R : 5' – C G G G G A T C C T T C A G C T A T T C G A T A A G C - 3'

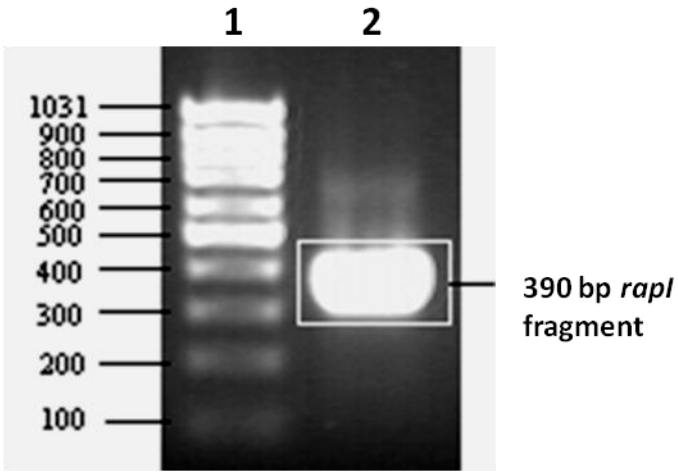


Figure 3.6: Amplification of 390 base pair long *rapI* gene fragment. MassRuler™ DNA Ladder, Low Range (lane 1). Amplified *rapI* gene fragment (lane 2).

Following 390 base pair long *rapI* internal gene fragment amplification by PCR, fragment was extracted from the gel and ligated into pDrive cloning vector using the standard ligation procedure. Then, pDrive vector carrying the *rapI* gene fragment was used to transform *E.coli* Top10F' cells. X-gal/IPTG-ampicillin containing agar plates were used as selectice medium in order to perform blue-white screening. White colonies were selected to be the transformants and those colonies were further processed through plasmid isolation to confirm the presence of recombinant pDrive vector. Furthermore, transformed plasmid was further subjected to sequence PCR and *yvfI* fragment was confirmed.

Digestion of the plasmid DNAs with *BamH*I and *Hind*III has resulted in 390 bp long fragment and 3,85 kbp pDrive vector. Hence, it was concluded that transformants included 390 bp long *rapI* gene fragment. The selected clone is shown in figure 3.7. Furthermore, transformed plasmid was further subjected to sequence PCR and *yvfI* fragment was confirmed. Moreover pMutinT3 plasmid was also subjected to double digestion with *Bam*HI and *Hind*III enzymes and linearized plasmid appeared as a single band on agarose gel (Figure 3.8).

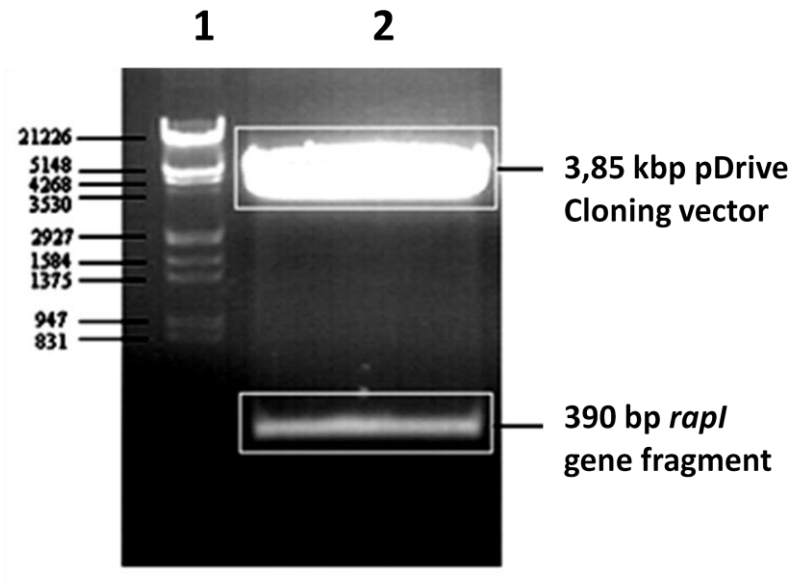


Figure 3.7 : Double digestion of selected transformant DNA (lane 2) 3851 bp pDrive cloning vector with 390 bp *rapI* insert. Marker 3: Lambda DNA / *Eco*RI+*Hind*III (lane 1).

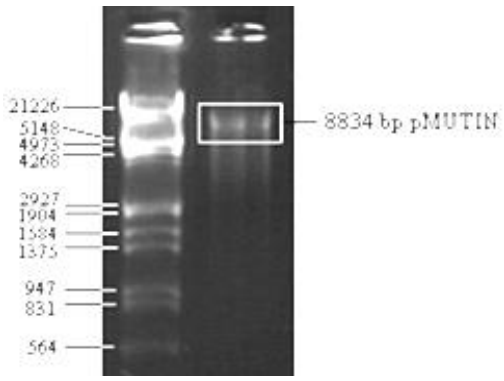


Figure 3.8 : Linearized 8834 bp pMUTIN integrative plasmid.

Cloned fragment in pDrive was used for the ligation into the pMutinT3 vector. Following this purpose, pMutinT3 vector DNA and previously constructed, rapI carrying recombinant vector were digested with *Hind*III and *BamH*I restriction endonucleases. Double digestion of the selected transformants has resulted in the visualization of a 390 bp DNA fragment, representing cloned *rapI* gene fragment and a 3.85 kbp fragment which standing for the lineer form of the pDrive. As a consequence of the double digestion of pMutinT3 vector, 8,83 linearized vector was obtained. Following extraction of both gene fragment and linearized pMutinT3, *rapI* gene fragment was inserted into linearized vector through ligation using T4 DNA ligase. Ensueing transformation of pMutinT3 vector containing *rapI* gene into the *E.coli* Top 10F'competent cells, *rapI* gene fragment inside pMutinT3 was further

confirmed through double digestion of the obtained plasmids with *Hind*III and *BamHI*. As a result of restriction analysis, it was proven that *rapI* gene was inserted into mentioned vector as shown in figure 3.9.

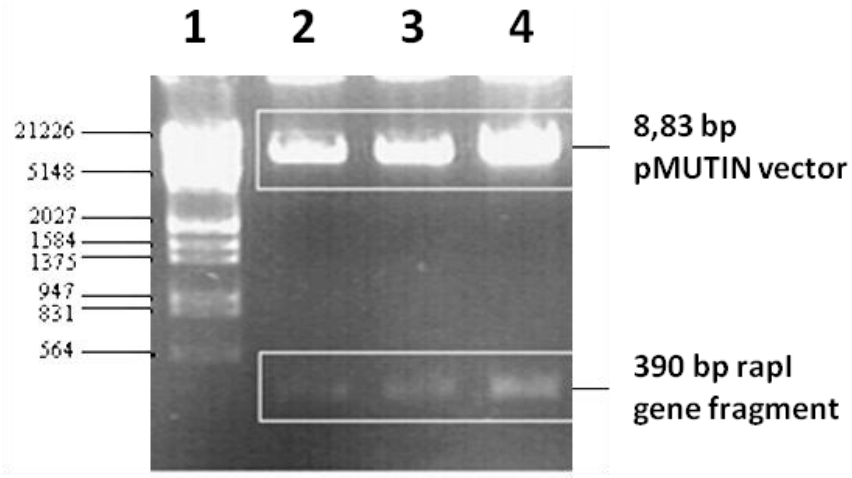


Figure 3.9 : 8,83 kbp pMUTIN plasmid containing 390 bp *rapI* gene fragment digested with *BamH*I and *Hind*III restriction endonucleases (lane 2-4) Marker 3: Lambda DNA / *Eco*RI+*Hind*III (lane 1).

As a further step, integrative plasmid pMUTIN containing rap*I* gene fragment was used to transform *B. subtilis* PY79 cells and TEK1 *yvfI::Tn10::spc* cells via single cross-over event known as Campbell-like integration method in order to construct *rapI*::*lacZ*::*erm* genotype in the chromosome of PY79 and TEK1 cells. Transformants were selected against erythromycin resistance gene (Erm^R) that is found on pMUTIN plasmid. The mutant strain constructed was named as *Bacillus subtilis* OYK2 (*rapI::lacZ::erm*) and OYK3 (*yvfI::Tn10::spc rapI*::*lacZ*::*erm)* strains. Chromosomal DNA isolation was performed using these transformant colonies and isolated chromosomal DNA's were subjected to PCR for validation purposes. Therefore two sets of primers were used for the verification process; primers that amplify *erm* cassette and *rapI* gene specific primers. In conclusion, PCR analysis confirmed the construction of recombinant pMutinT3 plasmids carrying *rapI* inserts (Figure 3.10).

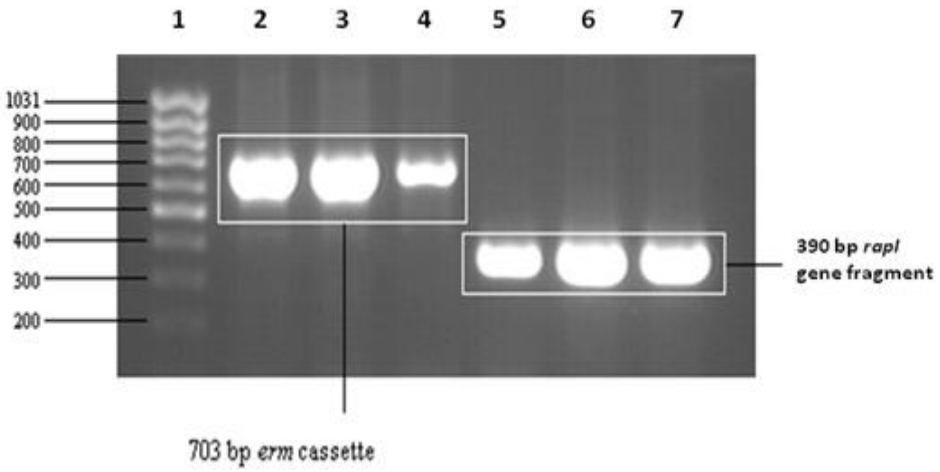


Figure 3.10 : PCR for the confirmation of the constructed recombinant pMutinT3 plasmids carrying *rapI* inserts. PCR products of 703 bp *erm* cassette and 390 bp *rapI* fragment. Lanes 1-2 indicate PCR products resulting from *erm* cassette amplification, OYK2 strain used as template. Lane 3 indicates PCR products resulting from *erm* cassette amplification, OYK3 strain used as template. Lanes 4-5 indicate PCR products resulting from *rapI* gene fragment amplification, OYK2 strain used as template. Lane 6 indicates PCR products resulting from *rapI* gene fragment amplification, OYK3 strain used as template. Marker: MassRuler™ DNA Ladder, Low Range (lane 1)

3.1.3 Construction of *spoIIE::lacZ::cat* mutant strains

In order to detect *spoIIE* directed-*lacZ* expression in wild type and *yvfI* mutant cells, *spoIIE::lacZ* bearing strain ML105 (*spoIIE::lacZ::cat*) supplied by A. D. Grossman was subjected to chromosomal DNA isolation and isolated chromosomal DNA was used to transform both natural competent cells of wild type PY79 and of the TEK1 (*yvfI::Tn10::spc*) strain. Transformants were selected for their ability to metabolize X-gal and obtained mutant strains were further used to measure gene expression activities.

3.2 Determination of the *yvfI* transcription start sites by 5'RACE-PCR

As mentioned in introduction part 1.6, a Conserved Domain Database (CDD) (Marchler-Bauer et al. 2005) search for YvfI protein has pointed out FadR C-terminal ligand binding (FCD) of YvfI protein which is located at the C-terminal of protein. Furthermore, YvfI protein with a 44 amino acid extended sequence (O07007; EMBL accession number CAB08003) was proposed (Kunst et al., 1997) and it revealed an incomplete but considerable match to the GntR wHTH domain at the N-terminus (Figure 3.12). CLUSTAL W algorithm analysis was applied to GntR and YvfI proteins and this incomplete match is given in Figure 3.11.

```
YvfI         ----------------------------MIKNGELKPGDKLDSVQALAESFQVSRSAVR 31
GntR         MLDSKDLLYPAKWLSKASTGVRVAYELRMRIVSGLIESG-TILSENTIAAEFSVSRSPVR 59
                                          * .* ::.* .: * :::* .*.****.**

YvfI         EALSALKAMGLVEMKQG--------EGTYLKEFELNQISQPLSAALLMKKEDVKQLLEVR 83
GntR         EALKILASEKIIRLERMGAVVIGLTEKKIAEIYDVRLLLETFVFERLVKIDIEPLVKDLS
119
             ***. * :  ::.:::         * .  : :::. : :.:    *:* :    : ::

YvfI         KLLEIGVASLAAEKRTEADLER----------------------IQDALKEMGSIEADGE 121
GntR         KILEMMKVSIKYEDADEFSFQDVLFHETIIRAIDHSYIQMIWNNLKPVMESFILLSMRVR 179
             *:**:  .*:  *.  * .::                       :: .::.:  :.   .

YvfI         LGEKAD-----FAFHLALADASQNELLKHLMNHVSSLLLETMRETRKIWLFSKKTSVQRL 176
GntR         LKEKYEDFTRILDNHELYIQAIKTKDRALMIQSLHQNFDDVQDKVEDLWLSQQMLAKGAE 239
             * ** :     :  *    :* :.:    ::: : . : :.  :...:** .:  :

YvfI         YEEH 180
GntR         YNND 243
             *::.
```

Figure 3.11 : Alignment of amino acid sequences of YvfI and GntR regulatory proteins using CLUSTAL W algorithm Sequences that are underlined represent α-helices while sequences that are encircled represent β-sheet configurations of protein N-terminus regions. * (asterisk) indicates positions with fully conserved residues, : (colon) indicates conservation between groups of strongly similar properties, . (period) indicates conservation between weakly similar groups.

Moreover, helix-turn-helix motif and GntR-type resembling domains are given in figure 3.12. Hence, 44 amino acid extended sequence was further accepted for YvfI protein which consists of 219 amino acids encoded by 657 bp nucleotide sequence (Kunst et al., 1997).

```
        10         20         30         40         50         60
MIKNGELKPG DKLDSVQALA ESFQVSRSAV REALSALKAM GLVEMKQGEG TYLKEFELNQ

        70         80         90        100        110        120
ISQPLSAALL MKKEDVKQLL EVRKLLEIGV ASLAAEKRTE ADLERIQDAL KEMGSIEADG

       130        140        150        160        170        180
ELGEKADFAF HLALADASQN ELLKHLMNHV SSLLLETMRE TRKIWLFSKK TSVQRLYEEH

       190        200        210
ERIYNAVAAG NGAQAEAAML AHLTNVEDVL SGYFEENVQ
```

Figure 3.12 : H-T-H helix-turn-helix motif and GntR-type resembling configuration of YvfI protein. Amino acid sequence that is underlined defines GntR-type resembling configuration which involves first 56 amino acids. Encircled amino acid sequence defines H-T-H motif which involves amino acids starting from 16th ending at 35th.

Furthermore, using Kyoto Encyclopedia of Genes and Genomes, SSDB motif search (Sato et al., 2001) was applied to YvfI protein and the outcomes has supported that YvfI protein gives high similarity with GntR family regulatory proteins on 1^{th} to 52^{th} amino acids, while 15th to 50th amino acids gives high similarity with HTH motifs found in database, 78th to 204th amino acids gives the highest homology with other represented motifs because the highest e value was calculated for this motif (Table 3.1). It should be noted that E-value is the expectation value. E-values approaching 1 represents what should be expected by chance. Thus, the lower an E-value, the more specific the search for domains are and threshold level for CDD Pfam queries is set to 10^{-6} (Marchler-Bauer et al. 2005).

Table 3.1 : SSDB motif search result for YvfI protein.

Motif id	From	To	Definition	E value*
pf:GntR	1	52	Bacterial regulatory proteins, gntR family	8.1e-17
pf:Rrf2	9	52	Transcriptional regulator	0.0002
pf:DUF4423	12	55		0.0015
pf:MarR_2	13	48	MarR family	0.00023
pf:DUF2250	14	49	Uncharacterized protein conserved in archaea (DUF2250)	0.027
pf:HTH_28	14	44		0.11
pf:HTH_11	15	50	HTH domain	1.4e-05
pf:HTH_27	15	49		0.014
pf:Fe_dep_repress	15	48	Iron dependent repressor, N-terminal DNA binding domain	0.0039
pf:HTH_IclR	15	44	IclR helix-turn-helix domain	0.0092
pf:Phage_rep_org_N	15	63	N-terminal phage replisome organiser (Phage_rep_org_N)	0.02
pf:HTH_24	15	44		0.045
pf:HTH_DeoR	15	44	DeoR-like helix-turn-helix domain	0.12
pf:HTH_20	15	45	Helix-turn-helix domain	0.89
pf:PaaX	15	48	PaaX-like protein	0.19
pf:TrmB	16	51	Sugar-specific transcriptional regulator TrmB	0.0003
pf:HTH_5	16	44	Bacterial regulatory protein, *arsR* family	0.00054
pf:HTH_Crp_2	16	48		0.017
pf:HrcA_DNA-bdg	18	48	Winged helix-turn-helix transcription repressor, HrcA DNA-binding	0.031
pf:Mo25	69	174	Mo25-like	0.069
pf:FCD	78	204	FCD domain	7.8e-26

On the basis of known YvfI sequence, *yvfI* transcription start site was enlightened by 5' RACE PCR technology. For this purpose, total RNA was isolated from *B. subtilis* TEK7 cells (*yvfI::yvfI-lacZ*) grown in PA medium to midexponential growth phase and to early stationary growth phase by using the RNeasy Midi RNA isolation kit (Qiagen). SMART^{TM} RACE cDNA Amplification kit (Clontech) was used through which an aliquot of 20–40 ng of total RNA was reverse transcribed into the 5'-RACE cDNA using 12 pmol of a genespecific primer (5'-. AAT ATC CCG AAA GCA CAT -3') complementray to nucleotides (nt) 623 to 640 downstream of the translational start point of *yvfI* and Universal Primer A mix (UPM) supplied with kit. Those converted cDNAs were served as templates for 5'-RACE PCR amplification reactions by using Advantage^{TM} 2 PCR Kit (Clontech) in the presence of UPM and the gene-specific primer mentioned above. One additional nested PCR reaction was performed in order to gain more distinct results by using Nested Universal Primer A (NUP) supplied with kit and a second gene-specific primer (5'-CAG AAG GGC GGC TGA GAG CGG CTG-3') complementray to nt 187 to 200 downstream of the translational start point of *yvfI*. Resulting PCR product were cloned in pDrive Cloning Vector (Qiagen) and sequenced.

5'-RACE-PCR analysis of *yvfI* transcription site revealed that transcription of *yvfI* was controlled from a σ^A-type promoter. This promoter described as P*yvfI* lies at position -136 to -115 with respect to *yvfI* translational start codon that seems to be a very strong promoter with a well confirmed consensus -10 (TATAAT) and consensus -35 (TTGGCG) sequences separated with 15 bp long space. Furthermore, at +12 downstream position, a sequence that shows a perfect match to the consensus Spo0A binding motif (5'-TGNCGAA) was identified (Fig 3.13). Additionally, three putative AbrB binding and one CodY binding sites were indicated on the sequence.

A

atgactgggtaataagaccagttacctagcattata**ttttgaaaaa**aatcaaagtacaaga**ctttttg**
AbrB AbrB

acaaaat**agtcaaaat**ctt**ttggcg**gcccacaaaacagag**tataat**agatcgt**A**aacagccagaccta
AbrB -35 σ^A -10 σ^A

caagttacggggtgaattgcattgaaatataaacagattaaaacaa**aaaaa**t**ata**t**ga**agaagtagcg
CodY

Spo0A
gatgccctattagat atg atc aaa aa**t ggc gaa** **ttg aag**_ccg ggg gat aaa ctg
M I K N G E L K P G D K L

gac **tct gtt ca**g gcg ct**t** gct gag agc ttt caa gtc agc cgt tca gcg gtt
D S V Q A L A E S F Q V S R S A V

cgc gaa gca ctt tct gcg cta aaa gcg atg ggg ctt gtc gaa atg aaa cag
R E A L S A L K A M G L V E M K Q

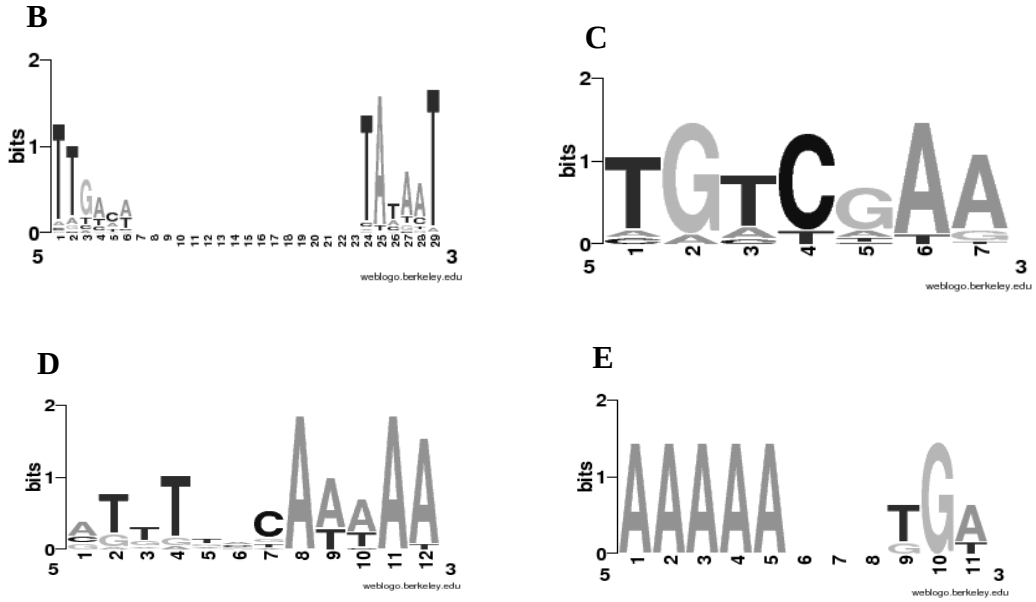


Figure 3.13 : (A) Mapping of transcription start sites by 5′-RACE-PCR using TEK7 (*yvfI*::*yvfI-lacZ erm*). The transcription start point of the *yvfI* promoters indicated by a bold uppercase letter. The −10 and −35 boxes of the two σ^A-type promoters controlling the *yvfI* transcription and binding sites of the transcriptional regulators, *spo0A*, *abrB*, *codY* are in bold characters and underlined. (B) Consensus sequence logo obtained from the compilation of 328 previously identified σA-binding sites (Makita et al., 2004). (C)Binding motif of *spo0A* bi-functional regulator obtained from 29 identified binding sequences (Makita et al., 2004). (D) Sequence logo of *abrB* binding site obtained from 36 previously determined sequences (Makita et al., 2004). (E) *codY* binding motif obtained from 16 identified sequences (Makita et al., 2004).

Hence, these results pointed out that *yvfI* gene is likely under the direct control of global regulatory system operated by Spo0A, CodY and AbrB.

3.3 Transcriptome studies

3.3.1 Identification of the genes regulated by YvfI during stationary phase

In order to identify the genes regulated by the transcriptional factor YvfI, gene expression levels were first analyzed on a genome-wide scale comparing the *yvfI* disrupted mutant TEK1 (*lutR::T10::spc*) samples to a sample of the *yvfI*$^{+}$ parent strain *B. subtilis* PY79 taken at the onset of stationary phase (OD_{600}7). Since transcriptional *yvfI-lacZ* analysis indicated that a relatively constant level of *yvfI-lacZ* expression during exponential growth phase increases rapidly to a maximal level upon entry into stationary phase but decreases rapidly to its basal level following within 2-4 hr (Fig. 3.14).

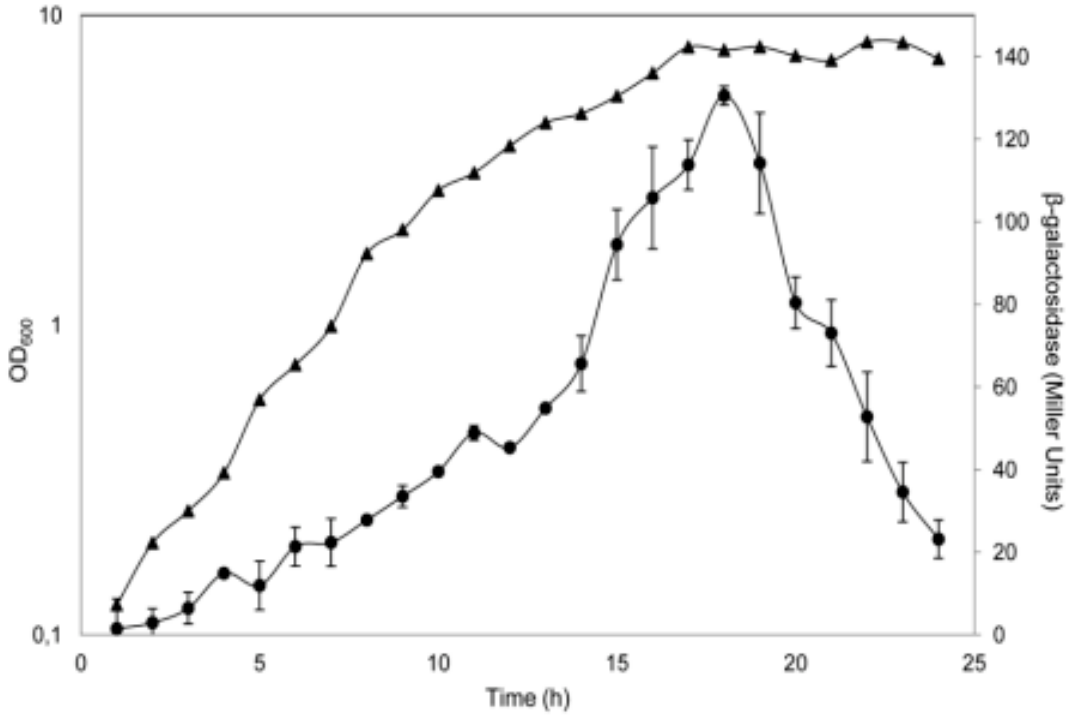


Figure 3.14 : Expression of transcriptional *yvfI::lacZ* fusion in PA medium. *B. subtilis* TEK7 (*yvfI*::*lacZ*::*erm*) was grown in PA medium and samples were taken for assay of β-galactosidase as described in Materials and methods. Specific activity was plotted as a function of time relative to the entry into stationary phase. Similar trends were obtained when the experiment was repeated in further occasions. (▲) growth profile of the strain TEK7; (●) β-galactosidase activity of TEK7 grown in PA medium

Microarrays used in this study contained probes for 4107 open reading frames of *B. subtilis*. For microarray analysis, RNA samples from TEK1 and wild type PY79 cells during stationary growth were reverse transcribed to cDNA's and labeled with Cy5 and Cy3 fluorescent dyes separately in order to be able to compare expression ratios of each individual gene. Fluorescent signals received from Cy3 and Cy5 dyes were quantified using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD) and transriptional data was processed and normalized using Micro-Prep in order to transform obtained raw data into processeable Excel data (van Hijum et al., 2003b). Following the acquisition of processable data, expression ratios were further processed using Cyber-T tool that works on t-test variant combined with a Bayesian statistical framework (Baldi and Long, 2001). Accepted Cyber-T (Bayes) p value was 0,01. DNA microarrays used in this study contained probes for 4107 open reading frames of *B. subtilis*.

It should be noted that evaluated data of microarray results usually demands verification through the usage of a complementary gene expression quantification methodology. For this purpose, Real-time PCR employment is the most commonly used method. Microarray technology has its own drawbacks and pitfalls such that data obtained using this methodology was previously proven to vary depending on the quality of RNA which is usually not constant, or the presence of contaminating factors including salts and alcohol that create a defect on the activity of reverse transcriptase, moreover, nonspecific or cross contamination of probes with non-targeted genes and many other variables (Freeman et al. 1999; Chuaqui et al. 2005; Yang et al. 2002). On the other hand, qPCR methodology has also many drawbacks including primer dimer formation or non-targeted annealing, amplification of misplacements, change in the efficiency of pPCR data when reaction proceeds, presence of contaminating factors for reverse transcriptase (Bustin, 2002; Freeman et al. 1999; Chuaqui et al. 2005). All the drawbacks aside, these two methodologies mainly differ in their data normalization procedures. Such that, gene expression studies using microarray requires a global normalization while RT-qPCR technology can only use reference genes so that all the evaluated genes can be normalized against expression ratios of these reference genes (Morey et al., 2006). Beside the facts that there is no absolute validation method, qPCR usually is employed for the verification of microarray data. In case of qPCR, once the mRNA has been reverse

transcribed into cDNA, several different methods are commonly used to quantify the cDNA, termed *absolute* and *relative quantification*. Absolute quantification employs an internal or external calibration curve to derive the input template copy number. Absolute quantification is important in case that the exact transcript copy number needs to be determined, however, relative quantification is sufficient for most physiological and pathological studies. Relative quantification relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in target sample versus reference samples. In other words, relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA (Pfaffl, 2004). This reference gene is often a housekeeping gene and in this study, *sigA*, *veg* and *ponA* genes were chosen to be reference genes thanks to their constitutive expression. As reference values, geometric mean of the Cp values of three given genes were used.

Relative quantification does not require standards with known concentrations and the reference can be any transcript, as long as its sequence is known. Relative quantification is based on the expression levels of a target gene versus a reference gene and in many experiments is adequate for investigating physiological changes in gene expression levels. To calculate the expression of a target gene in relation to an adequate reference gene various mathematical models are established. Calculations are based on the comparison of the distinct cycle determined by various methods, e.g., crossing points (CP) and threshold values (Ct) at a constant level of fluorescence; or CP acquisition according to established mathematic algorithm. In the LightCycler software the *"Second Derivative Maximum Method"* is performed where Cp is automatically identified and measured at the maximum acceleration of fluorescence. To date, several mathematical models that determine the relative expression ratio have been developed. Two types of relative quantification models are available and published: (1) without efficiency correction and (2) with kinetic PCR efficiency correction. In our study, we focused on the calculations without efficiency correction, because all the reaction efficiencies were kept between 1.8 and 2, where 1 is the minimum level and 2 is the 100% efficiency (Pfaffl, 2004). In this study, a calculation method was used which gives results for each sample that were expressed in N-fold changes in mutant strain target gene copies, normalized to

reference genes relative to the copy number of the target gene: $2^{-\Delta\Delta CP}$, ($2^{-[\Delta Cp\ sample - \Delta Cp\ control]}$).

Wild type *Bacillus subtilis* PY79 and *yvfI* distrupted mutant strain TEK1 (*yvfI*::*tn*10::*spc*) cells were grown in bacilysin biosynthetic medium (PA medium) to early-stationary phases. Sampling times was set in corcordance with microarray experiment. 1 x 10^9 were collected and total RNA's from these cells were isolated by using "the RNeasy Midi RNA Isolation Kit" (Qiagen). Reverse transcribed cDNA's were used as template for the reverse transcriptase quantitative PCR. "SYBR Green Master Mix Kit" (Roche) and Light Cycler 480 (Roche) instrument were used for the reverse transcriptase PCR analysis. All the primers used in this study were selectively chosen so that their Tm match in order to be able to work at the same temperature to obtain comparable data.

Hence, following the methodologies given above, genes showing at least 1,44 fold difference with reproducible characteristics in three independent experiments [displaying an acceptable Bayes P value ($p < 0,01$)] were subjected to RT-qPCR analysis. RT-qPCR was applied based on transcription units, meaning that first or several genes of operons that were identified to be affected were chosen to be validated by RT-qPCR. As a consequence, genes that are validated by these two methodologies were accepted as YvfI affected genes and the list of the total regulon was given listed in Table A.1. It should be noted that there were some exceptions that did not fit within the listed accepted conditions. For instance, five genes (*yjcM, yqgA, yqxIJ, ybyB* and *yotH*) identified in the microarray experiment were not further validated using RT-qPCR but still listed in Table A.1 in order not to underestimate possible YvfI-regulated genes. In this table, some genes with Bayesian P values that exceeded 0,01, were also placed under the respect that their significant differential expressions in the TEK1 strain were confirmed by RT-qPCR. These five genes are *pyrP*, *trkA*, *yokD*, *yvcA* and *abh* (Bayes P value >0,01). However, another gene with not acceptable Bayes P value was further confirmed through constructing *rapI-lacZ* fusion in order to measure gene expression level by beta-galactosidase assay. Furthermore, several genes (*czcD*, *citB*, *epsN*, *fabR*, *pbpE*, *sigW*, *spoIIE*, *yneN*, *yqxM*, *ytsC*, *ytsD*, *yvcA*, *ywfH* and the *ywfBCDEFG* operon) did not show at least 1,44 fold difference with reproducible characteristics in three independent experiments in the microarray analysis and thus their expression level were represented as ND in table

A.1 which stands for “no differential expression observed”. Once again, although these genes did not meet the chosen criteria, differential expreesions as a result of *yvfI* mutation for these cited genes (*czcD, citB, epsN, fabR, pbpE, sigW, spoIIE, yneN*, *ytsC*, *ytsD* and *yvcA*) were demonstrated by RT-qPCR analysis and for the expression of *spoIIE* gene, the path that was selected was to construct spoIIE-lacZ fusion in this study to monitor changes in gene expression via beta galactosidase assay. Additonally, RT-qPCR was applied based on transcription untis, therefore some of the genes of an operon that did not meet the criteria were also studied in order to obtain comparable data. To give an overall look, in total, 61 transcriptional units were identified to display altered expression in *yvfI* mutant compared to *yvfI*$^{+}$ wild type cells. Among these 61 transcriptional units, 19 of them were mono-cistronic genes while the remaining 42 consisted of operons that include 141 genes.

When these transcriptional units were grouped according to their known or putative functions, YvfI-affected genes were found to be involved in varied biological processes including cell-wall synthesis, membrane bioenergetics (ATP synthesis), nitrogen metabolism, protein synthesis and translocation, fatty acids and lipid metabolism, carbohydrate utilization and transport, sporulation delay and cannibalism, extracellular matrix production, antibiotic production and resistance, degradative enzyme production, transfer of mobile genetic element and phage infections.

3.3.1.1 Fatty acid and lipid metabolism

The first GntR subfamily, FadR, is the most crowded subfamily and it consists of a six or seven α-helices containing C-terminal domain depending on subgroups (Figure 1.5*a*). FadR-like proteins are associated to the regulation of amino acids metabolism or to various metabolic pathways such as aspartate (AnsR), pyruvate (PdhR), glycolate (GlcC), galactonate (DgoR), lactate (LldR), malonate (MatR), or gluconate (GntR). The FadR protein, the *E. coli* ortholog of YvfI (Figure 3.15), acting both as a repressor and an activator, and hence acts on regulating fatty acid biosynthetic and degradation genes (DiRusso et al., 1999).

```
FadR         MVIKAQSPAGFAEEYIIESIWNNRFPPGTILPAERELSELIGVTRTTLREVLQRLARDGW 60
YvfI         ------------------MIKNGELKPGDKLDSVQALAESFQVSRSAVREALSALKAMGL 42
                                * *..: **  * : : *:* : *:*:::**.*. *   *

FadR        LTIQHGKPTKVNNFWETSGLNILETLARLDHESVPQLIDNLLSVRTNISTIFIRTAFRQH 120
YvfI        VEMKQGEGTYLKEFELNQISQPLSAALLMKKEDVKQLLEVRKLLEIGVASLAAEKRTEAD 102
            : :::*: * :::*  ..  : *.:   :.:*.* **::    :. .::::  ..  . .

FadR        PDKAQEVLATANEVADHADAFAELDYNIFRGLAFASGNPIYGLILNGMKGLYTRIGRHYF 180
YvfI        LERIQDALKEMGSIEADGELGEKADFAFHLALADASQNELLKHLMNHVSSLLLETMR--- 159
             :: *:.*   ..:  ..:   : *: :. .** ** * :   ::* :..*  .  *

FadR        ANPEARSLALGFYHKLSALCSEGAHDQVYETVRRYG---HESGEIWHR--MQKNLPGDLA 235
YvfI        ---ETRKIWL-FSKKTSVQRLYEEHERIYNAVAAGNGAQAEAAMLAHLTNVEDVLSGYFE 215
               *:*.: * * :* *.      *:::*::*   .    *:. : *   ::. *.* :

FadR        IQGR 239
YvfI        ENVQ 219
                 : :
```

Figure 3.15 : Alignment of YvfI (from *Bacillus subtilis*) and FadR (from *Escherechia coli*) amino acid sequence using CLUSTAL W algorithm. Sequences that are underlined represent α-helices of protein N-terminus regions. * (asterisk) indicates positions with fully conserved residues, : (colon) indicates conservation between groups of strongly similar properties, . (period) indicates conservation between weakly similar groups.

The FadR protein functions as the repressor of fatty acid degradative (*fad*) genes (DiRusso et al., 1992). Furthermore, FadR directly induces lipid metabolism through the activation of *fab* genes (Black and DiRusso, 1994). As being an ortholog of FadR protein, YvfI as expectedly exerts a positive effect on the genes related to fatty acids and lipid metabolisms in *B. subtilis*. Expression levels of fatty acid and lipid metabolism related transcriptional units, *fabHB, fabRplsXfabDG, acpA, fabHAF* and *plsC* were found to be decreased in *yvf*I mutant strain (Table 3.2).

Table 3.2 : YvfI regulation on genes related to fatty acid and lipid metabolisms in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
bioW	1,99	1,46(±0,06)	6-carboxyhexanoate--CoA ligase	
bioA	1,38		adenosylmethionine--8-amino-7-oxononanoate transaminase	$\overleftarrow{bioI} \neq \overleftarrow{\boldsymbol{bioB}}\ \overleftarrow{\boldsymbol{bioD}}\ \overleftarrow{\boldsymbol{bioF}}\ \overleftarrow{\boldsymbol{bioA}}$
bioF	1,45		8-amino-7-oxononanoate synthase	$\overleftarrow{\boldsymbol{bioW}} \neq \overleftarrow{ytaP}\ \overrightarrow{msmR}$
bioD	2,06		dithiobiotin synthetase	
bioB	ND		biotin synthase	
lip(estA)	2,38	2,65(±0,09)	secreted alkaliphilic lipase	$\neq \overleftarrow{lmrB}\ \overleftarrow{lmrA}\ \overrightarrow{yccC} \neq \overrightarrow{\boldsymbol{lip}} \neq \overleftarrow{yczC} \neq$
fabHA	1,87	1,00(±0,12)	3-oxoacyl-(acyl carrier protein) synthase III	$\overrightarrow{comZ}\ \overleftarrow{yjzB}\ \overrightarrow{\boldsymbol{fabHA}}\ \overrightarrow{\boldsymbol{fabF}} \neq \overrightarrow{yjaZ}$
fabF	1,89		3-oxoacyl-(acyl carrier protein) synthase II	
ylpC(fabR)	1,36	1,16(±0,09)	fatty acid biosynthesis transcriptional factor	
plsX	1,66		putative glycerol-3-phosphate acyltransferase PlsX	$\overrightarrow{\boldsymbol{ylpC}}\ \overrightarrow{\boldsymbol{plsX}}\ \overrightarrow{\boldsymbol{fabD}}\ \overrightarrow{\boldsymbol{fabG}}\ \overrightarrow{\boldsymbol{acpA}} \neq \overrightarrow{rnc} \neq \overrightarrow{smc} \neq \overrightarrow{ftsY}$
fabD	2,42		malonyl CoA-acyl carrier protein transacylase	
fabG	2,28		beta-ketoacyl-acyl carrier protein reductase	
acpA	2,29		acyl carrier protein	
citB	-1,38	-2,21(±0,21)	aconitate hydratase	$\neq \overleftarrow{cotM}\ \overleftarrow{sspP}\ \overleftarrow{sspO}\ \overleftarrow{\boldsymbol{citB}} \neq \overrightarrow{yneN}$
citA	-1,50	-2,20(±0,30)	citrate synthase 1	$\neq \overleftarrow{citR}\ \overleftarrow{\boldsymbol{citA}}\ \overrightarrow{yhdF} \neq \overrightarrow{yhdG} \neq \overrightarrow{yhdH} \neq$

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "≠" indicates the termination sites

ND no differential expression observed

As seen in Table 3.2, YvfI stimulates the transcription of *fabHAF* and *fabRplsXfabHDacpA*. In *B. subtilis*, FabR is a transcriptional repressor known for fatty acid and phospholipid biosynthesis regulatory properties on transcription units including *fabRplsXfabHDacpA* and *fabHAF*. The FabR protein is controlled by the cellular levels of malonyl-CoA and intracellular concentration of this particular component was suggested to regulate FapR-mediated repression of target genes and operons (Schujman et al., 2003; Schujman et al., 2006). Hence, our study speculates that YvfI also affects indirectly the genes in the mentioned *fab* regulon via acting on FapR-mediated repression, probably through increasing the intracellular level of malonyl-CoA. Relieving FapR mediated repression can also be suggested to occur in a more indirect way since the pool of malonyl-CoA is regulated through feedback inhibition by the acyl-ACP end products (Davis and Cronan, 2001).

In this study, not only fatty acids biosynthetic genes, but also *lip* gene, that encodes for an extracellular lipase, and biotin biosynthesis operon (*bioWAFDB*) (Bower et al., 1996) were found to be upregulated. On the other hand, the expression of the *citB* (encoding aconitase) and the *citA* gene (encoding citrate synthase I) (Jin and Sonenshein, 1994), were found to be downregulated by YvfI. During the Krebs citric acid cycle, Acetyl-CoA is first converted to citrate by citrate synthetase and citrate to aconitate by aconitase (Hederstedt, 1993). Furthermore, in the presence of biotin, excess acetyl-CoA in the cell is shifted from Krebs citric acid cycle and converted to malonyl-CoA (an essential molecule for fatty acid biosynthesis) by acetyl-CoA carboxylase in a biotin-dependent enzyme. Therefore, as *citB* and *citA* are downregulated by YvfI, it should be expected that acetyl-CoA wouldn't be converted to aconitate and intracellular acetyl-CoA would be accumulated inside the cell. Hence, excessive amount of acetyl-CoA in the cell would lead to the promotion of malonyl-CoA synthesis. In toto, YvfI might be speculated to promote malonyl-CoA synthesis not only by inducing bioWAFDB operon, resulting in high amount of biotin synthesis, but also by repressing the first two enzymes of the Krebs citric acid cycle. Besides, YvfI induced lip gene expression might be expected to cause degradation of extracellular lipids which would eventually lead to acetyl-CoA accumulation and in turn to an increase in the intracellular level of malonyl-CoA, relieving FapR mediated repression on the *fab* genes.

B. subtilis genome encodes for seven extracytoplasmic σ factors σ^M, σ^W, σ^X, σ^Y, σ^Z, σ^V, and σ^{YlaC} (Huang and Helmann, 1998) that work in a harmony to display a control mechanism on many different functions (Helmann, 2002). Among these seven factors, the σ^M, σ^W, and σ^X regulons are widely studied and well-characterized. These three σ factors collectively control overlapping sets of genes related to resistance mechanism against cell envelope-active compounds and antibiotics (Helmann, 2002; Luo et al., 2010). When most of the cases are considered, genes that provide resistance are generally controlled by a single ECF sigma factor. To give examples, σ^W is responsible for controlling genes that provide resistance not only against fosfomycin which is a peptidoglycan synthesis inhibitor, but also against the toxic peptide SdpC, and the lantibiotic sublancin (Butcher and Helmann, 2006; Cao et al., 2001).

One of the operons that depend on σ^W is *fabHAfabF* operon and in case the membrane fluidity increases in a signal dependent manner, this sigma factor function in adapting membrane phospholipid composition (Kingston et al., 2011). *fabHA* becomes downregulated while *fabF* gets upregulated which in turn results in accumulation of straight chain fatty acids and in extension of the length of membrane phospholipids. Therefore, these adaptations lead to a increase in membrane viscosity and thus to an resistance to detergents and antibiotics produced by other strains (Kingston et al. 2011). Therefore, YvfI, through the upregulation of *fabHAfabF* operon, possibly could have led to a accumulation of branched chain fatty acids biosynthesis (Kingston et al. 2011). Hereafter, YvfI might have a crucial role in adjusting membrane phospholipid composition which is important in adaptation processes, during stationary phase, against stress signals and during cell differentiation.

3.3.1.2 Nitrogen metabolism

Nitrogen metabolism in bacteria is modulated by the intracellular levels of glutamine and glutamate, the two central intermediates in nitrogen metabolism that serve as the main nitrogen source in the cell (Fisher and Sonenshein, 1991). Glutamine is formed from glutamate and ammonium by glutamine synthetase (GS) and required directly for the biosynthesis of all of the major macromolecules of the cell: proteins (via the amino acids tryptophan, histidine, arginine, and glutamine itself), nucleic acids (via purines and pyrimidines), and cell-surface polymers (via glucosamine). An important characteristic for *B. subtilis* in ammonium assimilation is that it does not encode for an assimilatory glutamate dehydrogenase. Therefore, glutamate synthetase (GS) pathway is the sole pathway for ammonium assimilation through glutamate synthesis using Krebs cycle intermediate, α-ketoglutarate, and glutamine as substrates (Schreier, 1993) (Figure 3.16).

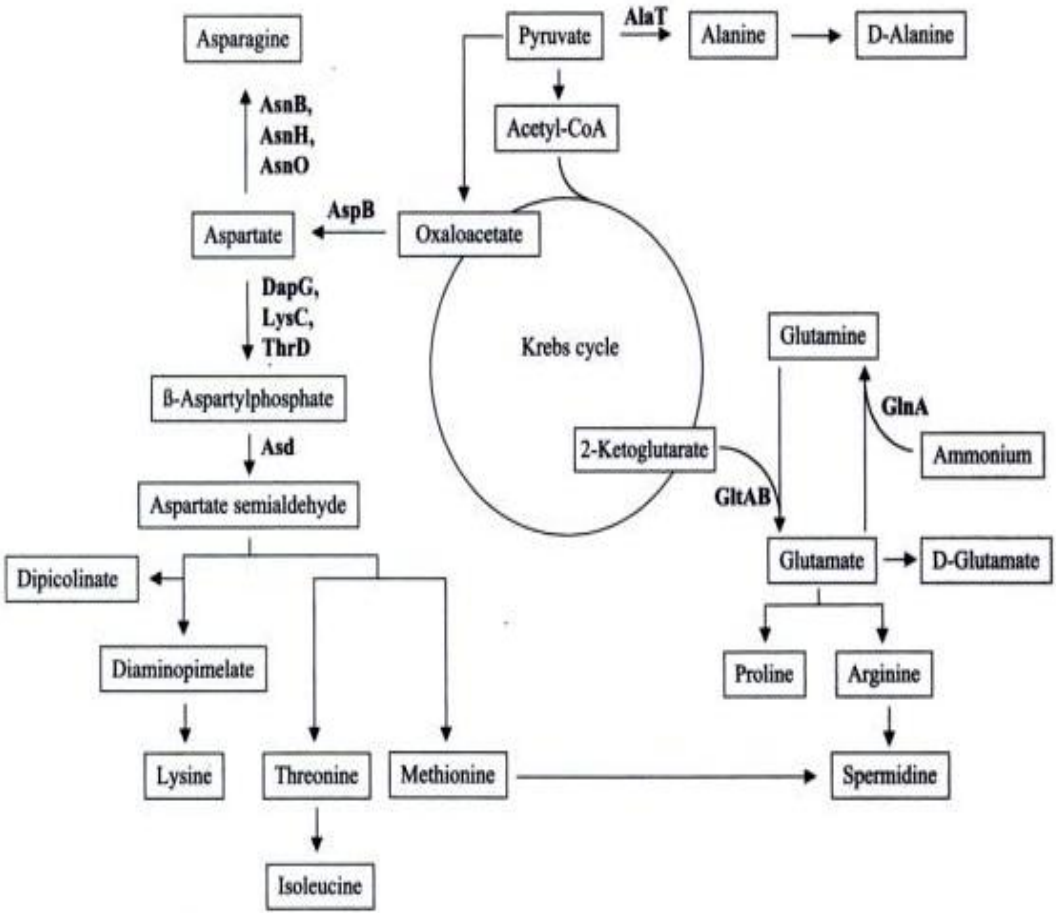


Figure 3.16 : Glutamate biosynthesis in *B. subtilis.*

Transcription level of genes for glutamate synthase, *gltA* (encoding glutamate synthase large subunit) and *gltB* (encoding glutamate synthase (NADPH) small chain) were found to be significantly increased in the *yvfI* mutant (Table 3.3). YvfI was also observed to act on *glnRA* operon (encoding nitrogen responsive regulator and glutamine synthetase, respectively) not as much as significantly as observed for *gltA* and *gltB* genes. However, it should be noted that expression levels of *glnRA* and *gltAB* operons are co-regulated by TnrA and GlnR under various nutritional conditions (Bohannon and Sonenshein, 1989; Fisher, 1999; Wray et al, 2001). Furthermore, in case of nitrogen limitation, the expression of *glnR, glnA* and *gltAB* are repressed by TnrA. Hence, the main role for TnrA was suggested to sense the level of glutamate and to control the production of sufficient levels of glutamate in the cell (Wray et al, 2001). In consistent with the regulatory role of TnrA, YvfI was found to act as another direct negative regulator of both *gltAB* and *glnRA* operons. It can be supposed that YvfI might directly adapt to changing carbon and/or nitrogen state of the cell through regulating transcriptions of *gltAB* and *glnRA* operons.

Table 3.3 : YvfI regulation on genes related to nitrogen metabolism in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
pyrR	2,52	2,79(±0,32)	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase	
*pyrP**	NR	3,48(±0,18)	uracil permease	
pyrB	1,37	4,34(±0,01)	aspartate carbamoyltransferase catalytic subunit	
pyrC	1,89		Dihydroorotase	$\neq \overrightarrow{\mathbf{pyrR}} \neq \overrightarrow{\mathbf{pyrP}} \neq \overrightarrow{\mathbf{pyrB}}\,\overrightarrow{\mathbf{pyrC}}$ $\overrightarrow{\mathbf{pyrAA}}\,\overrightarrow{\mathbf{pyrAB}}\,\overrightarrow{pyrK}\,\overrightarrow{pyrD}\,\overrightarrow{pyrF}$ $\overrightarrow{pyrE} \neq \overrightarrow{cysH}$
pyrAA	2,31	5,85(±0,20)	carbamoyl-phosphate synthetase (glutaminase subunit)	
pyrAB	2,00		carbamoyl-phosphate synthetase (catalytic subunit)	
pyrK	ND		dihdrooratate dehydrogenase	
pyrD	1,32		dihdrooratate dehydrogenase (catalytic subunit)	
pyrF	1,21		orotidine 5'-phosphate decarboxylase	
pyrE	1,08		orotate phosphoribosyl transferase	
argC	-1,63	-1,98(±0,22)	N-acetyl-gamma-glutamyl-phosphate reductase	
argJ	-1,89		bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	$\overrightarrow{yitY}\,\overrightarrow{yitZ} \neq \overrightarrow{\mathbf{argC}}\,\overrightarrow{\mathbf{argJ}}\,\overrightarrow{\mathbf{argB}}\,\overrightarrow{\mathbf{argD}}$ $\overrightarrow{\mathbf{carA}}\,\overrightarrow{\mathbf{carB}}\,\overrightarrow{\mathbf{argF}} \neq \overrightarrow{yjzC}$
argB	-2,08		acetylglutamate kinase	
argD	-2,13		acetylornithine aminotransferase	
carA	-2,22		carbamoyl phosphate synthase small subunit	
carB	-2,15		carbamoyl phosphate synthase large subunit	
argF	-2,16		ornithine carbamoyltransferase	
glnR	-1,31	-0,73(±0,10)	transcriptional regulator (nitrogen metabolism)	$\overrightarrow{spoVG} \neq \overrightarrow{ynbA}\,\overrightarrow{ynbB}\,\overrightarrow{\mathbf{glnR}}\,\overrightarrow{\mathbf{glnA}} \neq$
glnA	-1,19		glutamine synthase	
gltA	-1,31	-2,04(±0)	glutamate synthase (large subunit)	
gltB	-1,45		Glutamate synthase small chain	$\neq \overleftarrow{yofA}\,\overrightarrow{yogA} \neq \overleftarrow{\mathbf{gltB}}\,\overleftarrow{\mathbf{gltA}}\,\overrightarrow{gltC} \neq$ $\overleftarrow{proJ}\,\overleftarrow{proH}$
trpE	-1,9	-1,26(±0,05)	anthranilat synthase component I	
trpD	-2,1		anthranilate phosphoribosyltransferase	$\neq \overleftarrow{aroE}\,\overleftarrow{tyrA}\,\overleftarrow{hisC}\,\overleftarrow{\mathbf{trpA}}\,\overleftarrow{\mathbf{trpB}}$ $\overleftarrow{\mathbf{trpF}}\,\overleftarrow{\mathbf{trpC}}\,\overleftarrow{\mathbf{trpD}}\,\overleftarrow{\mathbf{trpE}}\,\overleftarrow{aroH}$
trpC	-2,62		indole-3-glycerol-phosphate synthase	
trpF	-2,22		N-(5'-phosphoribosyl) anthranilate isomerase	
trpB	-2,00		tryptophan synthase subunit beta	
trpA	-1,04		tryptophan synthase subunit α	

a. Numbers indicate the log 2 transformed expression ratio's.

b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),

c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "ǂ" indicates the termination sites

ND no differential expression observed

NR no reproducible data obtained

* Bayes P value higher than acceptable value (>0.01)

In harmony with the glutamate biosynthesis related genes, the transcription of all the genes related to arginine biosynthesis (*argCJBDcarABargF* and *argGH*) and tryptophane biosynthesis (*trpEDCGBA*) were found to be significantly down-regulated by YvfI (Table 3.3). Contrariwise, pyrimidine nucleotide biosynthetic (*pyr*) operon (*pyrB-pyrC-pyrAA-pyrAB-pyrK-pyrD-pyrF-pyrE*) was observed to be induced by YvfI (Table 3.3). In *B. subtilis*, *pyr* operon is controlled through transcription attenuation mechanism **(**Lu and Switzer, 1996). Besides to transcription attenuation, it is proposed in this study that *pyr* operon might also be subjected to positive control directed by YvfI. In conclusion, it might be speculated strongly that YvfI exerts important regulatory roles (both negatively and positively) on nitrogen metabolism.

3.3.1.3 Carbohydrate metabolism

In this study, YvfI was also demonstrated to repress the *acoABCL* operon, encoding acetoin dehydrogenase complex required for acetoin utilization (Huang et al., 1999) (Table 3.4). Acetoin is used as a carbon storage material that is synthesized and exported during exponential phase of growth; besides *B. subtilis* further consumes acetoin also during stationary phase of growth, so that it would act as a carbon and energy source during sporulation (Lopez and Thoms, 1976). Expression from this operon is subjected to catabolite repression by glucose through CcpA, a global regulator of carbon catabolite repression (Ali et al., 2001). This present study assigns another regulatory role to YvfI as being a negative regulator of *acoABCL* operon. In addition, *msmRE* operon encoding a putative regulatory protein belonging to LacI family (*msmR*) that consist of a HTH domain on its N terminal and encoding a putative binding proteins for the transport of multiple sugars (*msmE*) was directly downregulated by YvfI. These findings proposed that YvfI functions not only during carbohydrate utilization in the early stationary phase cell but also on the regulation of carbohydrate transport systems.

Table 3.4 : YvfI regulation on genes related to carbohydrate metabolism in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
acoA	-1,57	-3,07(±0,22)	acetoin dehydrogenase E1 component (TPP-dependent alpha subunit)	$\neq \overrightarrow{yfJM}\ \overrightarrow{yfJL} \neq \overrightarrow{\boldsymbol{acoA}}\ \overrightarrow{\boldsymbol{acoB}}\ \overrightarrow{\boldsymbol{acoC}}\ \overrightarrow{\boldsymbol{acoL}}$ $\neq \overrightarrow{acoR}\ \overrightarrow{sspH} \neq$
acoB	-1,54		acetoin dehydrogenase E1 component (TPP-dependent beta subunit)	
acoC	-1,56		branched-chain alpha-keto acid dehydrogenase subunit E2	
acoL	-1,36		dihydrolipoamide dehydrogenase	
msmR	-1,48	-1,43(±0,27)	transcriptional regulator (LacI family)	$\overleftarrow{bioW} \neq \overleftarrow{ytaP}\ \overrightarrow{\boldsymbol{msmR}}\ \overrightarrow{\boldsymbol{msmE}}\ \overrightarrow{amyD}$ $\overrightarrow{amyC}\ \overrightarrow{melA} \neq$
msmE	-1,51		multiple sugar-binding lipoprotein	

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "≠" indicates the termination sites

Microarray analysis revealed out that YvfI downregulates the transcription pattern of 6 genes which are known to be regulated by a stationary phase transition regulator, AbrB. It should be noted that AbrB is a stationary phase transition regulator, which is assigned during the stationary phase in *B. subtilis*. Furthermore, negative regulation pattern of AbrB regulatory protein on different genes, such as *pbpE*, and *sigW* that are transcribed through the extracytoplasmic sigma factor SigmaW dependent promoter was previously described (Strauch et al., 2007; Cao et al., 2002) This finding further supported the possibility that YvfI acts similarly with AbrB protein on effecting σ^W regulon by decreasing *sigW* gene transcription. Similar regulation pattern between AbrB and YvfI was further detected on *aprE* and *liaI* genes while varied regulation was observed for *sdpA*, *argC* and *citB* genes (Table 3.5). Additionally, the mRNA level of *citB* was significantly elevated in YvfI mutant. Expression of *citB* is under the complex regulation of CcpC, a citrate inhibited repressor and CodY, GTP-dependent repressor of stationary-phase cell, and AbrB, its transcription rapidly increases as a response to excessive citrate levels and decreases when intracellular pools of GTP and branched-chain amino acids boost up (Kim et al., 2003). Data given in this study revealed out that YvfI also participates to this complex regulation on *citB* through acting as a repressor.

Table 3.5 : Genes that are found in AbrB regulon and that become induced or repressed by YvfI during the stationary phase.

Gene	Expression ratio [a]	qPCR [b]	AbrB reg	Function
yvaW(sdpA)	0,7	1,08(±0,22)	-	Sporulation delay protein-export of killing factor
aprE	-3,78	-3,07(±0,91)	-	extracellular alkaline serine protease (subtilisin E)
argC	-1,39	-1,98(±0,22)	+	N-acetyl-gamma-glutamyl-phosphate reductase
citB	-0,89	-2,21(±0,21)	+	Aconitate hydratase
pbpE	-0,18	-3,44(±1,13)	-	penicillin-binding protein 4* (spore cortex)
sigW	-0,35	-4,31(±0,01)	-	RNA polymerase sigma factor sigW
yvqI (liaI)	-1,44	-1(±0,05)	-	permease

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),

In *B. subtilis*, *citB* encodes for an aconitase enzyme that catalyzes the reversible conversion of citrate and iso-citrate to aconitate during tricarboxylic acid branch of Krebs citric acid cycle and product of this gene is also known to be required for sporulation (Kim et al., 2003). Aconitase displays an additional role in sporulation which is independent from of its enzymatic activity required for the activation of Spo0A (Serio et al., 2006). This auxiliary activity provides the protein with a regulatory role for setting the proper timing of spore coat assembly and sporulation initiation (Craig et al, 1997; Serio et al., 2006). In consequence, YvfI regulatory behavior on *citB* has given more insights on the contribution of YvfI to sporulation process which would be explained in details in following sections.

Furthermore, it should be noted that, AbrB acts as a defined repressor on the biofilm formation in *Bacillus subtilis* during the transition from exponential growth to stationary phase (Chu et al., 2008). On the other hand, Abrb is not the sole regulator of the system, SinR repressor protein is also known to repress many genes involved in biofilm formation, including exopolysaccharide synthesis genes and *yqxM-sipW-tasA* genes that are also responsible for late spore processes (Branda et al., 2006; Chu et al., 2006). Another operon that was subjected to a regulation by SinR, was found to be responsible for lactate utilization during biofilm development and renamed as *lutABC*. Moreover, this operon was characterized to be under the cooperative dual control of two repressors SinR and GntR-type repressor, YvfI, renamed as LutR (Chai et al., 2009). Therefore, this data further strengthen the possibility for YvfI to act very similarly to AbrB, in case of both cooperating with SinR to act on architectural complexity of biofilms in the presence of L-lactate (Chai et al., 2009) and on effecting σ^w regulon.

3.3.1.4 Degradative enzyme production

In *Bacillus subtilis* nutrient limitations during the transition stage, enhance the production of a wide variety of degradative enzymes such as levansucrase, alkaline proteases and metalloproteases, α-amylase, β-glucanase, and xylanase in order to scavenge alternative nutrient sources (Stülke et al., 1993). Microarray studies conducted in this study, showed that the lipase encoding *lip* gene (Eggert et al 2000), and the *yhfEF* operon, encoding a putative endogluconase (Kunst et al., 1997), were upregulated by YvfI. YvfI was also shown to positively regulate the intracellular serine protease encoding gene *aprX* while repressing *aprE*, and *ispA* (Table 3.6). YvfI is not the first reported regulatory protein for these particular genes. The transcription of *ispA* and *aprE* were previously reported to be directly activated by DegS-DegU, a two component regulatory systems in *B. subtilis* (Msadek et al., 1991), and the transcription of *aprX* was known to be regulated by SinR as being the sole regulator (repressor) described for *aprX* (Valbuzzi et al., 1999). Herein, YvfI might be speculated to be the first positive regulator of *aprX*. The expression of the *aprE* gene that encodes for a extracellular alkaline serine protease, was tightly regulated by a combination of regulatory systems involving activation by DegU and repression by AbrB, ScoC and SinR (Msadek et al., 1991, Ogura et al., 2003, Ogura et al., 2004, Abe et al., 2009). This present study points out that YvfI is a novel transcription factor that regulates *aprE* along with the previously described regulators.

Table 3.6 : YvfI regulation on genes related to degradative enzyme production in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
aprX	1,57	2,98(±0,17)	serine protease	$\neq \overleftarrow{\boldsymbol{aprX}}\ \overrightarrow{ymaC}\ \overrightarrow{ymaD}\ \neq \overleftarrow{ebrB}$
yhfE	1,63	3,65(±0,04)	putative endoglucanase	$\neq \overleftarrow{fabHB}\ \overrightarrow{yhfC} \neq \overleftarrow{yhfD}\ \overrightarrow{\boldsymbol{yhfE}}\ \overrightarrow{\boldsymbol{yhfF}} \neq$
yhfF	1,40	2,18(±0,09)	hypothetical protein	
aprE	-2,35	-3,07(±0,91)	extracellular alkaline serine protease (subtilisin E)	$\neq \overleftarrow{yhfM}\ \overrightarrow{yhfN} \neq \overleftarrow{\boldsymbol{aprE}}\ \overrightarrow{yhfO}\ \overrightarrow{yhfP} \neq$
ispA	-2,01	-1,68(±0,22)	intracellular serine protease	$\neq \overleftarrow{metE} \neq \overleftarrow{\boldsymbol{ispA}}\ \overrightarrow{ykoB} \neq \overleftarrow{ykoC}\ \overrightarrow{ykoD}$

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "≠" indicates the termination sites

In *B. subtilis*, differing in auxiliary function from these degradative enzymes, IspA plays a critical role for the onset of efficient sporulation. IspA does not only function in creating proteolytic activity in sporulating cells, but it was also proposed to employ a regulatory pattern for sporulation, such that mutation of *ispA* resulted in delay of sporulation in *B. thuringiensis* (Chen et al., 2003). This data further strengthens the role of YvfI during sporulation process of *Bacillus subtilis.*

3.3.1.5 Antibiotic production and resistance

In this study YvfI has risen as an important regulatory protein that employs regulation on the expression of a number of varied types of antibiotics. For instance, YvfI positively controls the non-ribosomally synthesized lipopeptide antibiotic fengycin (pliapstatin) and polyketide antibiotic encoded from *pks* operon that is responsible for polyketide antibiotic biosynthesis and lipopeptide antibiotic fengycin-plipastatin production from *ppsABCDE* operon responsible for lipopeptide antibiotic fengycin-plipastatin production (Table 3.7). Lipopeptide antibiotics and polyketides are all related with lipid metabolism since lipopeptide antibiotics partly consist of β-hydroxyfattyacids and polyketides are usually biosynthesized via condensation of malonyl-CoA. In concordance with this data, during early stationary phase YvfI was suggested in the previous sections to be related with the expression of genes responsible for fatty acid and lipid biosynthesis like *fabHB,ylpC,plsX,fabHD,acpA, fahHAfabF* and *plsC, b*iotin biosynthesis *(bioWAFDB)* and extracellular lipase encoding gene, *lip*, together with polyketide and plipastatin biosynthetic operons.

Table 3.7 : YvfI regulation on genes related to antibiotic production and resistance in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
pksD	1,9	1,44(±0,06)	enzyme involved in polyketide synthesis	
pksE	1,59		enzyme involved in polyketide synthesis	$\overrightarrow{pksA} \neq \overrightarrow{pksB}\ \overrightarrow{pksC}\ \overrightarrow{\mathbf{pksD}}\ \overrightarrow{\mathbf{pksE}}\ \overrightarrow{acpK}$
acpK	1,77		acyl-carrier protein	$\overrightarrow{pksF} \neq \overrightarrow{\mathbf{pksG}}\ \overrightarrow{pksH}\ \overrightarrow{pksI}$
pksG	2,10	1,42(±0,18)	acetyl-S-AcpK beta-ketothioester polyketide intermediate transferase	$\overrightarrow{\mathbf{pksJ}}\ \overrightarrow{\mathbf{pksL}}\ \overrightarrow{\mathbf{pksM}}\overrightarrow{\mathbf{pksN}}\ \overrightarrow{\mathbf{pksR}} \neq \overleftarrow{pksS}\ \overrightarrow{ymzB}$
pksH	ND		enzyme involved in polyketide synthesis	
*pksI**	ND		enzyme involved in polyketide synthesis	
pksJ	2,73	4,14(±0,01)	polyketide synthase of type I	
pksL	3,95	6,19(±0,06)	polyketide synthase of type I	
pksM	2,17		polyketide synthase	
pksN	2,96		polyketide synthase of type I	
pksR	2,46		polyketide synthase	
*ppsA**	NR		plipastatin synthetase	
ppsB	1,1	1,09(±0,18)	plipastatin synthetase	$\neq \overleftarrow{\mathbf{ppsE}}\ \overleftarrow{ppsD}\ \overleftarrow{ppsC}\ \overleftarrow{\mathbf{ppsB}}\ \overleftarrow{\mathbf{ppsA}} \neq$
*ppsC**	NR		plipastatin synthetase	
ppsD	1,24		plipastatin synthetase	
ppsE	2,42		plipastatin synthetase	
yokD	NR	2,65(±0,25)	aminoglycosideN3'-acetyltransferase	$\neq \overrightarrow{yokF}\ \overrightarrow{yokE} \neq \overrightarrow{\mathbf{yokD}}\ \overleftarrow{yokC}$
ywfB(bacA)†	ND		bacilysin biosynthesis protein, dehidratase	
ywfC(bacB)	ND		isomerase component of bacilysin synthetase	$\neq ywfA \neq \overrightarrow{\mathbf{ywfB}}\ \overrightarrow{ywfC}\ \overrightarrow{ywfD}\ \overrightarrow{ywfE}$
*ywfD(bacC)**	NR		bacilysin biosynthesis oxidoreductase	$\overrightarrow{ywfF}\ \overrightarrow{ywfG} \neq \overleftarrow{\mathbf{ywfH}} \neq \overleftarrow{ywfI}$
ywfE(bacD)	ND		alanine-anticapsin ligase	
*ywfF(bacE)**	NR		efflux protein for bacilysin excretion, self-protection against bacilysin	
*ywfG**	NR		transaminase	
ywfH†	ND		carrier protein reductase of bacilysin biosynthesis	
yybN	1,76	2,05(±0,24)	hypothetical protein	
yybM	ND		integral inner membrane protein	
yybL	1,52	1,23(±0,12)	integral inner membrane protein	$\overrightarrow{\mathbf{yybN}} \neq \overrightarrow{\mathbf{yybM}}\ \overrightarrow{\mathbf{yybL}}\ \overrightarrow{\mathbf{yybK}}\ \overrightarrow{\mathbf{yybJ}}\ \overleftarrow{yybI}$ $\overleftarrow{yybH}\ \overrightarrow{yybG} \neq \overleftarrow{yybF}$
yybK	1,73		integral inner membrane protein	
yybJ	1,52		ATP-binding cassette protein	

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "+" indicates the termination sites

ND no differential expression observed
NR no reproducible data obtained
* Bayes P value higher than acceptable value (>0.01)
† Expression profiles were previously elucidated by *lacZ*-fusion analysis

It was previously reported that disruption of YvfI has resulted in the loss of dipeptide antibiotic bacilysin biosynthesis phenotype (Köroğlu et al., 2008). However,

microarray studies have failed to support this data since any significant differences in the expression of bacilysin biosynthetic cluster: *bacABCDEFywfG* operon and *ywfH* was not detected probably due to drawbacks of microarray studies. However, expressions of *bacABCDEywfG* operon (Köroglu et al., 2011) and *ywfH* gene (studied by our group, data not published) were previously studied using *lacZ* fusion assays. Results of these mentioned studies have revealed out that YvfI activity was crucial for the maximum level expression of *bac* operon (Köroglu et al., 2011) and *ywfH* (studied by our group, data not published). It should be noted that the unusual amino acid anticapsin from dipeptide bacilycin antibiotic is generated as a branching off from prephenate of the aromatic amino acid pathway through the action of BacA and BacB (Hilton et al., 1988, Mahlstedt and Walsh 2010). Then BacB is chosen as the substrate by YwfH, a short chain reductase that ultimately causes to promotion of tetrahydrotyrosine (H(4)Tyr) formation and ultimately to anticapsin synthesis through a unknown reaction (Figure 1.3) (Mahlstedt and Walsh 2010). An *yvfI* mutation was shown to decrease *ywfB-lacZ* expression to about 57% relative to wild type level during the stationary phase (Köroglu et al., 2011) while this mutation had effected *ywfH* expression more drastically.

This study also reports stimulation of *yybN-yybM-yybL-yybK* operon by YvfI during early stationary phase (Table 3.7). As described by Albano *et al* (2005), *yybN-yybM-yybL-yybK* cluster encodes for a protein with N-terminal transport signal sequence and three permease-like membrane proteins is involved in transport, respectively. In addition, another gene *yybJ*, located at the downstream of *yybK*, was also significantly affected by *yvfI* mutation and our transcriptional profiling data revealed out that *yybJ* is might br the last gene in this operon as being a putative ATP binding protein, considering similar induction profiles of the rest of the operon.

Under the light of these findings, it can be accepted as a fact that YvfI is involved in the regulation of antimicrobial product biosynthesis. However, YvfI also seems to function in providing resistance to bacterium against aminoglycoside antibiotics because it clearly induced *yokD* gene (encoding a putative aminoglycoside acetyltransferase as indicated by Hoffman et al., 2005) transcription significantly during late stage of growth (Table 3.7). This data is noticeable considering the fact that stationary phase cells and biofilm cells, because of their reduced permeability, are more tolerant to antibiotics and YvfI strikingly stimulates an aminoglycoside

resistance gene during stationary phase of growth which could be related to the regulatory behavior of YvfI on biofilm process that would be mentioned in section 3.3.1.9.

3.3.1.6 Regulation of *B. subtilis* mobile genetic element

Mobile genetic elements in bacteria contain a variety of genes with different functions from antibiotic resistance, symbiosis, to virulence; their high diversity and dissemination also stimulate bacterial evolution by conferring new genes and phenotypes to their recipients (Burrus and Waldor, 2004; Frost et al., 2005). Phages, plasmids, and integrative and conjugative elements (ICEs), also known as conjugative transposons are classified as the most frequently seen mobile genetic elements (Grohmann et al., 2003; Burrus et al., 2002). A class of ICE elements in *B. subtilis*, 20 kb ICEBs1 (Figure 3.17) gets excised from the chromosome when induced, and gains mobility to be transferred to recipient cells. When cells encounter unfavorable conditions like high cell density and overcrowded environment with cells lacking ICE*Bs1* and PhrI, a SOS signal is transmitted and ICEBs1 gene expression and excision get initiated (Auchtung et al., 2005).

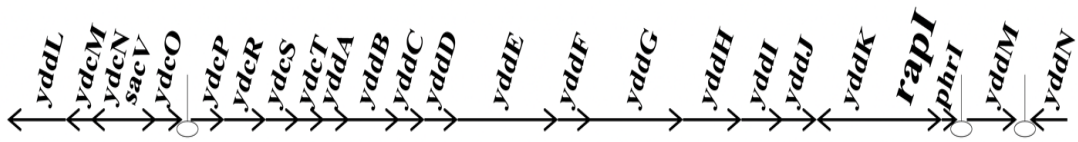


Figure 3.17 : Operonal organization of ICE*Bs1*. ICE*Bs1* is a 20-kb long element which contains app. 24 different ORFs (Auchtung et al., 2005).

Additionally, sensing population density, its excision and transfer are known to be induced by Rap protein, RapI and repressed by the pentapeptide PhrI both of which are encoded by ICEBs1 itself (Auchtung et al., 2005). It should drain some attention that *rapI* gene was processed to give an unreproducible expressional profile as a result of the microarray analysis, however the constructed *rapI-lacZ* trancriptional fusion implied that *rapI*, the major inducer of ICE*Bs1*, is significantly down regulated by YvfI during transition state (Figure 3.18), suggesting that YvfI represses *rapI* transcription directly.

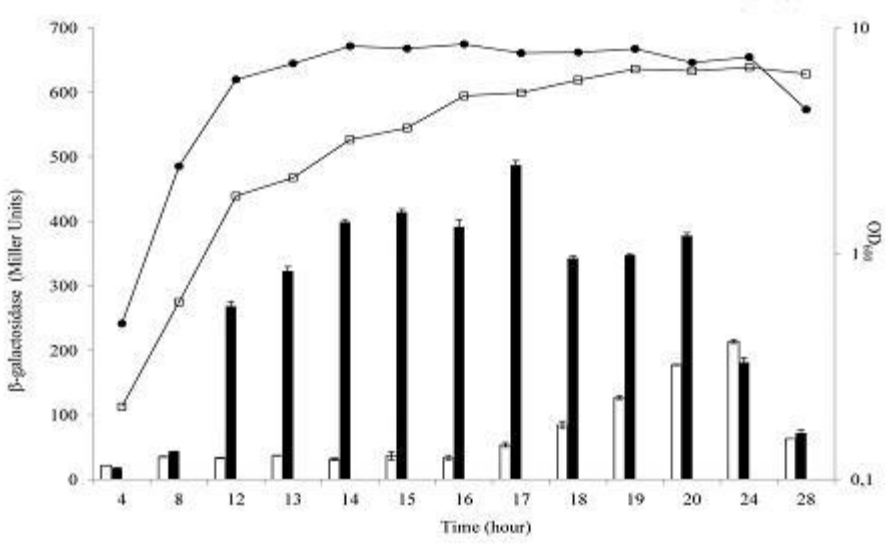


Figure 3.18 : Effect of *yvfI* mutation on the expression of *rapI* gene. Growth and β-galactosidase activity of *rapI*::*lacZ* fusion (white bars and white squares) and its congenic derivative *yvfI* mutant (*rapI*::*lacZ*, *yvfI*::*Tn10*::*spc*) (black bars and black circles). Error bars indicate the standard deviation of the mean of three independent experiments (n=3).

In Toto, these findings clearly contributed to the suggestion that YvfI contributes to the growth-phase dependent regulation of ICE*Bs1* in *B. subtilis* as a direct negative regulator for *rapI*.

3.3.1.7 SPP1 phage infection in *B. subtilis*

More than half of known phages are grouped by the presence of a long non-contractile tail, as is the case of SPP1 that consists of a 160-nm long tail. Bacteriophage SPP1, as a generalized transducing particle, is a virulent *Bacillus subtilis* phage that packages its genome in a 60-nm wide isometric capsid (Plisson et al., 2007; Tavares et al., 1992; Yasbin et al., 1974). Host infection of SPP1 is initiated by the binding of this 160-nm long tail to YueB, a membrane protein (Sao-Jose et al., 2004; Sao-Jose et al., 2006). This irreversible binding initiates a series of events resulting in release of phage genome into the *B. subtilis* cytoplasm. This membrane bound YueB protein of ~120 kDa shows great homology with other proteins from different species and one of these homologs is the protein Pip, which functions as a phage receptor in *Lactococcus lactis* (Sao-Jose et al., 2004).

Table 3.8: YvfI regulation on genes related to SPP1 phage infection in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
yukE	1,46	1,29(±0,06)	hypothetical protein	
yukD	ND		bacteriocin	
yukC	1,75		membrane-associated enzyme involved in bacteriocin production	≠ $\overleftarrow{yueD}$ $\overleftarrow{yueC}$ $\overleftarrow{yueB}$ $\overleftarrow{\boldsymbol{yukA}}$ $\overleftarrow{\boldsymbol{yukB}}$ $\overleftarrow{\boldsymbol{yukC}}$ $\overleftarrow{\boldsymbol{yukD}}$ $\overleftarrow{\boldsymbol{yukE}}$ $\overrightarrow{yukF}$ ≠
yukB	ND		putative cell division protein	
yukA	1,48		hypothetical protein	

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "≠" indicates the termination sites

ND no differential expression observed

Recently, *yueB* was reported to be a part of an operon including *yukE, yukD, yukC*, and *yukBA* transcription units (Sao-Jose et al., 2004). Another important observation in this study was that *yukE, yukC* and *yukAB* genes were significantly stimulated by YvfI (Table 3.8). Our overall data implied that YvfI is directly involved in the SPP1 infection in *B. subtilis*.

3.3.1.8 Protein translocation and folding

Most proteins that are secreted contain a signal peptide in order to direct these extracellularly functioning proteins to their final destination (Tjalsma et al., 2000). Chaperones and targeting factors generate machinery for protein transport the biggest of which is the Sec translocase that deals with preproteins in their unfolded state (Driessen et al., 2001). As it is reported, these secretory proteins are directed to Sec translocase by the signal recognition particle (SRP) (Hirose et al., 2000). This SRP complex is generated by Ffh (Fifty-four homolog) protein (Honda et al., 1993), a small cytoplasmic RNA (scRNA) (Nakamura et al., 1994; Nakamura et al., 1992), and a histone-like protein (HBsu) (Nakamura et al., 1999). SRP receptor-like protein FtsY and Ffh are among the widely conserved family of SRP-GTPases (Eichler and Moll, 2001). In *B. subtilis* RNase III encoded by *rnc* is responsible for the processing of small cytoplasmic RNA (scRNA) precursor. The upregulation of *rnc* and *ffh* transcription by YvfI (Table 3.9) reveals the possible effect of YvfI on the synthesis of SRP as an important component of the protein-secretion machinery of *B. subtilis*.

Table 3.9 : YvfI regulation on genes related to protein translocation and folding in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
ffh	1,51	2,91(±0,04)	signal recognition particle-like (SRP) GTPase	
rnc	2,41	1,64(±0,03)	ribonuclease III, cleaves both 5'- and 3'-sites of the small cytoplasmic RNA precursor	$\overrightarrow{acpA} \neq \overrightarrow{rnc} \neq \overrightarrow{smc} \neq \overrightarrow{ftsY} \neq$ ylqB

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "ǂ" indicates the termination sites

3.3.1.9 Sporulation, cannibalism and sporulation delay

B. subtilis initiates differentiation of specialized cell types when exposed to unfavorable environmental conditions in order to allow the bacteria survive in the increasingly hostile environment (Grossman, 1995; Phillips and Strauch, 2002; Errington, 2003; Piggot and Hilbert, 2004).). Production of antibiotics and macromolecular hydrolases, development of motility, chemotaxis and competance are among these responses (Kunst et al., 1997). Another response that makes *Bacillus subtilis* to form specialized cells is sporulation, *Bacillus subtilis* undergoes asymmetric cell division, followed by engulfment of the smaller forespore by the larger sibling, the mother cell (Errington, 2003; Levin and Grossman; 1998).

The process of sporulation, which represents a series of morphological and physiological events, that occurs through the sequential activation and silencing or blocking of genes (Errington, 2003). Spore development is a high energy requiring event and the pathway is usually completed before bacterium exit from this state. Thus, this bacterium has developed mechanisms to delay entry into sporulation and this event is called as cannibalism (Gonzalez-Pastor et al., 2003).

One of the most important finding of this project revealed out that the transcription of *sdpABC* (*yvaWXY*) operon, also called "sporulation delay operon" is controlled by Yvfl in a positive manner. In *B. subtilis*, nutritional limitation stimulates the spore production and the process of the sporulation is initiated by the transcriptional factor Spo0A which is activated through phosphorylation (Losick and Stragier, 1992). At the beginning of the sporulation it is discovered that Spo0A strongly induces two different operon groups; *skf* (sporulation killing factor) and *sdp* (sporulation delay)

(Fawcett et al., 2000). The other two member of the Skf operon, SkfE and SkfF provide the pumping out of the produced killing factor in order to protect the producer cell from the killing factor. Sdp operon, controlled by Spo0A, is responsible for the production of extracellular cell signal factor SdpC (weights 5 kD) which serves the purpose of signaling between bacteria. SdpC strongly controls *yvbA* and *yvaZ* genes which are under the *sdp* operon. In Spo0A-active cells, it is thought that transcriptional factor SdpR (YvbA), a member of ArsR family of regulators, strongly induces *yusLKJ* operon which is responsible for the lipid catabolism, and atp operon which is responsible for the production of ATP, for the purpose of delaying the onset of sporulation by increasing the energy level in the cell. In Spo0A-inactive cells, it is considered that through suppression of *sigW* gene, SdpR contributes to dissolving by Skf killing factor. It is thought that the cell is protected from the Skf killing factor effect by sigW, which is responsible for the development of detoxification and antimicrobial strength. Therefore, in Spo0A-inactive cells, it is accepted that SdpR and Skf killing factors move together to dissolve the cell so that Spo0A-active cells could use the released nutritions and consequently sporulation can be delayed and active growth can be maintained (Gonzalez-Pastor et al., 2003). From the aspect of YvfI, it is clearly seen that YvfI behaves parallel with SdpR and acts on regulating *sdp* operon causing induction in SdpC signal production and directly increasing the amount of SdpR in the cell and also by promoting *atp* operon and increasing the energy level in the cell at the same time, it appears that YvfI creates a possibility for a delayed pass into spore formation process. Furthermore, as an inducer of a putative endogluconase encoding gene, *yhfE* and an intracellular serine protease encoding gene, *acpX*, as described above, YvfI might also be speculated to delay the onset of sporulation by stimulating YhfE and AcpX and many other metabolic processes including protein biosynthesis through stimulating *rplJ* (50S ribosomal protein L1), *tsf* (Ts elongation factor), *frr* (ribosome recyclic factor) and *tig* trigger factor (prolyl isomerase), pyrimidine nucleotide biosynthesis (*pyrRPBCAAABKDFE)*, lipid and fatty acid biosynthesis (*fabHB; ylpC-plsX-fabDG-acpA; fabHA-fabF; plsC*) are observed to be induced by YvfI. Hence, in the transition to the stationary phase, YvfI regulatory factor also accelerates ATP synthesis and promotes many cellular activities necessary for active growth, including protein and nucleotide synthesis, cell wall synthesis, cell division, lipid and fatty acid biosynthesis and biotin biosynthesis. Therefore, all those mentioned limitations strengthen the possibility that “YvfI could

take a role in holding back the cell from sporulation and deccelerating initiation of the sporulation and support the cell growth at a certain time by promoting the metabolic activities in Spo0A-active cells during stationary phase".

Table 3.10 : YvfI regulation on genes related to cannibalism and sporulation delay in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
atpI	1,19	0,56(±0,03)	ATP synthase subunit I	
atpB	1,93		ATP synthase subunit A	
atpE	2,02	1,53(±0,15)	ATP synthase subunit C	
atpF	1,99		ATP synthase subunit B	$\neq \overleftarrow{atpC}\overleftarrow{atpD}\ \overleftarrow{\boldsymbol{atpG}}\ \overleftarrow{\boldsymbol{atpA}}\ \overleftarrow{\boldsymbol{atpH}}$
atpH	1,93		ATP synthase subunit delta	$\overleftarrow{\boldsymbol{atpF}}\ \overleftarrow{\boldsymbol{atpE}}\ \overleftarrow{\boldsymbol{atpB}}\overleftarrow{\boldsymbol{atpI}} \neq$
atpA	2,01	1,13(±0,10)	ATP synthase subunit alpha	
atpG	1,91		ATP synthase subunit gamma	
ftsE	1,11	1,94(±0,13)	cell-division ABC transporter (ATP-binding protein)	$\overleftarrow{yvjB} \neq \overleftarrow{\boldsymbol{ftsX}}\ \overleftarrow{\boldsymbol{ftsE}} \neq \overleftarrow{cccB}\ \overleftarrow{yvjA} \neq$
ftsX	1,68		cell-division ABC transporter	
rplJ	1,69	3,42(±0,30)	50S ribosomal protein L10	
rplL	1,66		50S ribosomal protein L7/L12	$\overrightarrow{nusG}\ \overrightarrow{rplK}\ \overrightarrow{rplA} \neq \overrightarrow{\boldsymbol{rplJ}}\ \overrightarrow{\boldsymbol{rplL}} \neq \overrightarrow{ybxB}$
spoIIE†	ND		serine phosphatease	$\neq \overrightarrow{\boldsymbol{spoIIE}}\ \overrightarrow{yabS}\ \overrightarrow{yabT}\ \overrightarrow{yacA}\ \overrightarrow{hprT}$
tig	1,68	1,11(±0,01)	trigger factor (prolyl isomerase), catalyze in vitro protein folding; essential for growth under starvation conditions	$\neq \overleftarrow{lonA} \neq \overleftarrow{lonB}\ \overleftarrow{clpX}\ \overleftarrow{\boldsymbol{tig}}\ \overleftarrow{ysoA} \neq$
tsf	1,69	1,31(±0,53)	Ts elongation factor	
pyrH	1,52	1,74(±0,15)	uridylate kinase	$\overrightarrow{sigD}\ \overrightarrow{ylxL}\ \overrightarrow{rpsB} \neq \overrightarrow{\boldsymbol{tsf}}\ \overrightarrow{\boldsymbol{pyrH}}\ \overrightarrow{\boldsymbol{frr}} \neq$
frr	1,55	1,11(±0,08)	ribosome recycling factor	
yvaW(sdpA)	1,26	1,08(±0,22)	sporulation delay protein-export of killing factor	
yvaX (sdpB)	2,2	1,68(±0,08)	Sporulation delay protein-exporter of killing factor SpbC	$\overrightarrow{yvaV}\ \overrightarrow{\boldsymbol{yvaW}}\ \overrightarrow{\boldsymbol{yvaX}}\ \overrightarrow{yvaY} \neq$
yvaY(sdpC)	ND		killing factor SdpC	
yvcE(cwlO)	2,24	1,70(±0,05)	secreted cell wall DL-endopeptidase	$\overleftarrow{yvcI}\ \overleftarrow{trxB}\ \overleftarrow{\boldsymbol{yvcE}} \neq \overleftarrow{yvcD}\ \overleftarrow{yvcC} \neq$
*Abh**	NR	-4,57(±0,28)	transcriptional regulator of transition state genes (AbrB-like)	$\neq \overleftarrow{ykpC}\ \overleftarrow{mreBH}\ \overleftarrow{\boldsymbol{abh}} \neq \overleftarrow{kinC}\ \overleftarrow{ykqA}$
pbpE	ND	-3,44(±1,13)	penicillin-binding protein 4* (spore cortex)	$\neq \overleftarrow{\boldsymbol{racX}}\ \overleftarrow{\boldsymbol{pbpE}}\ \overrightarrow{sacB} \neq \overrightarrow{yveB} \neq$
ytsC(bceA)	ND	-2,68(±0,32)	bacitracin ABC efflux transporter ATP-binding protein	$\neq \overleftarrow{yttB}\ \overrightarrow{yttA} \neq \overleftarrow{\boldsymbol{ytsD}}\ \overleftarrow{\boldsymbol{ytsC}}\ \overleftarrow{ytsB}$
ytsD(bceB)	ND	-3,77(±0,06)	Bacitracin export permease protein BceB	$\overleftarrow{ytsA} \neq \overleftarrow{ytrF}$

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "+" indicates the termination sites

ND no differential expression observed
NR no reproducible data obtained
* Bayes P value higher than acceptable value (>0.01)
† Expression profiles were previously elucidated by *lacZ*-fusion analysis

Beside its above mentioned functions, SdpR also induces cell death in Spo0A-inactive cells through repressing sigW (Gonzalez-Pastor et al., 2003), thus contributes to the loss of resistance against antibiotics and also stimulates the expression of *sdp* and *skf* operons indirectly since reducing σ^w dependent-transcription of abh, which encodes for an antimicrobial regulator, would induce the expression of the *skp* and *sdp* operons (Strauch et al., 2007; Chumsakul et al., 2011). This study has revealed that in addition to *sigW* gene itself, YvfI down regulates many genes regulated by σ^w, including *abh*, *bceAB* transcription unit that contributes to bacitracin resistance (Bernard et al., 2007), and also *pbpE* (encoding a penicillin binding protein, PBP4 endopeptidase) whose repression leads to reduced resistance against vancomycin, penicillin and bacitracin (Palomino et al., 2009). Therefore, it can be concluded that YvfI could also work similarly to SdpR by inducing cannibalism through Spo0A-inactive cells and this effect is more likely to be exerted by decreasing resistance of the cells against antimicrobial agents secreted by surrounding organisms and also against killing factor (SkF) produced by their own Spo0A-active, sporulating derivatives (Gonzalez-Pastor et al., 2003).

YvfI strikingly up regulated *ftsEX* operon in this study and therefore it can be speculated that in addition to its role in sporulation delay mechanism, YvfI should also have a role in setting the initial steps of sporulation. As Garti-Levi and co-workers (2008) suggested, FtsE and FtsX proteins encoded from this operon have crucial parts at the top of the sporulation phosphorelay cascade that leads to an efficient sporulation. A significant finding of this study was that *ftsEX* mutation on a wild type sporulating strain resulted in a expressional delay for a early sporulation, *spoIIE (*Garti-Levi et al., 2008). Under the light of this finding, since YvfI up regulated *ftsEX* operon, this study aimed to reveal whether *yvfI* mutation would alter *spoIIE-lacZ* expression or not. As a result, it was observed that *spoIIE* directed-*lacZ* expression was initiated in a 1 hour delayed manner when cells were grown in sporulation medium (DSM) (Figure 3.19), therefore this study further supported the proposition that YvfI acts on *ftsEX* and has a role on the regulation of sporulation initiation when it started. Spore count assay employed on *B. subtilis* TEK1 and wild type cells grown in DSM medium has revealed out that there were no difference on the sporulation percentages of both strains, suggesting that once sporulation initiated, no major defects are encountered when sporulation proceeds.

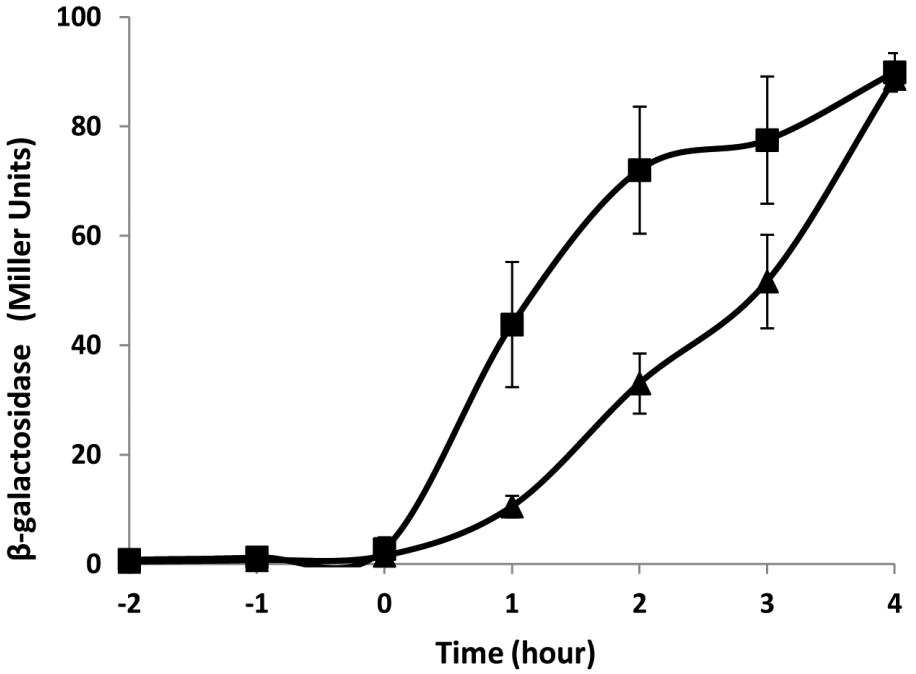


Figure 3.19 : Effect of *yvfI* mutation on the expression of *spoIIE.* Cells were grown in DSM medium at 37^{o}C and spollE-directed β-galactosidase synthesis was determined at the indicated times. Time zero represents initiation of sporulation in DSM. β-galactosidase activity of *spoIIE::lacz* fusion (squares) and its congenic derivative *yvfI* mutant (*spoIIE::lacz::cat, yvfI::Tn10::spc*) (triangle). Error bars indicate the standard deviation of the mean of three independent experiments (n=3).

3.3.1.10 Biofilm formation

B. subtilis is also known as a biofilm forming organism and through developing a biofilm, this bacterium gains the ability to behave as a multicellular community in which cells are held together tightly with the help of an extracellular matrix (Branda et al., 2005; Aguilar et al., 2007). Normally, in its natural habitat, *B. subtilis* stands as a flagellated, motile organism, however in case of biofilm formation; this motility is converted into a nonmotile state (Branda et al. 2001). This switch in motility is represented in figure 3.20 and the key components of biofilm development are visualized.

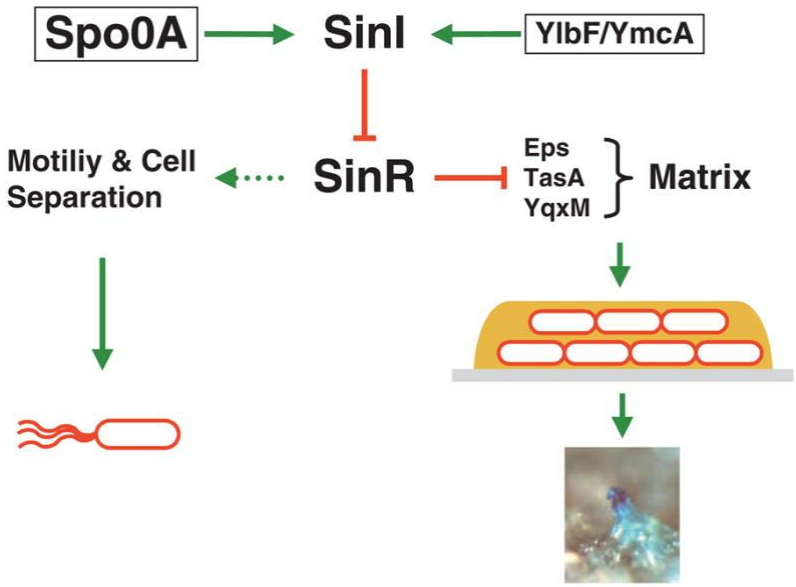


Figure 3.20 : Genetic circuitry governing the switch from motile cell to nonmotile cell in *B. subtilis* (Lemon et al., 2008).

The transcriptional repressor SinR was revealed to be the master repressor protein (Kearns et al. 2005) and in flagelleted cells, SinR represses the transcription of genes repress many genes involved in matrix formation, including exopolysaccharide synthesis genes and *tapA*(*yqxM)-sipW-tasA* genes that are also responsible for late spore processes (Branda et al., 2006; Chu, 2006). SinR is constitutively produced, and when conditions become favorable for biofilm formation, SinR activity is antagonized. SinI and two newly identified proteins, YlbF and YmcA, all serve to directly and/or indirectly antagonize SinR activity. Repressed SinR activity by SinI, YlbF and YmcA results in turn in the decrese of motility, cell chain formation, and matrix production (Branda et al. 2006). Furthermore, in nonmotile *B. subtilis*, regulation of cell surface proteins and extracellular matrix synthesis lead to the initiation of biofilm development (Gotz, 2002; Latasa et al. 2005). This matrix is composed of primarily exopolysaccharides and a protein, TasA which are encoded through *epsA-O* and *tapA-sipW-tasA* operons, respectively (Branda et al. 2006). EpsD, E, F, H, J, L, and M are all proposed to be glycosyl transferases, while EpsK is thought to be responsible for the export of saccharide (Branda et al. 2001). *tasA* and *tapA* (*yqxM)* mutants were also identified to produce cell chains that are incompetent to hold together and are defective for matrix production and *sipW* functions as a dedicated signal peptidase (Stover and Driks 1999a, 1999b).

In the microarray analysis of this sudy, it was demonstrated that *epsD, epsE*, *epsK* and *tapA-sipW-tasA* operon were induced by YvfI protein (Table 3.11), suggesting that YvfI might also have a role in regulation of biofilm formation next to cannibalism. Another supportive finding from this sudy was that a putative membrane-bound lipoprotein (*yvcA*) and a small cell wall associated protein (*yuaB*), that are further required for complex colony architecture development (Verhamme et al., 2007; Kovacs and Kupiers, 2011), were also significantly induced by YvfI.

Table 3.11 : YvfI regulation on genes related to biofilm formation in *B. subtilis.*

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
tapA (yqxM)	0,96	2,65(±0,06)	lipoprotein for biofilm formation	$\overrightarrow{sinI}\ \overrightarrow{sinR}\ \neq\neq\overleftarrow{\boldsymbol{tasA}}\ \overleftarrow{\boldsymbol{sipW}}\ \overleftarrow{\boldsymbol{yqxM}}\ \overrightarrow{yqzG}\ \overleftarrow{yqzE}$
sipW	2,12	1,42(±0,14)	type I signal peptidase	
tasA	1,50	2,65(±0,17)	major biofilm matrix component	
yuaB	3,33	2,70(±0,25)	hypothetical protein	$\overrightarrow{yuaC}\ \neq\overrightarrow{\boldsymbol{yuaB}}\ \neq\overrightarrow{yuaA}\ \overrightarrow{yubG}\ \neq$
*yvcA**	NR	1,56(±0,01)	Lipoprotein	$\overleftarrow{yvcC}\ \neq\overleftarrow{yvzA}\ \overleftarrow{yvcB}\ \overleftarrow{\boldsymbol{yvcA}}\ \neq\overleftarrow{hisI}$
yveN(epsD)	2,21	2,63(±0,05)	extracellular matrix biosynthesis enzyme	$\neq\overrightarrow{sigL}\ \overrightarrow{yvfG}\ \overleftarrow{\boldsymbol{yvfF}}\ \overleftarrow{\boldsymbol{yvfE}}\ \overleftarrow{yvfD}\ \overleftarrow{yvfC}\ \overleftarrow{yvfB}\ \overleftarrow{\boldsymbol{yvfA}}\ \overleftarrow{yveT}\ \overleftarrow{yveS}\ \overleftarrow{yveR}\ \overleftarrow{yveQ}\ \overleftarrow{yveP}\ \overleftarrow{\boldsymbol{yveO}}\ \overleftarrow{\boldsymbol{yveN}}\ \overleftarrow{yveM}\ \overleftarrow{yveL}\ \overleftarrow{yveK}\ \overrightarrow{slr}$
yveO(epsE)	2,08	1,34(±0,31)	Glycosyltransferase	
yvfA(epsK)	1,49	1,10(±0,21)	extracellular matrix component exporter	
yvfE(epsN)	0,79	1,02(±0,01)	aminotransferase	
yvfF(epsO)	1,79	0,94(±0,05)	pyruvyl transferase	

a. Numbers indicate the log 2 transformed expression ratio's.
b. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
c. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "ǂ" indicates the termination sites
* Bayes P value higher than acceptable value (>0.01)

It should be remarked that sporulation, cannibalism and biofilm production are tightly interlinked in *B. subtilis*, such that development of all three physiological states are initiated in a Spo0A dependent manner (Hamon and Lazazzera, 2001; Branda et al., 2004; Lopez et al., 2009). Cannibalism and biofilm development are observed to be initiated on the same colonies where Skf and Sdp toxins and the extracellular matrix of the biofilm were also produced (Lopez et al., 2009). Thus, above-mentioned roles of YvfI in cannibalism and biofilm formation are further strengthened as well as its ability to promote the growth of matrix-producing cells while delaying the sporulation. This growth inducing tendency finds more support considering that many genes regulated by YvfI are also involved in metabolism

directing pathways including degradative enzyme production, ATP production, lipid and fatty acid biosynthesis, carbon and nitrogen metabolism, nucleotide metabolism, cell wall synthesis, membrane transport, antibiotic production and resistance.

3.3.2 Identification of the genes regulated by YvfI during exponential phase

Previously mentioned study indicated that a maximum level of expression was observed following the entry into stationary phase (Figure 3.15). Hence, while aiming for the identification of all genes included in the regulon of YvfI transcriptional regulator, comparative transcriptome analysis of wild type control strain PY79 and *yvfI* disrupted TEK1 mutant, *yvfI::T10::spc* during exponential phase (~ OD 0,7) was studied using microarray analysis. *B. subtilis* PY79 and TEK1 strains were grown in PA medium until they reached OD 0,7 at A_{600}. In order to identify genes that are differentially expressed in PA medium during chosen growth phase, *B. subtilis* genomic DNA microarrays that harbors 4,107 open reading frames were preferred as described before.

It should be noted that exponential phase data was not expected to be in a great harmony with stationary phase expression data since YvfI expression level was significantly different for each phase. As discussed in previous sections the glutamate biosynthesis related genes, the transcription of all the genes related to arginine biosynthesis (*argCJBDcarABargF* and *argGH*) and tryptophane biosynthesis (*trpEDCGBA*) were significantly down-regulated by YvfI (Table 3.3). On the other hand, pyrimidine nucleotide biosynthetic (*pyr*) operon (*pyrB-pyrC-pyrAA-pyrAB-pyrK-pyrD-pyrF-pyrE*) was observed to be induced by YvfI during stationary phase of growth (Table 3.3). Contrariwise regulation of pyr operon by YvfI during log phase reveals the temporal regulatory effect of this Gntr-type protein (Table 3.12). In *B. subtilis*, *pyr* operon is controlled through transcription attenuation mechanism **(**Lu and Switzer, 1996). Besides to transcription attenuation, it is proposed in this study that *pyr* operon might also be subjected to dual control directed by YvfI in a growth phase-dependent manner. In conclusion, under the light of these findings, it might be speculated now even more strongly that YvfI exerts important regulatory roles (both negatively and positively) on nitrogen metabolism.

Interestingly, in our study, not only the expression of the gene for σ^W but also many genes known in "σ^W" regulon including *bceAB* (formerly *ytsCD*) locus responsible

from the bacitracin resistance (Bernard et al., 2007) were found to be down-regulated by YvfI during stationary phase; however our logarithmic phase study has shown the contrary effect of YvfI mutation on *bceB* gene, hence increasing bacitracin resistance (Table 3.13). Therefore, dual role of YvfI regulatory protein on different pathways become more striking, considering both *pyr* operon and *bceB* gene regulations by YvfI.

In a harmony with YvfI regulation during stationary phase, YvfI also exerts its inhibitory effect on carbohydrate metabolism during exponential phase. *acoABCL* operon, encoding acetoin dehydrogenase complex required for acetoin utilization (Huang et al., 1999), *msmRE* operon encoding a putative regulatory protein and a putative binding proteins for the transport of multiple sugars (*msmE*) was found to be downregulated by YvfI. Along with this data, three genes from *ydhMNOPQRST* operon that was assigned as Glucomannan utilization operon of *Bacillus subtilis* (Sadaie et al., 2007) and ywkA that encodes for a malic enzyme (Doan et al., 2003) were found to be repressed by YvfI protein (Table 3.12). It should be noted that *ydhM*, *ydhN*, and *ydhO* ORFs belonging to *ydhMNOPQRST* cluster encode for proteins homologous to the enzymes IIB, IIA, IIC of lactose-class phosphotransferase systems (PTSs) (Sadai et al., 2007). Strikingly, the fifth ORF (*ydhQ*) from this operon encodes for a DNA-binding protein that belongs to GntR family of transcriptional regulators (Aravind and Anantharaman, 2003). Nevertheless, this operon is negatively regulated by this GntR type repressor YdhQ and CcpA which is a global regulator of carbon catabolite repression (Ali et al., 2001). This study further propose YvfI as another regulatory protein that exerts repression on this operon that functions in sugar utilization. Putative sugar utilization gene cluster *yfnH*, *yfnG*, *yfnF*, *yfnE*, *yfnD* were found to induced by YvfI protein (Table 3.13), thus this data further strengten YvfI functioning during carbohydrate utilization in the exponential phase cell.

Table 3.12 : Transcription units that are down-regulated by YvfI during exponential phase of growth.

Gene	Expression ratio [a]	Function	Transcriptional Organisation[b]
pyrC	-1,75	dihydroorotase	$\neq \overrightarrow{pyrR} \neq \overrightarrow{pyrP} \neq \overrightarrow{pyrB}\overrightarrow{pyrC}\ \overrightarrow{pyrAA}\ \overrightarrow{pyrAB}\ \overrightarrow{pyrK}\ \overrightarrow{pyrD}\ \overrightarrow{pyrF}\ \overrightarrow{pyrE} \neq$
pyrB	-1,74	aspartate carbamoyltransferase	
pyrAA	-1,67	carbamoyl-phosphate synthetase (glutaminase subunit)	
pyrAB	-1,58	carbamoyl-phosphate synthetase (catalytic subunit)	
pyrD	-1,44	dihydroorotate dehydrogenase (catalytic subunit)	
pyrK	-0,93	dihydroorotate dehydrogenase (electron transfer subunit)	
pyrF	-0,89	orotidine 5'-phosphate decarboxylase	
pyrE	-0,81	orotate phosphoribosyltransferase	
ydhM	-2,34	Oligo-beta-mannoside-specific phosphotransferase enzyme IIB component	$\neq \overleftarrow{ydhL}\ \overrightarrow{ydhM}\ \overrightarrow{ydhN}\ \overrightarrow{ydhO}$
ydhN	-1,25	Oligo-beta-mannoside-specific phosphotransferase enzyme IIA component	
ydhO	-0,51	Oligo-beta-mannoside permease IIC component	
ywkA	-2,08	unknown; similar to malate dehydrogenase	$\overrightarrow{ywkA}\ \overrightarrow{ywkB} \neq \overleftarrow{ywkC} \neq$

a. Numbers indicate the log 2 transformed expression ratio's.
b. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "≠" indicates the termination sites

YvfI was found to significantly induce pyrimidin biosynthesis, ATP synthesis, glucose and lipid metabolisms as well. In accordance with this data, during exponential phase, transcription of butyrate-acetoasetate CoA transferase (*yodR*) and 3-oxoadipade CoA transferase (*yodS*) genes which participate in lipid metabolism was found to be increased in the presence of YvfI (Table 3.13). Therefore it should be noted that lipid metabolism genes were further induced by YvfI during late stages of growth. YvfI was found to repress the intracellular serine protease encoding gene *aprX* (Table 3.6) and the transcription of *aprX* was already known to be regulated by SinR and SinR was speculated as the only known regulator described for *aprX* (Valbuzzi et al., 1999). Hereafter, exponential phase data further supports and strengthens the roots of the suggestion that YvfI might be adressed as the first positive regulator of *aprX*.

Table 3.13 : Transcription units that are up-regulated by YvfI during exponential phase of growth.

Gene	Expression ratio [a]	Function	Transcriptional Organisation [b]
yodS	1,61	Probable coenzyme A transferase subunit alpha	$\neq \overrightarrow{yodT}\ \overrightarrow{yodS}\ \overrightarrow{yodR}\ \overrightarrow{yodQ}\ \overrightarrow{yodP}$ $\overrightarrow{kamA}\ \overrightarrow{yozE} \neq$
yodR	1,3	Probable coenzyme A transferase subunit beta	
yodP	1,74	Uncharacterized N-acetyltransferase	
aprX	1,72	intracellular alkaline serine protease	$\neq \overleftarrow{aprX}\ \overrightarrow{ymaC}\ \overrightarrow{ymaD} \neq$
yfnH	1,35	Probable glucose-1-phosphate cytidylyltransferase	$\neq \overrightarrow{yfnH}\ \overrightarrow{yfnG}\ \overrightarrow{yfnF}\ \overrightarrow{yfnE}\ \overrightarrow{yfnD} \neq$
yfnG	1,71	Putative sugar dehydratase/epimerase	
yfnF	1,57	Uncharacterized protein	
yfnE	1,6	Uncharacterized glycosyltransferase	
yfnD	1,68	Uncharacterized protein	
yoqL	2,47	SPBc2 prophage-derived putative	$\overleftarrow{yoqL}\ \overleftarrow{yoqK}\ \overleftarrow{yoqJ}$
ytsD	2,44	Bacitracin export permease protein	$\neq \overleftarrow{ytsD}\ \overleftarrow{ytsC}\ \overleftarrow{ytsB}\ \overleftarrow{ytsA} \neq$

a. Numbers indicate the log 2 transformed expression ratio's.
b. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "ǂ" indicates the termination sites

3.3.3 Transcriptome data evaluation of *amyE*::P_{spac}::*yvfI* strain

In order to identify the genes that are directly effected by YvfI transcriptional factor, comparative transcriptome analysis of IPTG inducible YvfI producer *B. subtilis* PY79 *amyE*::P_{spac}::*yvfI*, *yvfI::Tn10"* mutant strain between IPTG induced (*yvfI* gene expressed) and noninduced (*yvfI* gene not overexpressed) states was performed. *B. subtilis amyE*::P_{spac}::*yvfI* were grown in LB medium until they reached OD of 0,5 at 600 nm. 1 mM IPTG was used to induce cells and sampling was done at 30th and 60th minutes. Comparative studies were performed in order to define expressional differences between IPTG induced (*yvfI* gene overexpressed) "*B. subtilis* PY79 *amyE*::P_{spac}::*yvfI*, *yvfI::Tn10"* mutant strain and non-induced "*B. subtilis* PY79 *amyE*::P_{spac}::*yvfI*, *yvfI::Tn10"* mutant strain.

Initially, *yvfI* gene expression was examined from the microarray data in order to see if the overexpression was achieved or not. As can be seen in Table 3.14 and 3.15 *yvfI* expression ratio was observed to gradually increase while growth further continued. No striking or significant upregulation was found from 30th minute transcriptional profile analysis. On the other hand *trpC*, *ytmN* and *ykwD* genes were found to be repressed by YvfI protein. In harmony with the identified genes that are regulated by YvfI during stationary phase of growth (Table 3.3), tryptophan biosynthetic gene

trpC was also found to be repressed by YvfI protein which further support the role of YvfI on nitrogen metabolism (Table 3.14). Besides, data from 60 min IPTG induction experimental set pointed out another gene product HisC to be severely repressed by YvfI, when overexpression of YvfI is achieved (Table 3.15). Additionally, *ykwD* gene was suggested to encode a protein with an N-terminal signal peptide that provide the protein with a probable extracellular activity. YkwD was further propsed to function as calcium chelating serine protease (Keijse et al., 2007) while YtmN functions as L-cystine import ATP-binding protein (Auger et al., 2002). Hence, It can be concluded that YvfI is not only involved in nitrogen metabolism but also this protein is involved in transport and catabolism of amino acids.

Table 3.14. Regulated genes that are revealed out by comparative microarray analysis between 30th minute sampling of IPTG induced *B. subtilis* PY79 *amyE*::P_{spac}::*yvfI yvfI::Tn10* strain and 30th minute sampling of non-induced *B. subtilis* PY79 *amyE*::P_{spac}::*yvfI yvfI::Tn10* strain.

Gene	Expression ratio [a]	Function	Transcriptional Organisation [b]
yvfI	1,25	ATP synthase subunit C	ǂ$\overrightarrow{yvfH}$ $\overleftarrow{\boldsymbol{yvfI}}$ ǂ $\overleftarrow{lacR}$ ǂ $\overleftarrow{yvfK}$
trpC	-1,34	Indole-3-glycerol phosphate synthase	$\overleftarrow{trpA}$ $\overleftarrow{trpB}$ $\overleftarrow{trpF}$ $\overleftarrow{\boldsymbol{trpC}}$ $\overleftarrow{trpD}$ $\overleftarrow{trpE}$
ytmN	-1,43	L-cystine import ATP-binding protein	$\overleftarrow{ytnI}$ $\overleftarrow{ytmO}$ $\overleftarrow{\boldsymbol{ytmN}}$ $\overleftarrow{ytmM}$ $\overleftarrow{ytmL}$
ykwD	-1,56	proposed to be a Ca2+-chelating serine protease	ǂ $\overrightarrow{ykwC}$ ǂ $\overleftarrow{\boldsymbol{ykwD}}$ $\overrightarrow{ykuA}$ $\overrightarrow{kinA}$ $\overleftarrow{patA}$

a. Numbers indicate the log 2 transformed expression ratio's.
b. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "ǂ" indicates the termination sites

Findings of 60 min IPTG induction experimental set reveals out that effected gene profile has changed considerably. Different genes are effected and also direction of the effect has changed dependent on YvfI concentration. As mentioned in Section 3.3.1.6, phages, plasmids, and integrative and conjugative elements (ICEs), are the most frequently seen mobile genetic elements (Grohmann et al., 2003; Burrus et al., 2002). A class of ICE elements in *B. subtilis*, 20 kb ICEBs1 (Figure 3.19) contains phrI gene among 24 different ORFs that are transferred to recipient cells. Moreover, population density sensing by ICEBs1, and its conjugal transfer are repressed by the pentapeptide PhrI (antagonist of RapI) and induced by RapI (Auchtung et al., 2005). Expression analysis using the constructed *rapI-lacZ* trancriptional fusion has revealed out that, the major inducer of ICE*Bs1*, is significantly down regulated by

YvfI during transition state such that its expression was completely increased as a result of a mutation on *yvfI* gene (Figure 3.20), suggesting that YvfI represses *rapI* transcription directly. Hence during the exponential phase of growth, as being the antagonist of RapI, expression from *phrI* gene was found to severely repressed by YvfI protein (Table 3.15), pointing out that YvfI regulator protein induces indirectly the conjugal transfer of "ICEBs1" locus during logarithmic phase of growth.

Table 3.15. Regulated genes that are revealed out by comparative microarray analysis between 60th minute sampling of IPTG induced *B. subtilis* PY79 *amyE*::P_{spac}::*yvfI yvfI::Tn10* strain and 60th minute sampling of non-induced *B. subtilis* PY79 *amyE*::P_{spac}::*yvfI yvfI::Tn10* strain.

Gene	Expression ratio [a]	Function	Transcriptional Organisation [b]
yvfI	2,26	HTH-type transcriptional regulator	ǂ$\overrightarrow{yvfH}$ $\overleftarrow{\boldsymbol{yvfI}}$ ǂ $\overleftarrow{lacR}$ ǂ $\overleftarrow{yvfK}$
yqaF	2,56	yqaF-yqaN operon of the skin element Uncharacterized HTH-type transcriptional regulator	ǂ $\overrightarrow{yqaD}$ $\overleftarrow{yqaE}$ $\overrightarrow{\boldsymbol{yqaF}}$ ǂ $\overrightarrow{yqaA}$ $\overrightarrow{yqaG}$
*yomD**	1,75	SPBc2 prophage-derived uncharacterized protein	$\overrightarrow{yomF}$ ǂ $\overrightarrow{yomE}$ $\overrightarrow{\boldsymbol{yomD}}$ $\overrightarrow{bylA}$ $\overrightarrow{bhlA}$
*yoke**	1,71	SPBc2 prophage-derived uncharacterized protein	ǂ $\overleftarrow{yokH}$ $\overleftarrow{yokG}$ $\overrightarrow{yokF}$ $\overrightarrow{\boldsymbol{yokE}}$ ǂ $\overrightarrow{yokD}$
yoqS	1,68	SPBc2 prophage-derived uncharacterized protein yoqS	$\overleftarrow{yoqP}$ $\overleftarrow{yoqR}$ $\overleftarrow{\boldsymbol{yoqS}}$ $\overrightarrow{yoqT}$ $\overrightarrow{yoqU}$
*yorW**	1,49	SPBc2 prophage-derived uncharacterized protein yorW	$\overleftarrow{yorV}$ $\overleftarrow{\boldsymbol{yorW}}$ $\overleftarrow{yorX}$ $\overleftarrow{yorY}$ $\overleftarrow{yorZ}$
*phrI**	-1,46	potassium/proton-divalent cation antiporter	$\overleftarrow{yddK}$ $\overrightarrow{rapI}$ $\overrightarrow{\boldsymbol{phrI}}$ ǂ $\overrightarrow{yddM}$ ǂ
hisH	-2,87	Imidazole glycerol phosphate synthase subunit HisH	ǂ $\overleftarrow{hisI}$ $\overleftarrow{hisF}$ $\overleftarrow{hisA}$ $\overleftarrow{\boldsymbol{hisH}}$ $\overleftarrow{hisB}$
yorV	-1,54	SPBc2 prophage-derived uncharacterized protein yorV	$\overleftarrow{\boldsymbol{yorV}}$ $\overleftarrow{yorW}$ $\overleftarrow{yorX}$ $\overleftarrow{yorY}$ $\overleftarrow{yorZ}$
*yopT**	-3,01	SPBc2 prophage-derived uncharacterized protein	$\overleftarrow{yopV}$ $\overleftarrow{yopU}$ $\overleftarrow{yopT}$ $\overrightarrow{\boldsymbol{yopS}}$ ǂ $\overleftarrow{yopR}$

a. Numbers indicate the log 2 transformed expression ratio's.
b. Transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "ǂ" indicates the termination sites
c. * p value < 0,05

In section 3.3.1.7, it was mentioned that YvfI is directly involved in the SPP1 infection in *B. subtilis* through inducing an operon including *yukE, yukD, yukC*, and *yukBA* transcription units which also contains yueB genes which initiates a series of events resulting in release of phage genome into the *B. subtilis* cytoplasm (Sao-Jose et al., 2004). This membrane bound YueB protein of ~120 kDa shows great homology with other proteins from different species and one of these homologs is the protein Pip, which functions as a phage receptor in *Lactococcus lactis* (Sao-Jose et al., 2004).

Recently, *yueB* was reported to be a part of an operon including *yukE, yukD, yukC*, and *yukBA* transcription units (Sao-Jose et al., 2004). Another important observation in this study was that *yukE, yukC* and *yukAB* genes were significantly stimulated by YvfI (Table 3.8). Our overal data implied that YvfI is directly involved in the SPP1 infection in *B. subtilis*. Furthermore, *Bacillus subtilis* contains a number of prophage-like elements including PBSX, skin, and SP-β (Kunst et al., 1997). Several DNA repetitions are found within these regions that are called as prophages and these prophages are suggested to diverge from a common ancestor (Meadigue et al., 1995; Krogh et al., 1996). Our studies have revealed out that, along with SPP1 infection, YvfI can be speculated strongly to involve in SPβc2 prophage infections, considering repressed profiles observed from *yorV* and *yopT* genes and induced profiles of *yomD, yokE, yoqS, yorW* genes (Table 3.15). Moreover, one of the prophage-like elements, skin element, was found to be encoded through *yqaF-yqaN* operon (Kimura et al., 2010) and this present study further implies the involvement of YvfI on expression of prophage-like elements under the light of the fact that *yqaF* gene is among the severely induced genes by YvfI protein (Table 3.15).

4. CONCLUSION

The main purpose of this research was to achieve insight about the regulatory roles of YvfI protein and revealing the promoter region, putative regulatory protein binding sites and 5'-end of *yvfI* cDNA. Therefore a genome-wide comparative transcriptome analyses of wild type PY79 strain that produces YvfI transcriptional factor and *yvfI* disrupted TEK1 mutant, *yvfI::T10::spc* during exponential (OD_{600} 0.7) and stationary growth phase (~ OD_{600}7) was performed. Moreover, comparative studies were performed in order to define expressional differences between IPTG induced (*yvfI* gene overexpressed) "*B. subtilis* PY79 *amyE*::P_{spac}::*yvfI*, *yvfI::Tn10"* mutant strain and non-induced (*yvfI* gene not overexpressed) "*B. subtilis* PY79 *amyE*::P_{spac}::*yvfI*, *yvfI::Tn10"* mutant strain. Data obtained from microarray analysis were further validated by RT-qPCR and *lacZ* fusion analysis where necessary.

Comparative transcriptome analysis data indicated that YvfI could be involved in the regulation of a wide variety of cellular processes. Processable data from microarray analysis was further studied and genes showing at least 1,44 fold difference with reproducible characteristics, which means displaying an acceptable Bayes P value ($p < 0,01$) were subjected to RT-qPCR analysis. First or several genes of operons that were identified to be affected were chosen to be validated by RT-qPCR. Finally, genes that are formalized by applying these two methodologies were further accepted as YvfI affected genes and these genes were grouped according to their functional properties. As a result, degradative enzyme production, antibiotic production and resistance, carbohydrate utilization and transport, nitrogen metabolism, membrane transport, fatty acid and lipid metabolism, protein synthesis and translocation, cell-wall synthesis, energy production and membrane bioenergetics, mobile genetic element, phage infection, sporulation, delay of sporulation and cannibalism, biofilm formation were found to be subjected to regulation, either in a negative or in a positive manner, by YvfI protein during stationary phase.

In a harmony with YvfI regulation during stationary phase, YvfI was also found to inhibit carbohydrate metabolism and pyrimidin biosynthesis, induce resistance against bacitracin and participate in regulation of genes involved in lipid metabolism during exponential phase.

Comparative studies performed in order to define expressional differences between IPTG induced (*yvfI* gene overexpressed) mutant strain and non-induced (*yvfI* gene not overexpressed) mutant strain have revealed out that YvfI is not only involved in nitrogen metabolism but also this protein is involved in transport and catabolism of amino acids and when over expression is achieved, YvfI regulator protein probably induces indirectly the conjugal transfer of "ICEBs1" locus while it is involved in the expression of prophage-like elements.

In conclusion, YvfI regulatory protein was found to regulate complex interconnected regulatory circuits as a novel global regulator. This finding is in harmony with the fact that YvfI belongs to GntR family regulatory proteins and regulators from this family are consisted of a conserved N-terminal DNA binding domain (D-b domain) followed by C-terminal domain involved in the effector binding and/or oligomerization (E-b/O domain) and additionally they contain a winged helix-turn-helix motif which acts as the DNA recognition domain. Most importantly, these proteins exert their regulatory activiy by applying repressions and inductions on many different types of regulons. GntR family of transcriptional regulators consists of about 2000 different proteins, one of which is considered to be YvfI. Therefore, YvfI could be a critical regulator protein that responds to different stimuli, recieved as a result of changing environmental conditions. As a further study, the effects of this protein on mentioned pathways should be investigated in more details in order to understand if the effects can be followed phenotypically or not.

In this research, *yvfI* transcription site was also determined by 5'-RACE-PCR analysis and found that its transcription was controled from a σ^A-type promoter. This promoter region described as P*yvfI* was identified to lie at position -136 to -115 with respect to *yvfI* translational start codon with a well confirmed consensus -10 (TATAAT) and consensus -35 (TTGGCG) sequences separated with 15 bp long gap. Furthermore, at +12 downstream position, a sequence that shows a perfect match to Spo0A binding motif (5'-TGNCGAA), three putative AbrB binding and one CodY

binding sites were identified on the sequence. Hence, these data have shed more light to the regulation pattern of *yvfI* gene indicating that this gene is most probably directly regulated by global regulatory genes:Spo0A, AbrB and CodY in *B. subtilis.*

REFERENCES

Abe, S., Yasumura, A., and Tanaka, T. (2009). Regulation of *Bacillus subtilis* aprE Expression by glnA through Inhibition of scoC and sigma(D)-Dependent degR Expression, *Journal of Bacteriology **191***, 3050-3058.

Aguilar, C., Vlamakis, H., Losick, R., and Kolter, R. (2007). Thinking about *Bacillus subtilis* as a multicellular organism, *Current Opinion in Microbiology **10***, 638-643.

Albano, M., Smits, W. K., Ho, L. T. Y., Kraigher, B., Mandic-Mulec, I., Kuipers, O. P., and Dubnau, D. (2005). The Rok protein of *Bacillus subtilis* represses genes for cell surface and extracellular functions, *Journal of Bacteriology **187***, 2010-2019.

Ali, N. O., Bignon, J., Rapoport, G., and Debarbouille, M. (2001). Regulation of the acetoin catabolic pathway is controlled by sigma L in *Bacillus subtilis*, *Journal of Bacteriology **183***, 2497-2504.

Allison, S. L., and Phillips, A. T. (1990). Nucleotide-sequence of the gene encoding the repressor for the histidine utilization genes of *Pseudomonas putida*, *Journal of Bacteriology **172***, 5470-5476.

Aravind, L., and Anantharaman, V. (2003). HutC/FarR-like bacterial transcription factors of the GntR family contain a small molecule-binding domain of the chorismate lyase fold, *Fems Microbiology Letters **222***, 17-23.

Auchtung, J. M., Lee, C. A., Monson, R. E., Lehman, A. P., and Grossman, A. D. (2005). Regulation of a *Bacillus subtilis* mobile genetic element by intercellular signaling and the global DNA damage response, *Proceedings of the National Academy of Sciences of the United States of America **102***, 12554-12559.

Auger, S., Danchin, A., and Martin-Verstraete, I. (2002). Global expression profile of *Bacillus subtilis* grown in the presence of sulfate or methionine, *Journal of Bacteriology **184***, 5179-5186.

Baldi, P., and Long, A. D. (2001). A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes, *Bioinformatics **17***, 509-519.

Basalp, A., Ozcengiz, 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, *Current Microbiology **24***, 129-135.

Belitsky, B. R. (2004). *Bacillus subtilis* GabR, a protein with DNA-binding and aminotransferase domains, is a PLP-dependent transcriptional regulator, *Journal of Molecular Biology **340***, 655-664.

Belitsky, B. R., and Sonenshein, A. L. (2002). GabR, a member of a novel protein family, regulates the utilization of gamma-aminobutyrate in *Bacillus subtilis*, *Molecular Microbiology **45***, 569-583.

Bernard, R., Guiseppi, A., Chippaux, M., Foglino, M., and Denizot, F. (2007). Resistance to bacitracin in *Bacillus subtilis*: Unexpected requirement of the BceAB ABC transporter in the control of expression of its own structural genes, *Journal of Bacteriology **189***, 8636-8642.

Black, P. N., and Dirusso, C. C. (1994). Molecular and biochemical analyses of fatty-acid transport, metabolism, and gene regulation in *Escherichia coli*, *Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism **1210***, 123-145.

Bohannon, D. E., and Sonenshein, A. L. (1989). Positive regulation of glutamate biosynthesis in *Bacillus subtilis*, *Journal of Bacteriology **171***, 4718-4727.

Bower, S., Perkins, J. B., Yocum, R. R., Howitt, C. L., Rahaim, P., and Pero, J. (1996). Cloning, sequencing, and characterization of the *Bacillus subtilis* biotin biosynthetic operon, *Journal of Bacteriology **178***, 4122-4130.

Branda, S. S., Chu, F., Kearns, D. B., Losick, R., and Kolter, R. (2006). A major protein component of the *Bacillus subtilis* biofilm matrix, *Molecular Microbiology **59***, 1229-1238.

Branda, S. S., Gonzalez-Pastor, J. E., Ben-Yehuda, S., Losick, R., and Kolter, R. (2001). Fruiting body formation by *Bacillus subtilis*, *Proceedings of the National Academy of Sciences of the United States of America **98***, 11621-11626.

Branda, S. S., Gonzalez-Pastor, J. E., Dervyn, E., Ehrlich, S. D., Losick, R., and Kolter, R. (2004). Genes involved in formation of structured multicellular communities by *Bacillus subtilis*, *Journal of Bacteriology **186***, 3970-3979.

Branda, S. S., Vik, A., Friedman, L., and Kolter, R. (2005). Biofilms: the matrix revisited, *Trends in Microbiology **13***, 20-26.

Brennan, R. G., and Matthews, B. W. (1989). the helix-turn-helix dna-binding motif, *Journal of Biological Chemistry **264***, 1903-1906.

Burrus, V., Pavlovic, G., Decaris, B., and Guedon, G. (2002). Conjugative transposons: the tip of the iceberg, *Molecular Microbiology **46***, 601-610.

Burrus, V., and Waldor, M. K. (2004). Shaping bacterial genomes with integrative and conjugative elements, *Research in Microbiology **155***, 376-386.

Bustin, S. A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, *Journal of Molecular Endocrinology **29***.

Butcher, B. G., and Helmann, J. D. (2006). Identification of *Bacillus subtilis* sigma(W)-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli, *Molecular Microbiology **60***, 765-782.

Cao, M., T. Wang, R. Ye, and J. D. Helmann. (2002). Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σW and σM regulons., *Mol. Microbiol **45***, 1267-1276.

Cao, M., Bernat, B. A., Wang, Z. P., Armstrong, R. N., and Helmann, J. D. (2001). FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma(W), an extracytoplasmic-function sigma factor in *Bacillus subtilis*, *Journal of Bacteriology **183***, 2380-2383.

Carrillo, C., Teruel, J. A., Aranda, F. J., and Ortiz, A. (2003). Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin, *Biochimica Et Biophysica Acta-Biomembranes **1611***, 91-97.

Chai, Y., Kolter, R., and Losick, R. (2009). A Widely Conserved Gene Cluster Required for Lactate Utilization in *Bacillus subtilis* and Its Involvement in Biofilm Formation, *Journal of Bacteriology **191***, 2423-2430.

Chen, F. C., Shen, L. F., Tsai, M. C., and Chak, K. F. (2003). The IspA protease's involvement in the regulation of the sporulation process of Bacillus thuringiensis is revealed by proteomic analysis, *Biochemical and Biophysical Research Communications **312***, 708-715.

Chu, F., Kearns, D. B., Branda, S. S., Kolter, R., and Losick, R. (2006). Targets of the master regulator of biofilm formation in *Bacillus subtilis*, *Molecular Microbiology **59***, 1216-1228.

Chu, F., Kearns, D. B., McLoon, A., Chai, Y., Kolter, R., and Losick, R. (2008). A novel regulatory protein governing biofilm formation in *Bacillus subtilis*, *Molecular Microbiology **68***, 1117-1127.

Chuaqui, R. F., Bonner, R. F., Best, C. J. M., Gillespie, J. W., Flaig, M. J., Hewitt, S. M., Phillips, J. L., Krizman, D. B., Tangrea, M. A., Ahram, M., Linehan, W. M., Knezevic, V., and Emmert-Buck, M. R. (2002). Post-analysis follow-up and validation of microarray experiments, *Nature Genetics **32***, 509-514.

Chumsakul, O., Takahashi, H., Oshima, T., Hishimoto, T., Kanaya, S., Ogasawara, N., and Ishikawa, S. (2011). Genome-wide binding profiles of the *Bacillus subtilis* transition state regulator AbrB and its homolog Abh reveals their interactive role in transcriptional regulation, *Nucleic Acids Research **39***, 414-428.

Craig, J. E., Ford, M. J., Blaydon, D. C., and Sonenshein, A. L. (1997). A null mutation in the *Bacillus subtilis* aconitase gene causes a block in Spo0A-phosphate-dependent gene expression, *Journal of Bacteriology **179***, 7351-7359.

Cruzramos, H., Boursier, L., Moszer, I., Kunst, F., Danchin, A., and Glaser, P. (1995). Anaerobic transcription activation in *Bacillus subtilis* - Identification of distinct *fnr*-dependent and *fnr*-independent regulatory mechanisms, *Embo Journal **14***, 5984-5994.

Cutting, S. M., and Vander Horn, P. B. (1990) Genetic analysis, In *Molecular biological methods for Bacillus.* (Harwood, C. R., and Cutting, S. M., Eds.), pp 28–74, John Wiley & Sons, New York.

Davis, M. S., and Cronan, J. E. (2001). Inhibition of Escherichia coli acetyl coenzyme A carboxylase by acyl-acyl carrier protein, *Journal of Bacteriology **183***, 1499-1503.

DiRusso, C. C., Black, P. N., and Weimar, J. D. (1999). Molecular inroads into the regulation and metabolism of fatty acids, lessons from bacteria, *Progress in Lipid Research **38***, 129-197.

Dirusso, C. C., Heimert, T. L., and Metzger, A. K. (1992). Characterization of Fadr, a global transcriptional regulator of fatty-acid metabolism in *Escherichia coli* interaction with the *fadb* promoter is prevented by long-chain fatty acyl coenzyme-as, *Journal of Biological Chemistry **267***, 8685-8691.

Doan, T., Servant, P., Tojo, S., Yamaguchi, H., Lerondel, G., Yoshida, K., Fujita, Y., and Aymerich, S. (2003). The *Bacillus subtilis* ywkA gene encodes a malic enzyme and its transcription is activated by the YufL/YufM two-component system in response to malate, *Microbiology-Sgm **149***, 2331-2343.

Driessen, A. J. M., Manting, E. H., and van der Does, C. (2001). The structural basis of protein targeting and translocation in bacteria, *Nature Structural Biology **8***, 492-498.

Earl, A. M., Losick, R., and Kolter, R. (2008). Ecology and genomics of *Bacillus subtilis*, *Trends in Microbiology **16***, 269-275.

Eggert, T., Pencreac'h, G., Douchet, I., Verger, R., and Jaeger, K. E. (2000). A novel extracellular esterase from *Bacillus subtilis* and its conversion to a monoacylglycerol hydrolase, *European Journal of Biochemistry **267***, 6459-6469.

Eichler, J., and Moll, R. (2001). The signal recognition particle of Archaea, *Trends in Microbiology **9***, 130-136.

Errington, J. (2003). Regulation of endospore formation in *Bacillus subtilis*, *Nature Reviews Microbiology **1***, 117-126.

Fawcett, P., Eichenberger, P., Losick, R., and Youngman, P. (2000). The transcriptional profile of early to middle sporulation in *Bacillus subtilis*, *Proceedings of the National Academy of Sciences of the United States of America **97***, 8063-8068.

Fisher, S. H. (1999). Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference!, *Molecular Microbiology **32***, 223-232.

Fisher, S. H., and Sonenshein, A. L. (1991). Control of carbon and nitrogen-metabolism in *Bacillus subtilis*, *Annual Review of Microbiology **45***, 107-135.

Freeman, W. M., Walker, S. J., and Vrana, K. E. (1999). Quantitative RT-PCR: Pitfalls and potential, *Biotechniques **26***, 112-+.

Frost, L. S., Leplae, R., Summers, A. O., and Toussaint, A. (2005). Mobile genetic elements: The agents of open source evolution, *Nature Reviews Microbiology **3***, 722-732.

Fujita, Y., Fujita, T., Miwa, Y., Nihashi, J., and Aratani, Y. (1986). Organization and transcription of the gluconate operon, gnt, of *Bacillus subtilis*, *Journal of Biological Chemistry* ***261***, 3744-3753.

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.

Gallegos, M. T., Michan, C., and Ramos, J. L. (1993). The Xyls/Arac family of regulators, *Nucleic Acids Research* ***21***, 807-810.

Gallegos, M. T., Schleif, R., Bairoch, A., Hofmann, K., and Ramos, J. L. (1997). AraC/XylS family of transcriptional regulators, *Microbiology and Molecular Biology Reviews* ***61***, 393-&.

Garti-Levi, S., Hazan, R., Kain, J., Fujita, M., and Ben-Yehuda, S. (2008). The FtsEX ABC transporter directs cellular differentiation in *Bacillus subtilis*, *Molecular Microbiology* ***69***, 1018-1028.

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* ***177***, 1112-1115.

Gonzalez-Pastor, J. E., Hobbs, E. C., and Losick, R. (2003). Cannibalism by sporulating bacteria, *Science* ***301***, 510-513.

Gotz, F. (2002). Staphylococcus and biofilms, *Molecular Microbiology* ***43***, 1367-1378.

Grohmann, E., Muth, G., and Espinosa, M. (2003). Conjugative plasmid transfer in gram-positive bacteria, *Microbiology and Molecular Biology Reviews* ***67***, 277-+.

Grossman, A. D. (1995). Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*, *Annual Review of Genetics* ***29***, 477-508.

Haenssler, E., Mueller, T., Jessberger, N., Voelzke, A., Plassmeier, J., Kalinowski, J., Kraemer, R., and Burkovski, A. (2007). FarR, a putative regulator of amino acid metabolism in Corynebacterium glutamicum, *Applied Microbiology and Biotechnology* ***76***, 625-632.

Hagege, J., Pernodet, J. L., Sezonov, G., Gerbaud, C., Friedmann, A., and Guerineau, M. (1993). Transfer-functions of the conjugative integrating element psam2 from *Streptomyces ambofaciens* - characterization of a kil-kor system associated with transfer, *Journal of Bacteriology* ***175***, 5529-5538.

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

Hamon, M. A., and Lazazzera, B. A. (2001). The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*, *Molecular Microbiology* ***42***, 1199-1209.

Harrison, S. C. (1991). A structural taxonomy of dna-binding domains, *Nature **353***, 715-719.

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

Harwood, C. R., and Wipat, A. (1996). Sequencing and functional analysis of the genome of *Bacillus subtilis* strain 168, *Febs Letters **389***, 84-87.

Haydon, D. J., and Guest, J. R. (1991). A New family of bacterial regulatory proteins, *Fems Microbiology Letters **79***, 291-296.

Hederstedt, L. (1993) The Krebs citric acid cycle, In *Bacillus subtilis and other gram-positive bacteria: biochemistry, physiology, and molecular genetics.* (Sonenshein, A. L., Hoch, J. A., and Losick, R., Eds.), pp 181-197, American Society for Microbiology, Washington, D.C.

Helmann, J. D. (2002). The extracytoplasmic function (ECF) sigma factors, *Advances in Microbial Physiology, Vol 46 **46***, 47-110.

Henikoff, S., Haughn, G. W., Calvo, J. M., and Wallace, J. C. (1988). A large family of bacterial activator proteins, *Proceedings of the National Academy of Sciences of the United States of America **85***, 6602-6606.

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

Hirose, I., Sano, K., Shioda, I., Kumano, M., Nakamura, K., and Yamane, K. (2000). Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study, *Microbiology-Uk **146***, 65-75.

Hoch, J. A., and Losick, R. (1997). Genome sequencing - Panspermia, spores and the *Bacillus subtilis* genome, *Nature **390***, 237-238.

Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z. Y., Jones, R. A., and Miller, S. I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation, *Nature **436***, 1171-1175.

Honda, K., Nakamura, K., Nishiguchi, M., and Yamane, K. (1993). Cloning and characterization of a *Bacillus subtilis* gene encoding a homolog of the 54-kilodalton subunit of mammalian signal recognition particle and escherichia-coli ffh, *Journal of Bacteriology **175***, 4885-4894.

Hong, H. A., Duc, L. H., and Cutting, S. M. (2005). The use of bacterial spore formers as probiotics, *Fems Microbiology Reviews **29***, 813-835.

Huang, X. J., Gaballa, A., Cao, M., and Helmann, J. D. (1999). Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function sigma factor, sigma(W), *Molecular Microbiology **31***, 361-371.

Huang, X. J., and Helmann, J. D. (1998). Identification of target promoters for the *Bacillus subtilis* sigma(X) factor using a consensus-directed search, *Journal of Molecular Biology **279***, 165-173.

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*, *Journal of Biological Chemistry* ***278***, 2169-2176.

Jaacks, K. J., Healy, J., Losick, R., and Grossman, A. D. (1989). Identification and characterization of genes controlled by the sporulation-regulatory gene spooh in *Bacillus subtilis*, *Journal of Bacteriology* ***171***, 4121-4129.

Jin, S. F., and Sonenshein, A. L. (1994). Identification of 2 distinct *Bacillus subtilis* citrate synthase genes, *Journal of Bacteriology* ***176***, 4669-4679.

Karmirantzou, M., and Hamodrakas, S. J. (2001). A Web-based classification system of DNA-binding protein families, *Protein Engineering* ***14***, 465-472.

Kataoka, M., Tanaka, T., Kohno, T., and Kajiyama, Y. (2008). The Carboxyl-Terminal Domain of TraR, a Streptomyces HutC Family Repressor, Functions in Oligomerization, *Journal of Bacteriology* ***190***, 7164-7169.

Katz, E., and Demain, A. L. (1977). Peptide antibiotics of Bacillus - chemistry, biogenesis, and possible functions, *Bacteriological Reviews* ***41***, 449-474.

Kearns, D. B., Chu, F., Branda, S. S., Kolter, R., and Losick, R. (2005). A master regulator for biofilm formation by *Bacillus subtilis*, *Molecular Microbiology* ***55***, 739-749.

Keijser, B. J. F., Ter Beek, A., Rauwerda, H., Schuren, F., Montijn, R., van der Spek, H., and Brul, S. (2007). Analysis of temporal gene expression during *Bacillus subtilis* spore germination and outgrowth, *Journal of Bacteriology* ***189***, 3624-3634.

Kendall, K. J., and Cohen, S. N. (1988). Complete nucleotide-sequence of the streptomyces-lividans plasmid pij101 and correlation of the sequence with genetic properties, *Journal of Bacteriology* ***170***, 4634-4651.

Kenig, M., Vandamme, E., and 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.

Kim, H. J., Kim, S. I., Ratnayake-Lecamwasam, M., Tachikawa, K., Sonenshein, A. L., and Strauch, M. (2003). Complex regulation of the *Bacillus subtilis* aconitase gene, *Journal of Bacteriology* ***185***, 1672-1680.

Kimura, T., Amaya, Y., Kobayashi, K., Ogasawara, N., and Sato, T. (2010). Repression of sigK Intervening (skin) Element Gene Expression by the CI-Like Protein SknR and Effect of SknR Depletion on Growth of *Bacillus subtilis* Cells, *Journal of Bacteriology* ***192***, 6209-6216.

Kingston, A. W., Subramanian, C., Rock, C. O., and Helmann, J. D. (2011). A sigma(W)-dependent stress response in *Bacillus subtilis* that reduces membrane fluidity, *Molecular Microbiology* ***81***, 69-79.

Klein, C., Kaletta, C., Schnell, N., and Entian, K. D. (1992). Analysis of genes involved in biosynthesis of the lantibiotic subtilin, *Applied and Environmental Microbiology **58***, 132-142.

Ko, T. P., Wu, S. P., Yang, W. Z., Tsai, H., and Yuan, H. S. (1999). Crystallization and preliminary crystallographic analysis of the Escherichia coli tyrosine aminotransferase, *Acta Crystallographica Section D-Biological Crystallography **55***, 1474-1477.

Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S. C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L. C., Danchin, A., Debarbouille, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S. K., Dreesen, O., Errington, J., Fillinger, S., Foster, S. J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C. R., Hecker, M., Hosoya, D., Hullo, M. F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Masson, A., Mauel, C., Meima, R., Mellado, R. P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Z., Pooley, H. M., Rapoport, G., Rawlins, J. P., Rivas, L. A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H. H., Scanlan, E., Schumann, W., Seegers, J., Sekiguchi, J., Sekowska, A., Seror, S. J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H. B., Vagner, V., van Dijl, J. M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U., and Ogasawara, N. (2003). Essential *Bacillus subtilis* genes, *Proceedings of the National Academy of Sciences of the United States of America **100***, 4678-4683.

Kobayashi, K., and Ogasawara, N. (2002) Genome biology of a model bacterium, *Bacillus subtilis*, In *Genome Science: Towards a New Paradigm?* (Yoshikawa, H. O. N. S. N., Ed.), pp 15-25.

Koroglu, T. E., Kurt-Gur, G., Unlu, E. C., and Yazgan-Karatas, A. (2008). The novel gene *yvfI* in *Bacillus subtilis* is essential for bacilysin biosynthesis, *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology **94***, 471-479.

Koroglu, T. E., Ogulur, I., Mutlu, S., Yazgan-Karatas, A., and Ozcengiz, G. (2011). Global Regulatory Systems Operating in Bacilysin Biosynthesis in *Bacillus subtilis*, *Journal of Molecular Microbiology and Biotechnology **20***, 144-155.

Kovacs, A. T., and Kuipers, O. P. (2011). Rok Regulates yuaB Expression during Architecturally Complex Colony Development of *Bacillus subtilis* 168, *Journal of Bacteriology **193***, 998-1002.

Krogh, S., Oreilly, M., Nolan, N., and Devine, K. M. (1996). The phage-like element PBSX and part of the skin element, which are resident at different locations on the *Bacillus subtilis* chromosome, are highly homologous, *Microbiology-Uk* ***142***, 2031-2040.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, 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., Dusterhoft, 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, N., Ghim, S. Y., Glaser, P., Goffeau, A., Golightly, E. J., Grandi, G., Guiseppi, G., Guy, B. J., Haga, K., Haiech, J., Harwood, C. R., Henaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M. F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., KlaerrBlanchard, M., Klein, C., Kobayashi, Y., Koetter, P., 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., Medigue, C., Medina, N., Mellado, R. P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., Oreilly, 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.

Latasa, C., Roux, A., Toledo-Arana, A., Ghigo, J. M., Gamazo, C., Penades, J. R., and Lasa, I. (2005). BapA, a large secreted protein required for biofilm formation and host colonization of Salmonella enterica serovar Enteritidis, *Molecular Microbiology* ***58***, 1322-1339.

Lee, H. Y., An, J. H., and Kim, Y. S. (2000). Identification and characterization of a novel transcriptional regulator, MatR, for malonate metabolism in Rhizobium leguminosarum bv. trifolii, *European Journal of Biochemistry* ***267***, 7224-7229.

Lemon, K. P., and Grossman, A. D. (1998). Localization of bacterial DNA polymerase: Evidence for a factory model of replication, *Science* ***282***, 1516-1519.

Levin, P. A., and Grossman, A. D. (1998). Cell cycle and sporulation in *Bacillus subtilis*, *Current Opinion in Microbiology **1***, 630-635.

Li, J., Yang, Q., Zhao, L.-h., Zhang, S.-m., Wang, Y.-x., and Zhao, X.-y. (2009). Purification and characterization of a novel antifungal protein from *Bacillus subtilis* strain B29, *Journal of Zhejiang University-Science B **10***, 264-272.

Loeffler, W., Tschen, J. S. M., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T. F., and Wu, T. G. (1986). Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* f-29-3 a comparison with activities of other bacillus antibiotics, *Journal of Phytopathology-Phytopathologische Zeitschrift **115***, 204-213.

Lopez, D., Vlamakis, H., Losick, R., and Kolter, R. (2009). Cannibalism enhances biofilm development in *Bacillus subtilis*, *Molecular Microbiology **74***, 609-618.

Lopez, J. M., and Thoms, B. (1976). Relations between catabolite repression and sporulation in *Bacillus subtilis*, *Archives of Microbiology **109***, 181-186.

Losick, R., and Stragier, P. (1992). Crisscross regulation of cell-type-specific gene-expression during development in *Bacillus subtilis*, *Nature **355***, 601-604.

Lu, Y., and Switzer, R. L. (1996). Transcriptional attenuation of the *Bacillus subtilis* pyr Operon by the PyrR regulatory protein and uridine nucleotides in vitro, *Journal of Bacteriology **178***, 7206-7211.

Luo, J., Ran, W., Hu, J., Yang, X., Xu, Y., and Shen, Q. (2010). Application of Bio-Organic Fertilizer Significantly Affected Fungal Diversity of Soils, *Soil Science Society of America Journal **74***, 2039-2048.

Mahlstedt, S. A., and Walsh, C. T. (2010). Investigation of Anticapsin Biosynthesis Reveals a Four-Enzyme Pathway to Tetrahydrotyrosine in *Bacillus subtilis*, *Biochemistry **49***, 912-923.

Majumdar, S. K., and Bose, S. K. (1960). Amino acid sequence of mycobacillin, *Biochem. J. **74***, 596-599.

Makita, Y., Nakao, M., Ogasawara, N., and Nakai, K. (2004). DBTBS: database of transcriptional regulation in *Bacillus subtilis* and its contribution to comparative genomics, *Nucleic Acids Research **32***, D75-D77.

Marahiel, M. A. (1997). Protein templates for the biosynthesis of peptide antibiotics, *Chemistry & Biology **4***, 561-567.

Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., DeWweese-Scott, C., Geer, L. Y., Gwadz, M., He, S. Q., Hurwitz, D. I., Jackson, J. D., Ke, Z. X., Lanczycki, C. J., Liebert, C. A., Liu, C. L., Lu, F., Marchler, G. H., Mullokandov, M., Shoemaker, B. A., Simonyan, V., Song, J. S., Thiessen, P. A., Yamashita, R. A., Yin, J. J., Zhang, D. C., and Bryant, S. H. (2005). CDD: a conserved domain database for protein classification, *Nucleic Acids Research **33***, D192-D196.

Mascher, T., Zimmer, S. L., Smith, T. A., and Helmann, J. D. (2004). Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*, *Antimicrobial Agents and Chemotherapy* ***48***, 2888-2896.

Medigue, C., Moszer, I., Viari, A., and Danchin, A. (1995). Analysis of a B*acillus subtilis* genome fragment using a cooperative computer-system prototype (reprinted from gene-combis, vol 165, pg gc37-gc51, 1995), *Gene* ***165***, GC37-GC51.

Mercedes Palomino, M., Sanchez-Rivas, C., and Ruzal, S. M. (2009). High salt stress in *Bacillus subtilis*: involvement of PBP4*as a peptidoglycan hydrolase, *Research in Microbiology* ***160***, 117-124.

Morey, J. S., Ryan, J. C., and Van Dolah, F. M. (2006). Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR, *Biological Procedures Online*, 175-193.

Moszer, I. (1998). The complete genome of *Bacillus subtilis*: from sequence annotation to data management and analysis, *Febs Letters* ***430***, 28-36.

Msadek, T., Kunst, F., Klier, A., and Rapoport, G. (1991). DegS-DegU and ComP-comA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*, *Journal of Bacteriology* ***173***, 2366-2377.

Nakamura, K., Imai, Y., Nakamura, A., and Yamane, K. (1992). Small cytoplasmic rna of *Bacillus subtilis* - functional-relationship with human signal recognition particle 7s rna and *Escherichia coli* 4.5s rna, *Journal of Bacteriology* ***174***, 2185-2192.

Nakamura, K., Nishiguchi, M., Honda, K., and Yamane, K. (1994). The bacillus-subtilis srp54 homolog, ffh, has an intrinsic gtpase activity and forms a ribonucleoprotein complex with small cytoplasmic rna in-vivo, *Biochemical and Biophysical Research Communications* ***199***, 1394-1399.

Nakamura, K., Yahagi, S., Yamazaki, T., and Yamane, K. (1999). *Bacillus subtilis* histone-like protein, HBsu, is an integral component of a SRP-like particle that can bind the Alu domain of small cytoplasmic RNA, *Journal of Biological Chemistry* ***274***, 13569-13576.

Nasser, W., Reverchon, S., Condemine, G., and Robertbaudouy, J. (1994). Specific interactions of *Erwinia chrysanthemi* kdgr repressor with different operators of genes involved in pectinolysis, *Journal of Molecular Biology* ***236***, 427-440.

Nguyen, C. C., and Saier, M. H. (1995). Phylogenetic, structural and functional analyses of the LacI-GalR family of bacterial transcription factors, *Febs Letters* ***377***, 98-102.

Ogura, M., Matsuzawa, A., Yoshikawa, H., and Tanaka, T. (2004). *Bacillus subtilis* SalA (YbaL) negatively regulates expression of scoC, which encodes the repressor for the alkaline Exoprotease gene, aprE, *Journal of Bacteriology* ***186***, 3056-3064.

Ogura, M., Shimane, K., Asai, K., Ogasawara, N., and Tanaka, T. (2003). Binding of response regulator DegU to the aprE promoter is inhibited by RapG, which is counteracted by extracellular PhrG in *Bacillus subtilis*, *Molecular Microbiology **49***, 1685-1697.

Ortuno-Olea, L., and Duran-Vargas, S. (2000). The L-asparagine operon of Rhizobium etli contains a gene encoding an atypical asparaginase, *Fems Microbiology Letters **189***, 177-182.

O'Toole, G., Kaplan, H. B., and Kolter, R. (2000). Biofilm formation as microbial development, *Annual Review of Microbiology **54***, 49-79.

Ozcengiz, G., and Alaeddinoglu, N. G. (1991). Bacilysin production by *Bacillus subtilis* - effects of bacilysin, ph and temperature, *Folia Microbiologica **36***, 522-526.

Ozcengiz, G., and Alaeddinoglu, N. G. (1991). Bacilysin production and sporulation in *Bacillus subtilis*, *Current Microbiology **23***, 61-64.

Ozcengiz, G., Alaeddinoglu, N. G., and Demain, A. L. (1990). Regulation of biosynthesis of bacilysin by *Bacillus subtilis*, *Journal of Industrial Microbiology **6***, 91-100.

Pabo, C. O., and Sauer, R. T. (1992). Transcription factors - structural families and principles of dna recognition, *Annual Review of Biochemistry **61***, 1053-1095.

Perry, D., and Abraham, E. P. (1979). Transport and metabolism of bacilysin and other peptides by suspensions of *Staphylococcus aureus*, *Journal of General Microbiology **115***, 213-221.

Peypoux, F., Besson, F., Michel, G., Lenzen, C., Dierickx, L., and Delcambe, L. (1980). Characterization of a new antibiotic of iturin group - bacillomycin-d, *Journal of Antibiotics **33***, 1146-1149.

Peypoux, F., Bonmatin, J. M., and Wallach, J. (1999). Recent trends in the biochemistry of surfactin, *Applied Microbiology and Biotechnology **51***, 553-563.

Peypoux, F., Pommier, M. T., Marion, D., Ptak, M., Das, B. C., and Michel, G. (1986). Revised structure of mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*, *Journal of Antibiotics **39***, 636-641.

Pfaffl, M. W. (2004) Quantification strategies in real-time PCR., In *A-Z of quantitative PCR* (Bustin, S. A., Ed.), pp 87-112.

Phillips, Z. E. V., and Strauch, M. A. (2002). *Bacillus subtilis* sporulation and stationary phase gene expression, *Cellular and Molecular Life Sciences **59***, 392-402.

Piggot, P. J., and Hilbert, D. W. (2004). Sporulation of *Bacillus subtilis*, *Current Opinion in Microbiology **7***, 579-586.

Plisson, C., White, H. E., Auzat, I., Zafarani, A., Sao-Jose, C., Lhuillier, S., Tavares, P., and Orlova, E. V. (2007). Structure of bacteriophage SPP1 tail reveals trigger for DNA ejection, *Embo Journal **26***, 3720-3728.

Quail, M. A., Dempsey, C. E., and Guest, J. R. (1994). Identification of a fatty acyl responsive regulator (FarR) in *Escherichia coli*, *Febs Letters* ***356***, 183-187.

Raman, N., Black, P. N., and DiRusso, C. C. (1997). Characterization of the fatty acid-responsive transcription factor FadR - Biochemical and genetic analyses of the native conformation and functional domains, *Journal of Biological Chemistry* ***272***, 30645-30650.

Ramos, J. L., Martinez-Bueno, M., Molina-Henares, A. J., Teran, W., Watanabe, K., Zhang, X. D., Gallegos, M. T., Brennan, R., and Tobes, R. (2005). The TetR family of transcriptional repressors, *Microbiology and Molecular Biology Reviews* ***69***, 326-+.

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 & Development* ***15***, 1093-1103.

Reizer, A., Deutscher, J., Saier, M. H., and Reizer, J. (1991). Analysis of the gluconate (gnt) operon of *Bacillus subtilis*, *Molecular Microbiology* ***5***, 1081-1089.

Ren, D. C., Bedzyk, L. A., Setlow, P., Thomas, S. M., Ye, R. W., and Wood, T. K. (2004). Gene expression in *Bacillus subtilis* surface biofilms with and without sporulation and the importance of yveR for biofilm maintenance, *Biotechnology and Bioengineering* ***86***, 344-364.

Resch, M., Schiltz, E., Titgemeyer, F., and Muller, Y. A. (2010). Insight into the induction mechanism of the GntR/HutC bacterial transcription regulator YvoA, *Nucleic Acids Research* ***38***, 2485-2497.

Rigali, S., Derouaux, A., Giannotta, F., and Dusart, J. (2002). Subdivision of the helix-turn-helix GntR family of bacterial regulators in the FadR, HutC, MocR, and YtrA subfamilies, *Journal of Biological Chemistry* ***277***, 12507-12515.

Rigali, S., Nothaft, H., Noens, E. E. E., Schlicht, M., Colson, S., Mueller, M., Joris, B., Koerten, H. K., Hopwood, D. A., Titgemeyer, F., and van Wezel, G. P. (2006). The sugar phosphotransferase system of Streptomyces coelicolor is regulated by the GntR-family regulator DasR and links N-acetylglucosamine metabolism to the control of development, *Molecular Microbiology* ***61***, 1237-1251.

Rocha, E. P. C., Guerdoux-Jamet, P., Moszer, I., Viari, A., and Danchin, A. (2000). Implication of gene distribution in the bacterial chromosome for the bacterial cell factory, *Journal of Biotechnology* ***78***, 209-219.

Rosinski, J. A., and Atchley, W. R. (1999). Molecular evolution of helix-turn-helix proteins, *Journal of Molecular Evolution* ***49***, 301-309.

Rossbach, S., Kulpa, D. A., Rossbach, U., and Debruijn, F. J. (1994). Molecular and genetic-characterization of the rhizopine catabolism (mocabrc) genes of *Rhizobium meliloti* l5-30, *Molecular & General Genetics* ***245***, 11-24.

Sadaie, Y., Nakadate, H., Fukui, R., Yee, L. M., and Asai, K. (2007). Glucomannan utilization operon of *Bacillus subtilis*, *Fems Microbiology Letters* ***279***, 103-109.

Sao-Jose, C., Baptista, C., and Santos, M. A. (2004). *Bacillus subtilis* operon encoding a membrane receptor for bacteriophage SPP1, *Journal of Bacteriology* ***186***, 8337-8346.

Sao-Jose, C., Lhuillier, S., Lurz, R., Melki, R., Lepault, J., Santos, M. A., and Tavares, P. (2006). The ectodomain of the viral receptor YueB forms a fiber that triggers ejection of bacteriophage SPP1 DNA, *Journal of Biological Chemistry* ***281***, 11464-11470.

Sato, Y., Nakaya, A., Shiraishi, K., Kawashima, S., Goto, S., Kanehisa, M. (2001). SSDB: Sequence Similarity Database in KEGG, *Genome Informatics* ***12***, 230–231.

Schock, F., and Dahl, M. K. (1996). Expression of the tre operon of *Bacillus subtilis* 168 is regulated by the repressor TreR, *Journal of Bacteriology* ***178***, 4576-4581.

Schock, F., and Dahl, M. K. (1996). Analysis of DNA flanking the treA gene of *Bacillus subtilis* reveals genes encoding a putative specific enzyme IITre and a potential regulator of the trehalose operon, *Gene* ***175***, 59-63.

Schreier, H. J. (1993) Biosynthesis of glutamine and glutamate and the assimilation of ammonia., In *Bacillus subtilis and other gram-positive bacteria.* (Sonenshein, A. L., Hoch, J. A., and Losick, R., Eds.), pp 281-298, American Society for Microbiology,Washington, D.C.

Schujman, G. E., Paoletti, L., Grossman, A.D., and De Mendoza, D. (2003). FapR, a bacterial transcription factor involved in global regulation of membrane lipid biosynthesis, *Dev Cell* ***4***, 663-672.

Schujman, G. E., Guerin, M., Buschiazzo, A., Schaeffer, F., Llarrull, L. I., Reh, G., Vila, A. J., Alzari, P. M., and de Mendoza, D. (2006). Structural basis of lipid biosynthesis regulation in Gram-positive bacteria, *Embo Journal* ***25***, 4074-4083.

Sengupta, S., Banerjee, A. B., and Bose, S. K. (1971). Gamma-glutamyl and D- or L-peptide linkages in mycobacillin, a cyclic peptide antibiotic, *Biochem J.* ***121***, 839-846.

Seo, J. W., Ohnishi, Y., Hirata, A., and Horinouchi, S. (2002). ATP-binding cassette transport system involved in regulation of morphological differentiation in response to glucose in Streptomyces griseus, *Journal of Bacteriology* ***184***, 91-103.

Serio, A. W., Pechter, K. B., and Sonenshein, A. L. (2007). *Bacillus subtilis* aconitase is required for efficient late-sporulation gene expression (vol 188, pg 6396, 2006), *Journal of Bacteriology* ***189***, 5403-5403.

Shimotsu, H., and Henner, D. J. (1986). Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*, *Gene* ***43***, 85-94.

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

Sonenshein, A. L., Hoch, J. A., and Losick, R. (1993) In *Bacillus subtilis And Other Gram-Positive Bacteria: Biochemistry, Physiology And Molecular Genetics*, Washington, DC: American Society For Microbiology.

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

Steinborn, G., Hajirezaei, M. R., and Hofemeister, J. (2005). *bac* genes for recombinant bacilysin and anticapsin production in Bacillus host strains, *Archives of Microbiology **183***, 71-79.

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.

Stoodley, P., Sauer, K., Davies, D. G., and Costerton, J. W. (2002). Biofilms as complex differentiated communities, *Annual Review of Microbiology **56***, 187-209.

Stover, A. G., and Driks, A. (1999). Secretion, localization, and antibacterial activity of TasA, a *Bacillus subtilis* spore-associated protein, *Journal of Bacteriology **181***, 1664-1672.

Stover, A. G., and Driks, A. (1999). Regulation of synthesis of the *Bacillus subtilis* transition-phase, spore-associated antibacterial protein TasA, *Journal of Bacteriology **181***, 5476-5481.

Strauch, M. A., Bobay, B. G., Cavanagh, J., Yao, F., Wilson, A., and Le Breton, Y. (2007). Abh and AbrB control of *Bacillus subtilis* antimicrobial gene expression, *Journal of Bacteriology **189***, 7720-7732.

Stulke, J., Hanschke, R., and Hecker, M. (1993). TEMPORAL ACTIVATION OF BETA-GLUCANASE SYNTHESIS IN BACILLUS-SUBTILIS IS MEDIATED BY THE GTP POOL, *Journal of General Microbiology **139***, 2041-2045.

Sung, M. H., Tanizawa, K., Tanaka, H., Kuramitsu, S., Kagamiyama, H., Hirotsu, K., Okamoto, A., Higuchi, T., and Soda, K. (1991). Thermostable aspartate - aminotransferase from a thermophilic *Bacillus* species - gene cloning, sequence determination, and preliminary- x-ray characterization, *Journal of Biological Chemistry **266***, 2567-2572.

Sutherland, I. W. (2001). The biofilm matrix - an immobilized but dynamic microbial environment, *Trends in Microbiology **9***, 222-227.

Tavares, P., Santos, M. A., Lurz, R., Morelli, G., Delencastre, H., and Trautner, T. A. (1992). Identification of a gene in *Bacillussubtilis* bacteriophage spp1 determining the amount of packaged dna, *Journal of Molecular Biology **225***, 81-92.

Tjalsma, H., Bolhuis, A., Jongbloed, J. D. H., Bron, S., and van Dijl, J. M. (2000). Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome, *Microbiology and Molecular Biology Reviews* ***64***, 515-+.

Trun, N., and Trempy, J. (2004) In *Fundemantal Bacterial Genetics*, Victoria: Blackwell Publishing.

Tsuge, K., Akiyama, T., and Shoda, M. (2001). Cloning, sequencing, and characterization of the iturin A operon, *Journal of Bacteriology* ***183***, 6265-6273.

Ulrich, L. E., Koonin, E. V., and Zhulin, I. B. (2005). One-component systems dominate signal transduction in prokaryotes, *Trends in Microbiology* ***13***, 52-56.

Vagner, V., Dervyn, E., and Ehrlich, S. D. (1998). A vector for the systematic gene inactivation in *Bacillus subtilis*, *Microbiology -Uk* ***144***, 3097-3104.

Valbuzzi, A., Ferrari, E., and Albertini, A. M. (1999). A novel member of the subtilisin-like protease family from *Bacillus subtilis*, *Microbiology-Uk* ***145***, 3121-3127.

van Aalten, D. M. F., Dirusso, C. C., and Knudsen, J. (2001). The structural basis of acyl coenzyme A-dependent regulation of the transcription factor FadR, *Embo Journal* ***20***, 2041-2050.

van Aalten, D. M. F., DiRusso, C. C., Knudsen, J., and Wierenga, R. K. (2000). Crystal structure of FadR, a fatty acid-responsive transcription factor with a novel acyl coenzyme A-binding fold, *Embo Journal* ***19***, 5167-5177.

van Hijum, S., de Jong, A., Baerends, R. J. S., Karsens, H. A., Kramer, N. E., Larsen, R., den Hengst, C. D., Albers, C. J., Kok, J., and Kuipers, O. P. (2005). A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data, *Bmc Genomics* ***6***.

van Hijum, S., de Jong, A., Buist, G., Kok, J., and Kuipers, O. P. (2003). UniFrag and GenomePrimer: selection of primers for genome-wide production of unique amplicons, *Bioinformatics* ***19***, 1580-1582.

van Hijum, S. A., Garcia de la Nava, J., Trelles, O., Kok, J., & Kuipers, O.P. (2003). MicroPreP: a cDNA microarray data pre-processing framework., *Appl Bioinformatics* ***2***, 241-244.

Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K. H., Ehrenreich, P., Baumer, S., Henne, A., Liesegang, H., Merkl, R., Ehrenreich, A., and Gottschalk, G. (2004). The complete genome sequence of Bacillus licheniformis DSM13, an organism with great industrial potential, *Journal of Molecular Microbiology and Biotechnology* ***7***, 204-211.

Verhamme, D. T., Kiley, T. B., and Stanley-Wall, N. R. (2007). DegU co-ordinates multicellular behaviour exhibited by *Bacillus subtilis*, *Molecular Microbiology* ***65***, 554-568.

von Dohren, H., Dieckmann, R., and Pavela-Vrancic, M. (1999). The nonribosomal code, *Chemistry & Biology **6***, R273-R279.

Walker, J. E., and Abraham, E. P. (1970). The structure of bacilysin and other products of *Bacillus subtilis*, *Biochemistry Journal. **84***, 1148-1151.

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

Weickert, M. J., and Adhya, S. (1992). A family of bacterial regulators homologous to gal and lac repressors, *Journal of Biological Chemistry **267***, 15869-15874.

Wiethaus, J., Schubert, B., Pfaender, Y., Narberhaus, F., and Masepohl, B. (2008). The GntR-like regulator TauR activates expression of taurine utilization genes in Rhodobacter capsulatus, *Journal of Bacteriology **190***, 487-493.

Williams, P. (2007). Bacillus subtifis: A shocking message from a Probiotic, *Cell Host & Microbe **1***, 248-249.

Wintjens, R., and Rooman, M. (1996). Structural classification of HTH DNA-binding domains and protein - DNA interaction modes, *Journal of Molecular Biology **262***, 294-313.

Wray, L. V., Zalieckas, J. M., and Fisher, S. H. (2001). *Bacillus subtilis* glutamine synthetase controls gene expression through a protein-protein interaction with transcription factor TnrA, *Cell **107***, 427-435.

Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation, *Nucleic Acids Research **30***.

Yansura, D. G., and Henner, D. J. (1984). Use of the *Escherichia coli* lac repressor and operator to control gene expression in *Bacillus subtilis*, *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences **81***, 439-443.

Yasbin, R. E., and Young, F. E. (1974). Transduction in *Bacillus subtilis* by bacteriophage spp1, *Journal of Virology **14***, 1343-1348.

Yazgan, A., Ozcengiz, G., and Marahiel, M. A. (2001). Tn10 insertional mutations of *Bacillus subtilis* strains that block the biosynthesis of bacilysin, *Biochimica Et Biophysica Acta - Gene Structure and Expression **1518***, 87-94.

Yazgan-Karatas, A., Cetin, S., and Ozcengiz, G. (2003). The effects of insertional mutations in comQ, comP, srfA, spo0H, spo0A and abrB genes on bacilysin biosynthesis in *Bacillus subtilis*, *Biochimica Et Biophysica Acta-Gene Structure and Expression **1626***, 51-56.

Yoshida, K. I., Fujita, Y., and Sarai, A. (1993). Missense mutations in the *Bacillus subtilis* GNT repressor that diminish operator binding ability, *Journal of Molecular Biology **231***, 167-174.

Yoshida, K. I., Ohmori, H., Miwa, Y., and Fujita, Y. (1995). *Bacillus subtilis GNT* repressor mutants that diminish gluconate-binding ability, *Journal of Bacteriology **177***, 4813-4816.

APPENDICES

APPENDIX A : Overall look to genes that are affected by YvfI regulatory protein during stationary phase

APPENDIX B : List of primers used in this study

APPENDIX C : Composition and Preperation of Culture Media

APPENDIX D : Composition and Preparation of Buffers and Solutions

APPENDIX E : Chemicals and Enzymes

APPENDIX F : Markers

APPENDIX G : Laboratory equipments

APPENDIX A

Table A.1 : Genes that are up (A)- and down (B)- regulated by YvfI during early-stationary phase (OD_{600}7)

A.

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
aprX	1,57	2,98(±0,17)	serine protease	≠ **aprX** ymaC ymaD ≠ ebrB
atpI	1,19	0,56(±0,03)	ATP synthase subunit I	≠ atpC atpD **atpG atpA atpH atpF atpE atpB atpI** ≠
atpB	1,93		ATP synthase subunit A	
atpE	2,02	1,53(±0,15)	ATP synthase subunit C	
atpF	1,99		ATP synthase subunit B	
atpH	1,93		ATP synthase subunit delta	
atpA	2,01	1,13(±0,10)	ATP synthase subunit alpha	
atpG	1,91		ATP synthase subunit gamma	
bioW	1,99	1,46(±0,06)	6-carboxyhexanoate--CoA ligase	bioI ≠ **bioB bioD bioF bioA bioW** ≠ ytaP msmR
bioA	1,38		adenosylmethionine--8-amino-7-oxononanoate transaminase	
bioF	1,45		8-amino-7-oxononanoate synthase	
bioD	2,06		dithiobiotin synthetase	
bioB	0,36		biotin synthase	
*czcD**	NR	2,25(±0,22)	potassium/proton-divalent cation antiporter	yrdR yrdQ ≠ **trkA czcD** ≠ yrdN ≠
*trkA(czcO)**	1,76	1,02(±0,06)	potassium uptake oxidoreductase	
lip(estA)	2,38	2,65(±0,09)	secreted alkaliphilic lipase	≠ lmrB lmrA yccC ≠ **lip** ≠ yczC ≠
fabHA	1,87	1,00(±0,12)	3-oxoacyl-(acyl carrier protein) synthase III	comZ yjzB **fabHA fabF** ≠ yjaZ
fabF	1,89		3-oxoacyl-(acyl carrier protein) synthase II	
fabHB	3,00	1,98(±0,05)	3-oxoacyl-(acyl carrier protein) synthase III	yhgE ≠ **fabHB** yhfC ≠ yhfD yhfE
ylxM	1,32		hypothetical protein, DNA-binding protein	rnc ≠ smc ftsY ≠ ylqB ylxM **ffh** ≠
Ffh	1,51	2,91(±0,04)	signal recognition particle-like (SRP) GTPase	
ftsE	1,11	1,94(±0,13)	cell-division ABC transporter (ATP-binding protein)	yvjB ≠ **ftsX ftsE** ≠ cccB yvjA ≠
ftsX	1,68		cell-division ABC transporter	
hepS	1,35	0,91(±0,18)	Heptaprenyl diphosphate synthase component I	≠ **ndk hepT** menH **hepS** mtrB mtrA ≠ hbs ≠ spoIVA ≠
hepT	1,52		Heptaprenyl diphosphate synthase component II	
Ndk	1,37	1,01(±0,24)	nucleoside diphosphate kinase (purine nucleotide biosynthesis)	
mraY	1,45	1,20(±0,06)	phospho-N-acetylmuramoyl-pentapeptide-transferase	≠ spoVD murE **mraY murD** ≠ spoVE
murD	1,53		UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	
pksD	1,9	1,44(±0,06)	enzyme involved in polyketide synthesis	pksA ≠ pksB pksC **pksD pksE** acpK pksF ≠ **pksG** pksH pksI **pksJ pksL pksM pksN pksR** ≠
pksE	1,59		enzyme involved in polyketide synthesis	
acpK	1,77		acyl-carrier protein	
pksG	2,10	1,42(±0,18)	acetyl-S-AcpK beta-ketothioester polyketide intermediate transferase	
pksH	0,42		enzyme involved in polyketide synthesis	

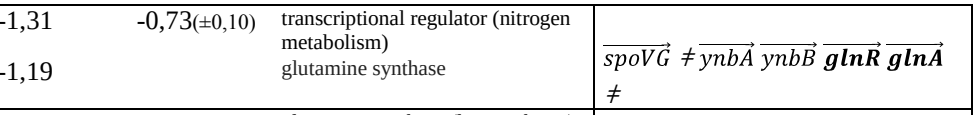
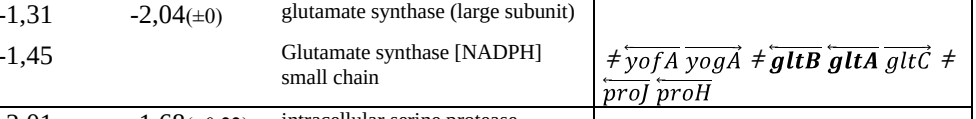
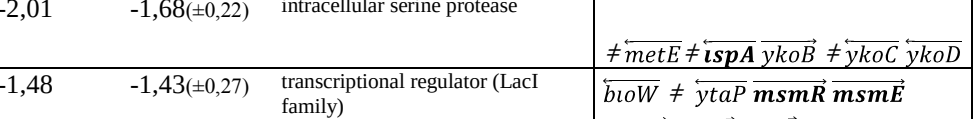
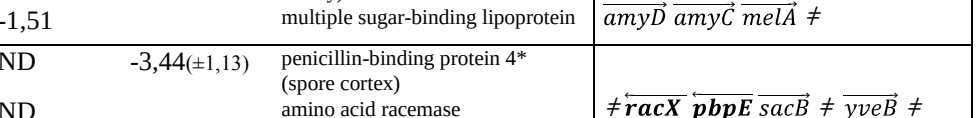
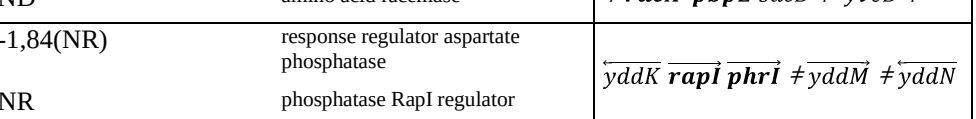
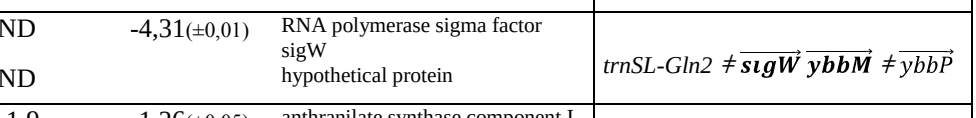
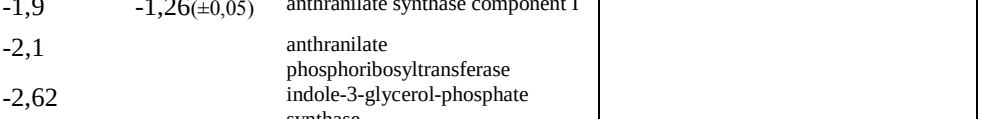
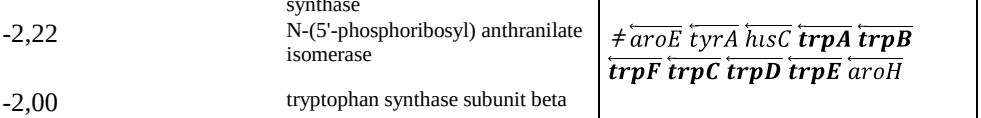
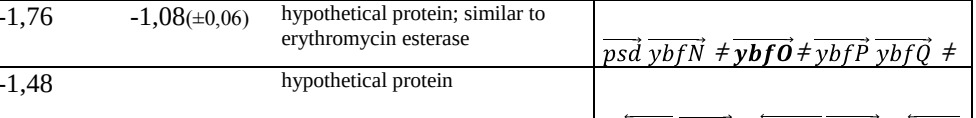
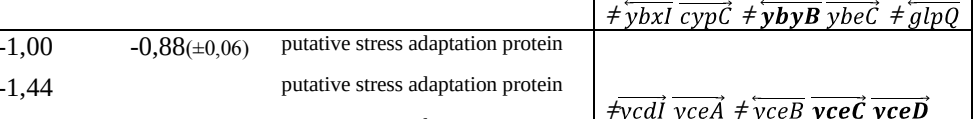
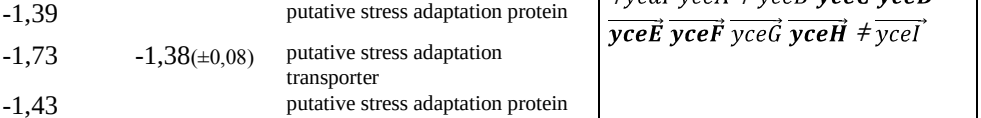
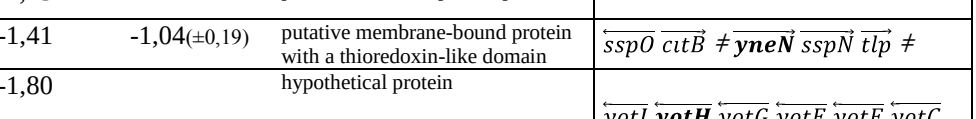
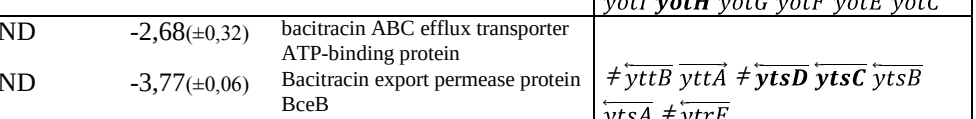
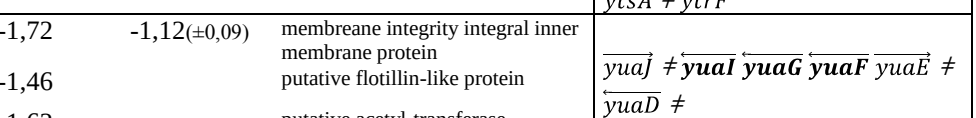
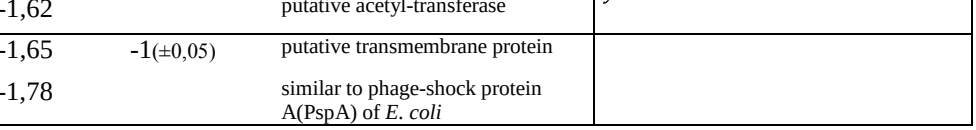
*pksI**	0,36		enzyme involved in polyketide synthesis	$\overleftarrow{pksS}\ \overrightarrow{ymzB}$
pksJ	2,73	4,14(±0,01)	polyketide synthase of type I	
pksL	3,95	6,19(±0,06)	polyketide synthase of type I	
pksM	2,17		polyketide synthase	
pksN	2,96		polyketide synthase of type I	
pksR	2,46		polyketide synthase	
*ppsA**	NR		plipastatin synthetase	$\neq \overleftarrow{\boldsymbol{ppsE}}\ \overleftarrow{ppsD}\ \overleftarrow{ppsC}\ \overleftarrow{\boldsymbol{ppsB}}\ \overleftarrow{\boldsymbol{ppsA}} \neq$
ppsB	1,1	1,09(±0,18)	plipastatin synthetase	
*ppsC**	NR		plipastatin synthetase	
ppsD	1,24		plipastatin synthetase	
ppsE	2,42		plipastatin synthetase	
pyrR	2,52	2,79(±0,32)	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase	$\neq \overrightarrow{\boldsymbol{pyrR}} \neq \overrightarrow{\boldsymbol{pyrP}} \neq \overrightarrow{\boldsymbol{pyrB}}\ \overrightarrow{\boldsymbol{pyrC}}\ \overrightarrow{\boldsymbol{pyrAA}}\ \overrightarrow{\boldsymbol{pyrAB}}\ \overrightarrow{pyrK}\ \overrightarrow{pyrD}\ \overrightarrow{pyrF}\ \overrightarrow{pyrE} \neq \overrightarrow{cysH}$
*pyrP**	NR	3,48(±0,18)	uracil permease	
pyrB	1,37	4,34(±0,01)	aspartate carbamoyltransferase catalytic subunit	
pyrC	1,89		Dihydroorotase	
pyrAA	2,31	5,85(±0,20)	carbamoyl-phosphate synthetase (glutaminase subunit)	
pyrAB	2,00		carbamoyl-phosphate synthetase (catalytic subunit)	
pyrK	0,85		dihdrooratate dehydrogenase	
pyrD	1,32		dihdrooratate dehydrogenase (catalytic subunit)	
pyrF	1,21		orotidine 5'-phosphate decarboxylase	
pyrE	1,08		orotate phosphoribosyl transferase	
Rnc	2,41	1,64(±0,03)	ribonuclease III, cleaves both 5'- and 3'-sites of the small cytoplasmic RNA precursor	$\overrightarrow{acpA} \neq \overrightarrow{\boldsymbol{rnc}} \neq \overrightarrow{\boldsymbol{smc}} \neq \overrightarrow{ftsY} \neq$ ylqB
smc	1,42		chromosome condensation and segregation SMC ATPase	
ftsY	1,08		signal recognition particle	
rplJ	1,69	3,42(±0,30)	50S ribosomal protein L10	$\overrightarrow{nusG}\ \overrightarrow{rplK}\ \overrightarrow{rplA} \neq \overrightarrow{\boldsymbol{rplJ}}\ \overrightarrow{\boldsymbol{rplL}} \neq \overrightarrow{ybxB}$
rplL	1,66		50S ribosomal protein L7/L12	
spoIIE†	ND		serine phosphatease	$\neq \overrightarrow{\boldsymbol{spoIIE}}\ \overrightarrow{yabS}\ \overrightarrow{yabT}\ \overrightarrow{yacA}\ \overrightarrow{hprT}$
Tig	1,68	1,11(±0,01)	trigger factor (prolyl isomerase), catalyze in vitro protein folding; essential for growth under starvation conditions	$\neq \overleftarrow{lonA} \neq \overleftarrow{lonB}\ \overleftarrow{clpX}\ \overleftarrow{\boldsymbol{tig}}\ \overleftarrow{ysoA} \neq$
Tsf	1,69	1,31(±0,53)	Ts elongation factor	$\overrightarrow{sigD}\ \overrightarrow{ylxL}\ \overrightarrow{rpsB} \neq \overrightarrow{\boldsymbol{tsf}}\ \overrightarrow{\boldsymbol{pyrH}}\ \overrightarrow{\boldsymbol{frr}} \neq$
pyrH	1,52	1,74(±0,15)	uridylate kinase	
frr	1,55	1,11(±0,08)	ribosome recycling factor	
ydjM	2,33		hypothetical protein	$\neq \overleftarrow{ydjL}\ \overrightarrow{\boldsymbol{ydjM}} \neq \overrightarrow{\boldsymbol{ydjN}} \neq \overleftarrow{ydjO}$
ydjN	1,48		hypothetical protein	
yhdN	ND		aldo/keto reductase	$\overrightarrow{yhdN} \neq \overrightarrow{\boldsymbol{yhdO}} \neq \overleftarrow{yhdP}\ \overrightarrow{yhdQ}\ \overrightarrow{yhdR} \neq$
yhdO(plsC)	1,49	1,05(±0,14)	1-acyl-sn-glycerol-3-phosphate acyltransferase (lipid metabolism)	

yhfE	1,63	3,65(±0,04)	putative endoglucanase	
yhfF	1,40	2,18(±0,09)	hypothetical protein	≠ *fabHB yhfC* ≠ *yhfD* **yhfE yhfF** ≠
yjcM	1,48		hypothetical protein	≠ *yjcK yjcL* ≠ **yjcM** *yjcN* ≠ *yjcO*
ylpC(fapR)	1,36	1,16(±0,09)	fatty acid biosynthesis transcriptional factor	
plsX	1,66		putative glycerol-3-phosphate acyltransferase PlsX	
fabD	2,42		malonyl CoA-acyl carrier protein transacylase	**ylpC plsX fabD fabG acpA** ≠ *rnc* ≠ *smc* ≠ *ftsY*
fabG	2,28		beta-ketoacyl-acyl carrier protein reductase	
acpA	2,29		acyl carrier protein	
yokD	NR	2,65(±0,25)	aminoglycosideN3'-acetyltransferase	≠ *yokF yokE* ≠ **yokD** *yokC*
yqgA	1,80		hypothetical protein	*yqgC yqgB* ≠ **yqgA** *yqfZ yqfY* ≠
yqxI	1,31		hypothetical protein	≠ *yqzI yqcG yqcF* ≠ **yqxJ yqxI**
yqxJ	1,78		hypothetical protein	
yqxM	0,96	2,65(±0,06)	lipoprotein for biofilm formation	
sipW	2,12	1,42(±0,14)	type I signal peptidase	*sınI sınR* ≠≠ **tasA sipW yqxM** *yqzG yqzE*
tasA	1,50	2,65(±0,17)	major biofilm matrix component	
yuaB	3,33	2,70(±0,25)	hypothetical protein	*yuaC* ≠ **yuaB** ≠ *yuaA yubG* ≠
yukE	1,46	1,29(±0,06)	hypothetical protein	
yukD	0,85		bacteriocin	
yukC	1,75		membrane-associated enzyme involved in bacteriocin production	≠ *yueD yueC yueB* **yukA yukB** **yukC yukD yukE** *yukF* ≠
yukB	0,46		putative cell division protein	
yukA	1,48		hypothetical protein	
yvaW(sdpA)	1,26	1,08(±0,22)	sporulation delay protein-export of killing factor	
yvaX (sdpB)	2,2	1,68(±0,08)	Sporulation delay protein-exporter of killing factor SpbC	*yvaV* **yvaW yvaX** *yvaY* ≠
yvaY(sdpC)	061		killing factor SdpC	
*yvcA**	NR	1,56(±0,01)	Lipoprotein	*yvcC* ≠ *yvzA yvcB* **yvcA** ≠ *hısI*
yvcE(cwlO)	2,24	1,70(±0,05)	secreted cell wall DL-endopeptidase	*yvcI trxB* **yvcE** ≠ *yvcD yvcC* ≠
yveN(epsD)	2,21	2,63(±0,05)	extracellular matrix biosynthesis enzyme	
yveO(epsE)	2,08	1,34(±0,31)	Glycosyltransferase	≠ *sıgL yvfG* **yvfF yvfE** *yvfD* *yvfC yvfB* **yvfA** *yveT yveS yveR* *yveQ yveP* **yveO yveN** *yveM* *yveL yveK slr*
yvfA(epsK)	1,49	1,10(±0,21)	extracellular matrix component exporter	
yvfE(epsN)	0,79	1,02(±0,01)	aminotransferase	
yvfF(epsO)	1,79	0,94(±0,05)	pyruvyl transferase	
ywcD	1,52	2,04(±0,06)	integral inner membrane protein	≠ *galT galK* **ywcD** *ywcC ywcB*
ywfB(bacA) †	ND		bacilysin biosynthesis protein, dehidratase	
ywfC(bacB)	ND		isomerase component of bacilysin synthetase	
ywfD(bacC) *	NR		bacilysin biosynthesis oxidoreductase	≠ *ywfA* ≠ **ywfB** *ywfC ywfD* *ywfE ywfF ywfG* ≠ **ywfH** ≠ *ywfI*
ywfE(bacD)	ND		alanine-anticapsin ligase	

ywfF(bacE) *	NR		efflux protein for bacilysin excretion, self-protection against bacilysin	
*ywfG**	NR		transaminase	
ywfH†	ND		carrier protein reductase of bacilysin biosynthesis	
yybN	1,76	2,05(±0,24)	hypothetical protein	
yybM	0,87		integral inner membrane protein	
yybL	1,52	1,23(±0,12)	integral inner membrane protein	
yybK	1,73		integral inner membrane protein	
yybJ	1,52		ATP-binding cassette protein	
yydF	ND		biactive peptide eliciting cell envelope stress sensed by the LiaRS TCS	
yydG	1,93	1,68(±0,09)	putative AdoMet radical enzyme	
yydH	0,62		membrane-embedded protease	
yydI	0,51		YydIJ: an ATP-binding cassette (ABC) transporter	
yydJ	0,73			

B.

Gene	Fold difference [a]	qPCR [b]	Function	Transcriptional Organisation [c]
*abh**	NR	-4,57(±0,28)	transcriptional regulator of transition state genes (AbrB-like)	
acoA	-1,57	-3,07(±0,22)	acetoin dehydrogenase E1 component (TPP-dependent alpha subunit)	
acoB	-1,54		acetoin dehydrogenase E1 component (TPP-dependent beta subunit)	
acoC	-1,56		branched-chain alpha-keto acid dehydrogenase subunit E2	
acoL	-1,36		dihydrolipoamide dehydrogenase	
aprE	-2,35	-3,07(±0,91)	extracellular alkaline serine protease (subtilisin E)	
argC	-1,63	-1,98(±0,22)	N-acetyl-gamma-glutamyl-phosphate reductase	
argJ	-1,89		bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	
argB	-2,08		acetylglutamate kinase	
argD	-2,13		acetylornithine aminotransferase	
carA	-2,22		carbamoyl phosphate synthase small subunit	
carB	-2,15		carbamoyl phosphate synthase large subunit	
argF	-2,16		ornithine carbamoyltransferase	
argG	-1,68		argininosuccinate synthase	
argH	-2,04		argininosuccinate lyase	
citA	-1,50	-2,20(±0,30)	citrate synthase 1	
citB	-1,38	-2,21(±0,21)	aconitate hydratase	

glnR	-1,31	-0,73(±0,10)	transcriptional regulator (nitrogen metabolism)	
glnA	-1,19		glutamine synthase	
gltA	-1,31	-2,04(±0)	glutamate synthase (large subunit)	
gltB	-1,45		Glutamate synthase [NADPH] small chain	
ispA	-2,01	-1,68(±0,22)	intracellular serine protease	
msmR	-1,48	-1,43(±0,27)	transcriptional regulator (LacI family)	
msmE	-1,51		multiple sugar-binding lipoprotein	
pbpE	ND	-3,44(±1,13)	penicillin-binding protein 4* (spore cortex)	
racX	ND		amino acid racemase	
*rapI**†	-1,84(NR)		response regulator aspartate phosphatase	
*phrI**	NR		phosphatase RapI regulator	
sigW	ND	-4,31(±0,01)	RNA polymerase sigma factor sigW	
ybbM	ND		hypothetical protein	
trpE	-1,9	-1,26(±0,05)	anthranilate synthase component I	
trpD	-2,1		anthranilate phosphoribosyltransferase	
trpC	-2,62		indole-3-glycerol-phosphate synthase	
trpF	-2,22		N-(5'-phosphoribosyl) anthranilate isomerase	
trpB	-2,00		tryptophan synthase subunit beta	
trpA	-1,04		tryptophan synthase subunit alpha	
ybfO	-1,76	-1,08(±0,06)	hypothetical protein; similar to erythromycin esterase	
ybyB	-1,48		hypothetical protein	
yceC	-1,00	-0,88(±0,06)	putative stress adaptation protein	
yceD	-1,44		putative stress adaptation protein	
yceE	-1,39		putative stress adaptation protein	
yceF	-1,73	-1,38(±0,08)	putative stress adaptation transporter	
yceH	-1,43		putative stress adaptation protein	
yneN	-1,41	-1,04(±0,19)	putative membrane-bound protein with a thioredoxin-like domain	
yotH	-1,80		hypothetical protein	
ytsC(bceA)	ND	-2,68(±0,32)	bacitracin ABC efflux transporter ATP-binding protein	
ytsD(bceB)	ND	-3,77(±0,06)	Bacitracin export permease protein BceB	
yuaF	-1,72	-1,12(±0,09)	membreane integrity integral inner membrane protein	
yuaG	-1,46		putative flotillin-like protein	
yuaI	-1,62		putative acetyl-transferase	
yvqI (liaI)	-1,65	-1(±0,05)	putative transmembrane protein	
yvqH(*liaH*)	-1,78		similar to phage-shock protein A(PspA) of *E. coli*	

yvqG(liaG)	-1,31	putative membrane-anchored hypothetical protein	$\overleftarrow{yvqG}$ $\overleftarrow{\boldsymbol{yvqH}}$ $\overleftarrow{\boldsymbol{yvqI}}$ $\neq\overleftarrow{yvqJ}$ $\neq\overleftarrow{yvqK}$
yvqF(liaF)	-1,05	membrane protein	
yvqE(liaS)	-1,02	LiaRS: two component regulatory system	
yvqC(liaR)	-0,85		

Genes in an operon are grouped together

d. Numbers indicate the log 2 transformed expression ratio's.
e. Numbers indicate the log 2 transformed expression ratio's. (The mean of minimum three independent replicate experiments is given and standart deviation of the mean is shown in paranthesis),
f. transcriptional organization retrieved from http://genolist.pasteur.fr/SubtiList/, "≠" indicates the termination sites

* Bayes P value higher than acceptable value (>0.01)

NR no reproducible data obtained

ND no differential expression observed

† Expression profiles were elucidated by *lacZ*-fusion analysis

APPENDIX B

Table B.1 : List of primers used in this study

abh forward 5’ gat gaa tta ggc cgc att 3’
abh reverse 5’ ggc ttg aat ttc ttc gag 3’
acoA forward 5’ ctg gag atc agg ggc ttt 3’
acoE reverse 5’ aca gcc ttt ggc gat aca 3’
aprE Forward 5’ gcg ttc agc aac atg tct 3’
aprE Reverse 5’cac ata tgc aac gct cgg 3’
aprX F 5’ tgg act cct tgc ttc ttg 3’
aprX R 5’ tgc tga agg agt aac ctc 3’
argC forward 5’ agc gag ggt tat cct cat 3’
argC reverse 5’ cag atc acc tga cag atc 3’
argG forward 5’ gag ggc aaa gat ttg gca 3’
argG reverse 5’ acg aac ctg gtc att tcc 3’
atpA reverse 5’acc aac agg aac ctc cat 3’
atpA forward 5’gtc atc caa gtc ggt gac 3’
atpE reverse 5’gaa tgc gat aac gac agc
atpE forward 5’gca gct gcg att gca att 3’
atpI forward 5’ ttg gca gtg tat gta ctg ggt-3’
atpI reverse 5’ cgg gaa ttc cat tct tct gac gag cag taa-3’
bioW forward 5’gtc aga atg agg gct tca 3’
bioW reverse 5’ cac agg caa tgg ctg aat 3’
citA forward 5’ agc ttt gaa gaa gcg gct 3’
citA reverse 5’cgg atg gaa tgt gta cgt 3’
citB forward 5’ aag gtt tcg aag ctt cct 3’
citB reverse 5’ cag tga agc cag atc tac 3’
czcD forward 5’ tct gat gca ggc cat atg ctg 3’
czcD reverse 5’ aag cat gcc ggt tgt tgc tac 3’
fabHA forward 5’ gac gag tgg att cgt aca 3’
fabHA reverse 5’ gcc gag ttg ttc ttg aat 3’
fabHB forward 5’ gaa tgg atc gtt cag cgc 3’
fabHB reverse 5’ gct ttc cca gcc gaa ata 3’
ffh forward 5’ atg atg cgt gag gtc cgt ctt-3’
ffh reverse 5’ aac ttt aat gac ctg ctg gcc-3’
frr forward 5’ tat tac gga gcg cag aca 3’
frr reverse 3’ccg tct ctc ttc tgt tag 3’
ftsE forward 5’ tat gtt gtt ggt ccg agc 3’
ftsE reverse 5’cac ttc aag ggc aaa tgc 3’
glnR forward 5’ cca gcc aga agt gaa gga 3’
glnR reverse 5’ tct cag ttc gtc atc gga 3’
gltA forward 5’ atc ggc cta tat gca cac 3’
gltA reverse 5’tcc tac ccc gta acg ttc 3’

hepS forward 5’ gcg aag cat att tct gcg 3’
hepS reverse 5’ gag aag agt caa ttg gcg 3’
ispA forward 5’ aag gcg cca gaa atg tgg 3’
ispA reverse 5’ agc tgc aat tgt tcc ggc 3’
lip forward 5’ gtc gtt atg gtt cac ggt 3’
lip reverse 5’ cat gct gtg agc gac aat 3’
mraY forward 5’gga ccg aaa tca cat cag 3’
mraY reverse 5’gcg ctt cat gac aac ctt 3’
msmR forward 5’ ctt tct gtt gcg ggc gaa 3’
msmR reverse 5’ ccc ttt ccg aat gga aga 3’
ndk forward 5’ gtc caa cgt cag ctc att 3’
ndk reverse 5’ cag ctg tct cgt cac ttc 3’
pbpE forwrd 5’- cag ttt aac ggg acg gtt-3’
pbpE reverse 5’-ctg ata cgg aaa acc ggg-3’
pksD forward 5’ cga atc ggc aca tcc att 3’
pksD reverse 5’ cca gac tga tcc caa tac 3’
pksG forward 5’ ctt gat gtc atg gag ctg 3’
pksG reverse 5’ gtt gcg gtt gag acc taa 3’
pksJ forward 5’ ccg gct cca tta gcc gtt 3’
pksJ reverse 5’ tcc cgt act gcc tga agt 3’
pksL forward 5’ aag gct gac atg cac gca 3’
pksL reverse 5’ caa atg att ggc acc cac 3’
ppsB forward 5’ aat cta tgc gtc gac tcg 3’
ppsB reverse 5’agc atc aat cag cgt ctg 3’
pyrAA forward 5’tct tac tgc gga cag atc 3’
pyrAA reverse 5’ atc aat tcc ctg gag tcc 3’
pyrB forward 5’ttc gaa ccg agc acg aga 3’
pyrB reverse 5’ ctg gct gac aag ctc ttc 3’
pyrH forward 5’atc gct gag ctt gaa gtc gaa 3’
pyrH reverse 5’gga tgt ttg cac tct gga 3’
pyrP forward 5’gtc gga atg agt cct gct 3’
pyrP reverse 5’ caa taa gga aat cag ccc 3’
pyrR forward 5’agg att gct cac gaa atg 3’
pyrR reverse 5’ tac cgg aat atc tgc acc 3’
rapI reverse 5’-cgt gac taa gtc gta cgg aat-3’
rapI BamHI reverse 5’- cgg gga tcc ttc agc tat tcg ata agc-3’
rapI HindIII forward 5’- gcc aag ctt ttg cgg ggt gtt ttc tta-3’
rnc forward 5’-caa gaa cgg att tcg gtt cac-3’
rnc reverse 5’- cct ttc att atc ttc ata cgg-3’
rplJ forward 5’-cgc gga ctt aac gtt tct gaa-3’
rplJ reverse 5’-aag ctc agc ttg ttc aac cgc-3’
sdpA BamHI forward 5’- gcc ggatcc ttg atg cca aca ttg ccg aga 3’
sdpA EcoRI reverse 5’-cgg gaattc aat gtt ttc ttc tgt agg gct-3’

sigW forward 5’- gcg gac atc gta gat att-3’
sigW reverse 5’-aga ata cat ggt caa gcc-3’
sipW forward 5’- tca gtt ctg tca ggt tcg atg-3’
sipW reverse 5’-aac aat tct gtg ggt gac cgc-3’
tasA forward 5’-aag ccg gga gat aag ttg aca -3’
tasA reverse 5’-ctg gct gag gaa atc ttc tgg-3’
tig forward 5’- caa gtt tca att cct gga ttc-3’
tig reverse 5’- agg gta ttc tac agg aag agg-3’
trkA(czcO) forward 5’ ata gta atc ggg gct ggt 3’
trkA(czcO) reverse 5’ tcc ttc aag atg cat tcc 3’
trpE forward 5’ gag aag ctt gac agg gag 3’
trpE reverse 5’ agg aat gcc aag ctc agg 3’
tsf forward 5’ gcg tta act gaa act gac gga 3’
tsf reverse 5’ tgc aag aag gtg gtc agc taa 3’
ybfO forward 5’ gaa cga cat gca cag cct 3’
ybfO reverse 5’ atc agg aaa gcc gga ttc 3’
yceC forward 5’ aaa ttg atg gtc ggt ctc 3’
yceC reverse 5’ cag gtt gtc gcc tgt atg 3’
yceF forward 5’ ggt ttg atc ggt tcc ctt 3’
yceF reverse 5’cac ctt gat cca cca gaa 3’
yhdO forward 5’ atc gcg tgt aca cat tcc 3’
yhdO reverse 5’ cgg cgt ttt aat act gct 3’
yhE reverse 5'-tcc cga ggc ggt ttc aat ttg-3’
yhfE forward 5’-gaa acg gtt cgg aac cac aag -3’
yhfF forward 5’- ctg att cag cag atc ctt gcc-3’
yhfF reverse 5’-aat tcg gtc tcc att gcc ctc-3’
ylpC forward 5’ gat gaa gaa cta gcg ggt 3’
ylpC reverse 5’ aat gga tat cgc ctg atc 3’
yneN forward 5’ gtc ggt tat acg gga tgg 3’
yneN reverse 5’ ttg cag ctt ttc cat cgc 3’
yokD forward 5’aat ggt gga gct gtt gc 3’
yokD reverse 5’ ggc tgg cat act ttc tct 3’
yqxM forward 5’ gcc gca ata tgc tta caa-3’
yqxM reverse 5’ctt aag ttt ctc acc tgt-3’
ytsC forward 5’- ggc gaa ttc gtc agt att-3’
ytsC reverse 5’-gat cga taa agg cag aag-3’
ytsD forward 5’ gtc acg ctg cag tat gat-3’
ytsD reverse 5’ tac ccc gat cgc taa tga 3’
yuaB forward 5’- gca cct aca gct tct ttc-3’
yuaB reverse 5’-aaa tcc gct tgg caa tgt-3’
yuaF forward 5’ ccg aca tta gtg ctc tca 3’
yuaF reverse 5’tcc tct gag atc atc ttc 3’
yukE forward 5’atg gca cag gag gta atg 3’

yukE reverse 5 aag gtt tga gct gct cgt 3’
yvaW (sdpA) Forward (40-144) 5’-agc aat att tca cct cag aa-3’
yvaX(sdpB) forward 5’-aga agt tta ctt ggt ttc tca-3’
yvaX(sdpB) reverse 5’-agc ggt agg gat ata gac att-3’
yvcA reverse 5’ tgt ctt agg ttc att cgc-3’
yvcA forward 5’ gaa gag gaa cca gga tat-3’
yvcE(cwlO) reverse 5’ gtt gct tgt atc aag cgc 3’
yvcE(cwlO) forward 5’ gca tcg gcg gaa aca tta 3’
yveN forward 5’- aag ctg ccg tat gtg gat gag-3’
yveN reverse 5’- gtg cgc tgt gtc cag cac ctt-3’
yveO Forward 5’-tgc gat gat gcg tca aca-3’
yveO Reverse 5’-aac cac ctg ata gtg tcg gtg-3’
yvfA forward 5’ ctc acg gct ttt ctc ttg tct 3’
yvfA reverse 5’ ata cgc att tgc ttt ctc ccg 3’
yvfE forward 5’gaa gaa cag ctg gca gaa cga 3’
yvfE reverse 5’ cat att cca cgt atc agg ctc 3’
yvfF forward 5’ cgc tgg aat cca gac aat ttc 3’
yvfF reverse 5’cgt ccg ctt gag gtt gtc ttt 3’
yvfInested reverse 5' cag aag ggc ggc tga gag cgg ctg 3’
yvfI race reverse 5' aat atc ccg aaa gca cat 3’
yvfI BamHI reverse 5’- cgg gga tcc aat atc ccg aaa gca cat
yvfI HindIII forward 5’- gcc aag ctt atg aaa cag gga gaa ggc 3’
yvqI forward 5’ atc ggg agc ttg atg aca 3’
yvqI reverse 5’ aga agc cgg ttc agg atc 3’
ywcD forward 5’ gtt gcc gct tgg att ttg 3’
ywcD reverse 5’ ctg gcc gac aag aat gat 3’
yybL forward 5’ ttc aac cag aac cac act 3’
yybL reverse 5’ acg ctt aac cat cct cat 3’
yybN forward 5’ gta cct tat ggc tat ggt 3’
yybN reverse 5’ tcc ctg gct att tgc atg 3’
yydG forward 5’gag tta gta act gaa ttt gca 3’
yydG reverse 5’act tat agt cag tgc tat cac 3’

APPENDIX C

Luria Bertani (LB) Agar Medium (1000 mL)

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

2xYT Medium (1000mL)

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

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 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

$MgCl_2$ (1M) (filter steriled) 50 µL

Freshly prepared and completed up to 20 mL with sterile distilled H_2O.

Perry and Abraham (PA) Medium (pH 7.4)

KH_2PO_4 1 g/L

KCl 0.2 g/L

$MgSO_4.7H_2O$* 0.5 g/L

Glutamate.Na.H_2O 4 g/L

Sucrose* 10 g/L Autoclave separately

Ferric citrate** 0.15 g/L Filter sterilization

Trace elements** 1 mL Filter sterilization

$CoCl_2.6H_2O$ 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

DSM Medium (Difco's Sporulation Medium)

Nutrient Broth 8 g

KCl (10 % w/v) 10 mL

MgSO4.7H2O (1.2 %) 10 mL

NaOH (1 M) 0.5 mL

Autoclaved for 30 min. and cooled down to 50 °C.

Ca(NO3)4 (1 M) 1 mL

MnCl2 (0.01 M) 1 mL

FeSO4 (1 mM) 1 mL (resuspended before use)

APPENDIX D

P1 Buffer (pH 8)

Tris-base	6.06 gr
EDTA.2H2O	3.72 gr

Dissolve Tris-base and EDTA with 800 mL dH2O. Adjust pH to 8 with HCl. Adjust volume to 1 lt dH2O. Add 100 mg RNase A per liter of P1.

P2 Buffer

NaOH	8 gr
SDS solution (20%)	50 mL

Dissolve NaOH in 950 mL dH2O.

Add 50 mL SDS solution.

P3 Buffer (pH 5.5)

Potassium acetate	294.5 gr

Dissolve in 500 mL dH2O.

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)	57.1 mL
EDTA (100mL 0.5M)	100 mL (0.5 M, pH 8.0)

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

Low Melting Agarose Gel (1%)

Agarose 0.5 g

TAE buffer (1X) 50 mL

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

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

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

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

$NaCl_2$ 8.5 g

Dissolve in 1000 mL distilled water and autoclave.

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

Tris (hydromethyl)aminomethane 3.03 g

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

Z Buffer (500 mL, pH 7.0)

$Na_2HPO_4.7H_2O$ (60mM) 5.33 g

NaH_2PO_4 (40 mM) 3.12 g

KCl_2 (10 mM) 0.373 g

$MgSO_4.7H_2O$ (1 Mm) 0.123 g

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

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

Lysozyme final concentration: 2.5 mg/mL

ONPG final concentration: 4.0 mg/mL

APPPENDIX E

Chemicals	**Suppliers**
Agar Bacteriological	AppliChem
Agarose	Prona
Ammonium persulfate	Carlo Erba
Arginine	Merck
$CaCl_2.2H_2O$	Merck
Casamino acid	AppliChem
Cloramphenicol	Sigma
CoCl2.6H2O	Carlo Erba
CuSO4.5H2O	Carlo Erba
CTAB	Sigma
EDTA	Sigma
Ethanol	Botafarma
Ethidium bromide	Sigma
Glucose	Riedel deHaen
Glycerol	Merck
HCl	Carlo Erba
Histidine	Merck
IPTG	Sigma
KH2PO4	J.T.Baker
K2HPO4	J.T.Baker
KCl	Carlo Erba
Mercaptoethanol	Merck
MgCl2.6H2O	Carlo Erba
MgSO4.7H2O	Carlo Erba
MnCl2.4H2O	Carlo Erba
Na2CO3	Riedel deHaen
Na3citrate.2H2O	Riedel deHaen
NaCl	Carlo Erba
NaOH	Carla Erba
2-Nitrophyl β-D-galacto pyranoside (ONPG)	Sigma
Nutrient Broth	Merck

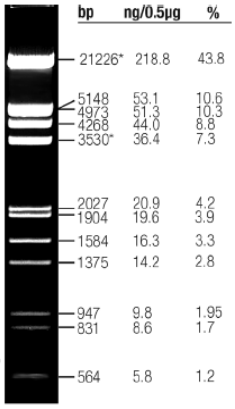
PEG 8000	Merck
Phenol-chloroform-isoamylalcohol	Fluka
Phenylalanin	Merck
SDS	Merck
Spermine	Fluka
Sucrose	Merck
Tris-base	Merck
Triton-X100	Sigma
Trypton	Acumedia
Tryptophan	Merck
X-Gal	Sigma
Yeast Extract	Acumedia

Enzymes

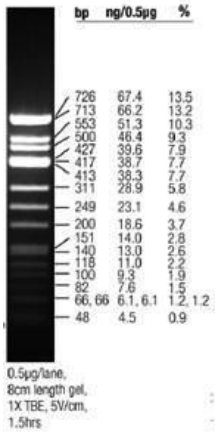
*Bam*HI	Fermentas
*Hin*dIII	Fermentas
Lysozyme	AppliChem
Proteinase K	Sigma
RNAse A	Sigma
Taq DNA Polymerase	Fermentas
T4 DNA Ligase	Roche

APPENDIX F

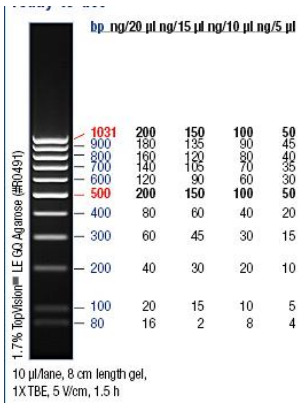
Marker	**Supplier**
Lambda DNA/*EcoR*I+ *Hind*III Marker (Marker 3)	MBI Fermentas



ΦX174 DNA/HinfI Marker (Marker 10)	MBI Fermentas



MassRuler™ DNA Ladder, Low range, ready-to-use	MBI Fermentas



APPENDIX G

LABORATORY EQUIPMENT

Autoclave: Tuttnauer Systec Autoclave (2540 mL)

Balances: Precisa 620C SCS

Precisa 125 A SCS

Centrifuge: Beckman Coulter, Microfuge 18

Centrifuge rotor: F241.5P

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

-20°C Arçelik 209lt

+4°C Arçelik

Electrophoresis equipments: E – C mini cell primo EC320

Gel documentation system: UVI PHotoMW Version 99.05 for Windows

Incubators: Nüve EN400

Nüve EN500

Orbital shaker incubators: Sertomat S – 2

Thermo 430

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

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

Eppendorf research 2,5 µL 10 µL, 20 µL, 200 µL, 1000 µL

pH meter: Mettler Toledo MP220

Spectrophotometer: PerkinElmer Lambda25 UV/VIS Spectrometer

Thermomixer: Eppendorf thermomixer comfort (1.5 mL)

Transillumunator: Biorad UV transilluminator 2000

Vortexing machine: Heidolph Raax top

Waterbaths: Memmert wb-22

Ultrafiltration tube: VIVASPIN

Power supply: Bio-Rad

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PUBLICATIONS

A. THESIS:

İrigül, Ö., February 2006, Functional role of *yvgW* gene during sporulation in *B. subtilis*, ***M.Sc. Thesis***, Department of Advanced Technologies, Molecular Biology-Genetics and Biotechnology Programme, Istanbul Technical University

B. PAPERS

1) İrigül, Ö. ve A. Yazgan Karataş, "Sporulation Specific Expression of the *yvgW* (*cadA*) Gene and the Effect of Blockage on Spore Properties in *Bacillus subtilis", Gene* (Section Functional Genomics), **382**, 71-78 (2006).

C. CONGRESS ABSTRACTS

1. Köroğlu, T.E., **İrigül, Ö**., Özcengiz, G. And Yazgan-Karataş, A. Identification of new genes related with bacilysin biosynthesis by Tn10 mutagenesis method in *B.subtilis*. 3rd International Conference on Analysis of Microbial Cells at the Single Cell Level (26-29 May, 2005, Semering, Austria). Book of Abstracts, PIII.15, p.71

2. Ülgen, A.G., **İrigül, Ö.,** Dereli, A., Hekimoğlu, B. ve A. Yazgan Karataş," Characterization of *yvgW* as a Noval Sporulation Gene in *B. subtilis*", *3rd İnternational Conference on Analysis of Microbial Cells at the Single Cell Level*, Semmering, Book of Abstracts, PIII.12, 68, Austria, 2005.

3. İrigül, Ö., Ülgen, A.G. ve A. Yazgan Karataş, "The Effect of Metal Ions of Cd, Zn, Co, Cr, Ni, Ag, Pb, H_2O_2 and Ethanol on *yvgW* Expression in *B. subtilis*", *3rd İnternational Conference on Analysis of Microbial Cells at the Single Cell Level*, Semmering, Book of Abstracts,PIII.13, 69, Austria, 2005.

4. Köroğlu, T.E., **İrigül, Ö**, Özcengiz, G. ve A. Yazgan Karataş, "Identificatipn of New Genes Related with Bacilysin Biosynthesis by Tn10 mutagenesis method in *B. subtilis*", *3rd İnternational Conference on Analysis of Microbial Cells at the Single Cell Level*, Semmering, Book of Abstracts, PIII.15, 71, Austria, 2005.

5. İrigül, Ö., Ülgen, A.G. ve A. Yazgan Karatas, "Induction Pattern of *yvgW*:Cpx-type ATPase", *8th BAGECO Conference of Bacterial Genetics and Ecology*, Lyon, Book of Abstracts, P11, 78, France, 2005.

6. İrigül, Ö., Karslı, G. ve A. Yazgan Karataş, "Properties of *yvgW* Spores in *B. subtilis*", *2nd FEMS Congress of European Microbiologist,* Madrid, Abstract Book, P.GEN.23, 241, Spain, 2006.

7. İrigül, Ö. ve A. Yazgan Karataş, "Effect of Sporulation Sigma Factors on the Expression of *yvgW* Gene in *B. subtilis", . 2nd FEMS Congress of European Microbiologist,* Madrid, Abstract Book, P.GEN.24, 241, Spain, 2006.

8. İrigül, Ö., Öztürk MA., Tokman N., Akman S. and Karatas A., "Heavy metal and mineral contents in *yvgW* mutant spores of *Bacillus subtilis*", *32nd FEBS Congress Molecular Machines*, Viyana, P222, 2007, Austria

9. İrigül, Ö. ve A. Yazgan Karataş, "SASP content and spore coat protein profile in *yvgW*spores", *ECB13, 13th European Congress on Biotechnology*, Barcelona, S252-S253, Spain, 2007

10 Ünlü E. C., Köroğlu T. E., **İrigül, Ö.**, Berkyürek A.C., Özcengiz G. Yazgan-Karataş A., The effects of Spo0A-AbrB regulation system on the expression of *yvfI* gene in *B.subtilis*. IUMS XII. International Congress of Bacteriology and Applied Microbiology, 5-9 August 2008, Istanbul. Absract Book BP-32, p.61

11. İrigül, Ö., Köroğlu T.E, Ünlü E.C., Özcengiz G., Yazgan-Karataş A. The regulatory effects of CodY and AbrB on the expression of *yvfI* in *B.subtilis*. IUMS XII. International Congress of Bacteriology and Applied Microbiology, 5-9 August 2008, Istanbul. Absract Book BP-39, p.64.

12. Köroğlu T.E., Ünlü E.C., **İrigül Ö.**, Özcengiz G., Yazgan-Karataş A. The effects of *spo0H* and *oppA* genes on the expression of *yvfI* gene in *B.subtilis*. IUMS XII. International Congress of Bacteriology and Applied Microbiology, 5-9 August 2008, Istanbul.

13. Karatas A., **İrigül Ö**, Köroğlu TE. The effects of *phr* peptides on the expression of yvfI gene in B.subtilis. 11th International Symposium on the Genetics of Industrial Microorganisms (GIM), 28 June - 1 July 2010, Melbourne, Australia.

14. İrigül Ö, Köroğlu TE, Tayran H, Karataş, A. Elucidation of regulatory behavior of Spo0A protein on *bac* operon. 35th FEBS Congress: Molecules of life, 26 June-1 July 2010, Gothenburg, Sweden.

15. Saygılı, E., Arslan, E., **İrigül, Ö**., Karataş, A. Identification of SASP and spore coat protein profiles in *bacA* spores. 35th FEBS Congress: Molecules of life, 26 June-1 July 2010, Gothenburg, Sweden

16. Arslan, E., Saygılı, E., **İrigul, Ö.,** Karataş, A. SASP content and spore coat protein profile in *yvfI* spores. 35th FEBS Congress: Molecules of life, 26 June-1 July 2010, Gothenburg, Sweden.

17. Gün, T., **İrigül, Ö.**, Karataş, A. The effect of ComK transcriptional regulator on the expression of *yvfI* gene in *Bacillus subtilis.* 35th FEBS Congress: Molecules of life, 26 June-1 July 2010, Gothenburg, Sweden.

18. İrigül, Ö., Köroğlu, T.E., Karataş, A. *B.subtilis*'te Quorum Sensing- Hücre Yoğunluğu Regülasyon Ağının *yvfI*- gen Ekspresyonu Üzerine Olan Etkileri. 1. Ulusal Moleküler Biyoloji ve Biyoteknoloji Kongresi, 26-29 Ekim 2010, Antalya.

19. Arslan, E., **İrigül, Ö.,** Köroğlu, T.E., Karataş, A. *yvfI* Mutasyonunun Sporlanma ile İlişkisi. 1. Ulusal Moleküler Biyoloji ve Biyoteknoloji Kongresi, 26-29 Ekim 2010, Antalya