CELL ENVELOPE STRESS RESPONSE AND ANTIMICROBIAL RESISTANCE IN *BACILLUS SUBTILIS*

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The cell envelope of bacteria is of pivotal importance for growth and survival, and hence it is often the target of antimicrobial compounds. One of the main components involved in CESRs are extracytoplasmic function (ECF) σ factors. The genome of *B. subtilis* encodes for seven ECF σ factors, σ^M, σ^W, σ^X, σ^Y, σ^V, σ^Z and σ^{YlaC}. Several studies have been conducted to understand the role that these ECF σ factors play in CESR in *B. subtilis*, one of the challenges found is that they display significant redundancy within their regulons.

In this study, we have performed an in depth analysis of one of the ECF σ factors of *B. subtilis*, σ^V, which had been previously uncharacterized. We have described the regulon of σ^V, the role that it plays in lysozyme resistance, and provided evidence for a novel promoter element important for σ^V recognition. Additionally, we have studied the role that σ^M plays in moenomycin resistance, and discovered a previously uncharacterized gene, *ypmB*, that seems to play an important role in cell envelope synthesis. Altogether, this dissertation takes further steps into understanding of the role that ECF σ factors play in regulating the stress response triggered by cell envelope acting antimicrobials in *B. subtilis*.

BIOGRAPHICAL SKETCH

Veronica Guariglia-Oropeza was born on July 10th, 1982, in Caracas, Venezuela. Even from her early years, Veronica was exposed to science and academia. With her mother going through graduate school for a doctorate in Botany, and her father in charge of the botanical gardens of the University, Veronica spent most of her childhood on campus and in research laboratories.

When the time came, it was an easy decision for Veronica to major in Biology at the Central University of Venezuela, in Caracas, and follow her parent's steps, with the difference of choosing to minor in Genetics and Biochemistry instead. Since then, she has been interested in the molecular mechanisms that rule the insides of cells.

For her undergraduate thesis project, she joined Dr. Guillermina Alonso's lab, where she studied the conjugational properties of plasmids found in bacteria isolated from natural environments in Venezuela, and graduated in December 2006.

During her college years, Veronica had the opportunity of doing a summer internship at the Fred Hutchinson Cancer Research Center in Seattle. She worked with Dr. Susan Parkhurst on a genetic screen for Capu, Rho and Spir interactions in *Drosophila melanogaster*. It was at this moment she decided to go to graduate school in the USA.

It took a bit of time, and saving up some money, for Veronica to realize her dreams. In the meantime, she worked as a research assistant under Dr. Maria E. Cavazza at the Biomedicine Institute in the Vargas Hospital in Caracas, Venezuela, screening *Helicobacter pylori cagA* and *vacA* genotypes in Cuban and Venezuelan populations.

Finally, in the summer of 2008, she arrived at Cornell to pursue her PhD in Microbiology. The move to the US, and adaptation to the new culture, went very smoothly,

especially with an amazing support group of family and friends. A year later, Veronica joined Dr. John Helmann's lab, to study extracytoplasmic function sigma factors and their role in cell envelope stress in *Bacillus subtilis*. This dissertation summarizes her major findings.

ACKNOWLEDGMENTS

The five years I have spent at Cornell have left me filled with incredible moments and experiences. The completion of this work would not have been possible without the help and support of the people I would like to acknowledge here.

I am incredibly grateful for my supervisor and mentor John Helmann. His passion for science and unbelievable knowledge inspires everyone to always dig deeper and keep up with recent findings. He has been great in guiding me through my projects but always leaving me room to experiment on my own. He was always available to talk and very approachable. This work would have not been the same without him. I am also thankful to my two minor committee members, Joe Peters (Genetics) and David Stern (Biochemistry) for their advice, availability, and encouragement.

John is a great leader in the lab, but the second in command, senior research associate Ahmed Gaballa, is the go-to guy for everything else. From technical details of protocols, little tricks to make everything work, how to repair a spectrophotometer or optimize *in vitro* transcription reactions, from biochemistry, genetics and microbiology to politics and football (soccer), Ahmed knows it all. I truly enjoyed having him in lab, and this work would not have been possible without him.

The Helmann lab as a whole has offered a fun, relaxed and overall great work environment, always filled with good energy and passion for research. Both current and past member have influenced my development as a person and a scientist, and it has been a pleasure to work with all of them.

The department of Microbiology is filled with wonderful people that need their recognition as well. I would like to thank the Winans lab members for their friendship and emergency supply of reagents. They were also the best lunch hosts and threw the best happy hours on Fridays. The secretaries of the department, especially Shirley and Patti, were supportive of me from day one, literally, offering a ride the airport and a place to stay my first night in Ithaca. Throughout the years, they have always continued to offer their help me, and I do not know how the department would function without them.

Throughout my years in Ithaca, I've made a great group of friends who have made my stay here enjoyable. My classmates and colleagues in Wing Hall, the CFC staff, the Spanish Catholic community of Ithaca, and the lovely ladies of the Ithaca Women's Soccer League. Without them, my years in Ithaca would not have been as fun and fulfilling.

Last but not least, none of this would have been possible without the support and love of my family. My mom and dad are very much a part of all my successes as a professional and as a person, and even though my dad does not get to live this moment, I know he is proud of me in heaven. And Luis who is not only my husband, my best friend, my soul mate and my soccer coach, but also my number one fan and supporter; without him, the long winters would not have been as warm.

To my parents

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CHAPTER 1

INTRODUCTION

1.1 Cell envelope structure and synthesis in *Bacillus subtilis*.

Bacillus subtilis is the best characterized Firmicute (77), and as such, serves as a model for understanding a wide range of physiological, developmental and molecular processes. The cell envelope of *B. subtilis* is composed of a cell membrane surrounded by a thick peptidoglycan (PG) layer and associated anionic polymers (30, 76) (Figure 1.1). The cell envelope as a whole serves of a barrier to counteract changes in the outside environment, provides shape, and actively and selectively allows for the exchange of molecules inside and out of the cell (45, 90). Therefore, it is no surprise that the cell envelope is a major target for antibiotics.

The cell membrane of *B. subtilis* is complex and composed of a predominantly phospholipid bilayer with embedded membrane proteins, lipoproteins, and lipid anchored cell wall components. Overall the cell membrane is negatively charged due to the abundance of anionic lipids (Phosphatidylglycerol and cardiolipin) which contributes to the ability of cationic antimicrobial peptides (CAMPs) to disrupt bacterial membrane function (23, 63). Additionally, *B. subtilis* contains teichoic acids (TA) which are either membrane associated (lipoteichoic acid, LTA) or PG-associated (wall teichoic acid, WTA) (Figure 1.1) (11). The composition of these anionic polymers varies widely between species and can change significantly in response to growth conditions (63).

PG consists of long glycan strands cross-linked via peptide side chains. The abundant crosslinking gives rise to a large macromolecule with a high degree of mechanical strength (30).

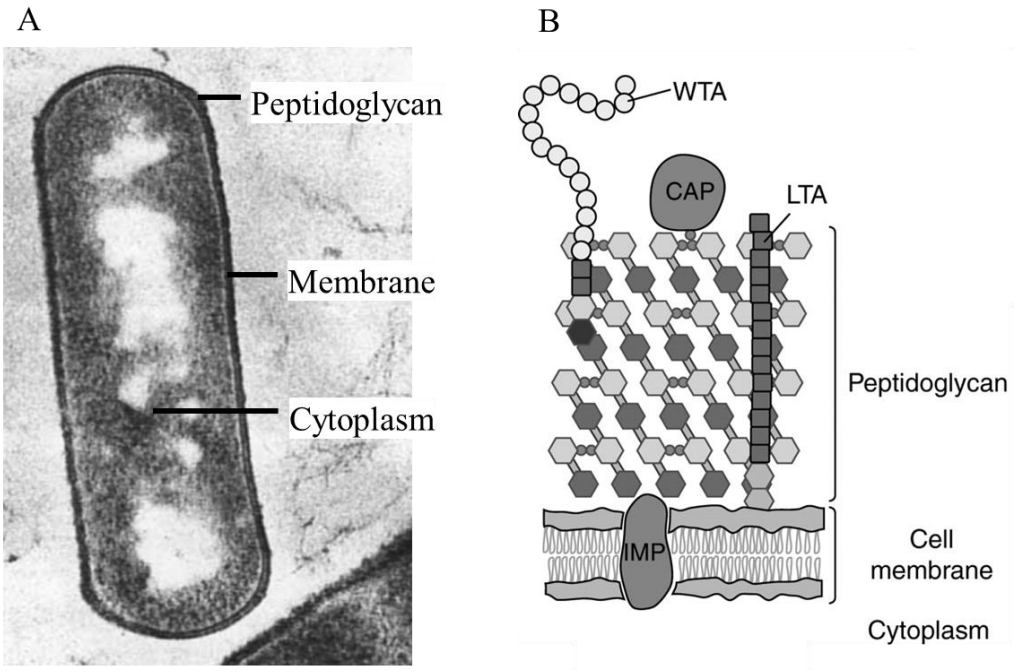

Figure1.1. *Bacillus subtilis* cell envelope structure. (A) Electron micrograph of a *B. subtilis* cell. Modified from (27). (B) Gram positive cell envelope structure. WTA, Wall Teichoic acid. LTA, LipoTeichoic Acid. CAP, Covalently Attached Protein. IMP, Integral Membrane Protein.

Hence, the PG layer is the major determinant of bacterial cell shape (45). The glycan strands are made up of repeating disaccharide residues of N-acetyl glucosamine (GlcNAc) and N-acetylmuramic (MurNAc) that are crosslinked by peptide side chains. The glycan chain lengths vary between species (95).

Being the major component of the cell wall and due to its great importance, PG synthesis is a highly regulated, complex pathway. The first step is the formation of uridine diphosphate-N-acetyl-muramic acid from uridine diphosphate -N-acetyl-glucosamine. In a series of steps, a pentapeptide is added to the MurNAc molecule (47). In *B. subtilis*, this pentapeptide is composed of L-Ala-D-Glu-mDAP-D-Ala-D-Ala. When the UDP-MurNAc-pentapeptide is covalently bonded to the membrane bound undecaprenyl-phosphate, Lipid I is formed. When an additional GlcNAc is attached to Lipid I, Lipid II is formed. Lipid II is then translocated to the extra-cytoplasmic face, where the GlcNAc-MurNAc-pentapeptide is incorporated into the nascent PG strand by the action of a transglycosylase (TG). Additionally, the pentapeptide side chain is cross-linked by a transpeptidase (TP). Many of the enzymes and intermediate products are targets of antibiotics (Figure 1.2).

As the glycan strands grow and polymerize the cross-linking between adjacent strands creates a three dimensional meshwork (6, 30, 64). The rigid sugar chains cross-linked with flexible peptide bridges allows for the PG layer to be a strong but also elastic stress bearing structure, which permits the constant assembly and disassembly that comes with cellular growth and division (47).

PG synthesis, breakdown and assembly require high level coordination of multi-protein complexes (25). Rod-shaped bacteria, like *B. subtilis*, alternate their machineries between division and elongation in a process that is spatially and temporally regulated. Several lines of

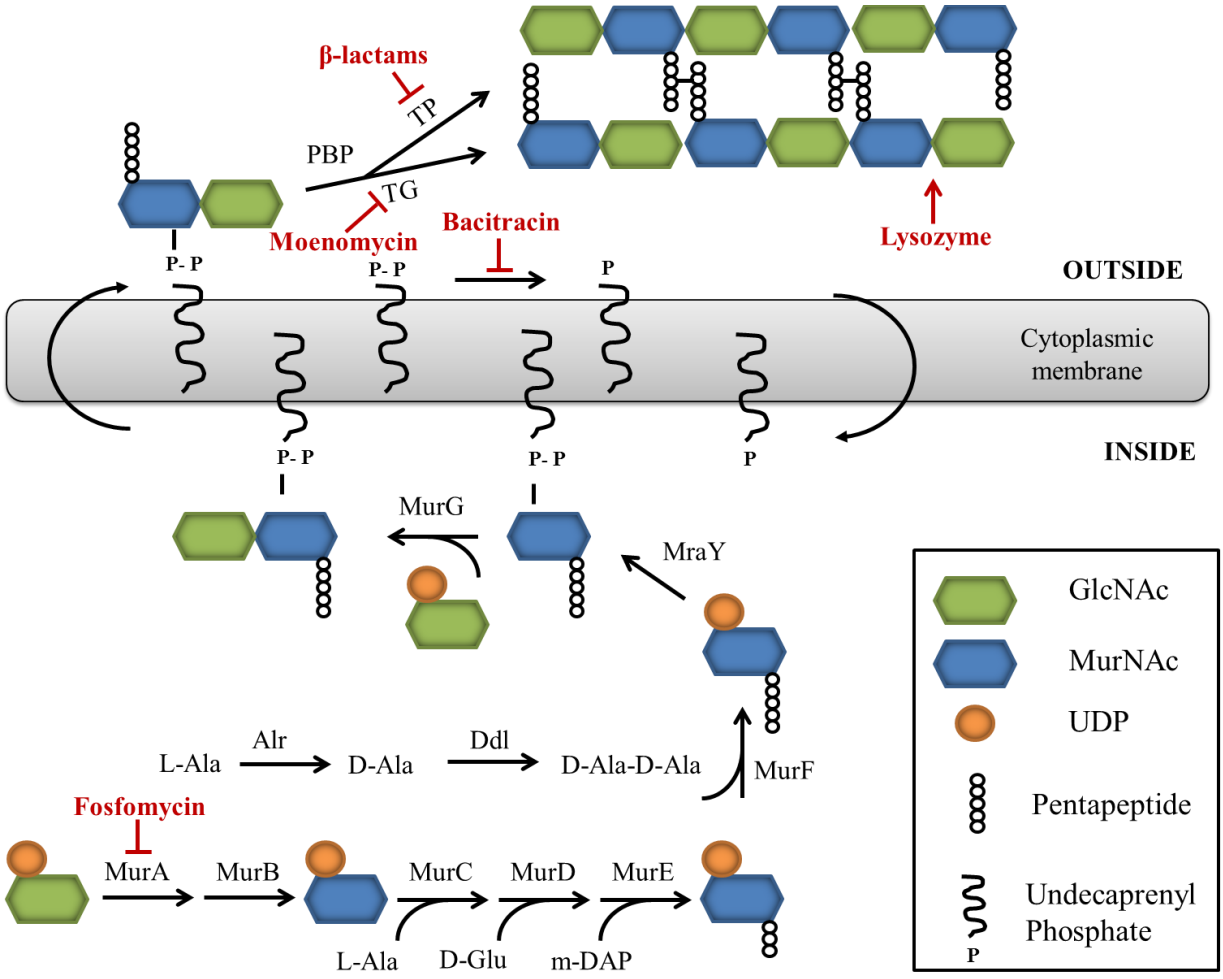

Figure 1.2. Peptidoglycan synthesis in *B. subtilis*. Antimicrobials relevant to this study are in red, the antibiotics with a —| on the step they inhibit, and lysozyme with an arrow where it cleaves the PG. GlcNAc, N-Acetyl-glucosamine. MurNAc, N-Acetyl-muramic acid. UDP, Uridine diphosphate. m-DAP, *meso*-diaminopimelic. TP, transpeptidation. TG, transglycosylation. Adapted from (47).

evidence suggest that the actin like protein, MreB, plays a central role in this regulation (97). Furthermore, the synthesis and maturation of PG in cell elongation is scaffolded on MreB, and coupled with the tubulin-like protein FtsZ at mid-cell during division (Figure 1.3).

In accordance to its important role, MreB and its homologs (Mbl and MreBH) recruit and interact, directly or indirectly, with proteins involved in cell shape and division (e.g. RodA, MreC, MreD, DivIVA), PG biosynthesis and hydrolysis (e.g. MurF, PBP1, PBP2a, PbpH and LytE), TA biosynthesis (e.g. TagU and TagT), and a few uncharacterized proteins (e.g. YpmB and YerH) (48).

MreB has been long thought to form continuous helical filaments along the length of the cell which has been evidenced shown in multiple studies using fluorescence microscopy (18, 19, 24, 26, 46). However, recent advances in microscopy techniques and novel biochemical data suggest that MreB might function differently than previously thought (97). It is now believed that MreB and a few PG elongation proteins are added in short patches as opposed to long helical filaments (83, 87). Additionally, this movement has been shown to be bidirectional and follows along after synthesis, rather than guiding and determining PG synthesis, as previously thought (34).

On the other hand, FtsZ polymerizes into an oligomeric structure that forms the initial ring at midcell, and recruits over a dozen proteins to form the divisome. This multi-protein complex carries out the processes of preseptal elongation, septum formation and cell separation (2, 29, 74).

1.2 Cell envelope degrading enzymes and antibiotics.

The pivotal importance of the cell envelope for bacterial growth and survival makes it a perfect

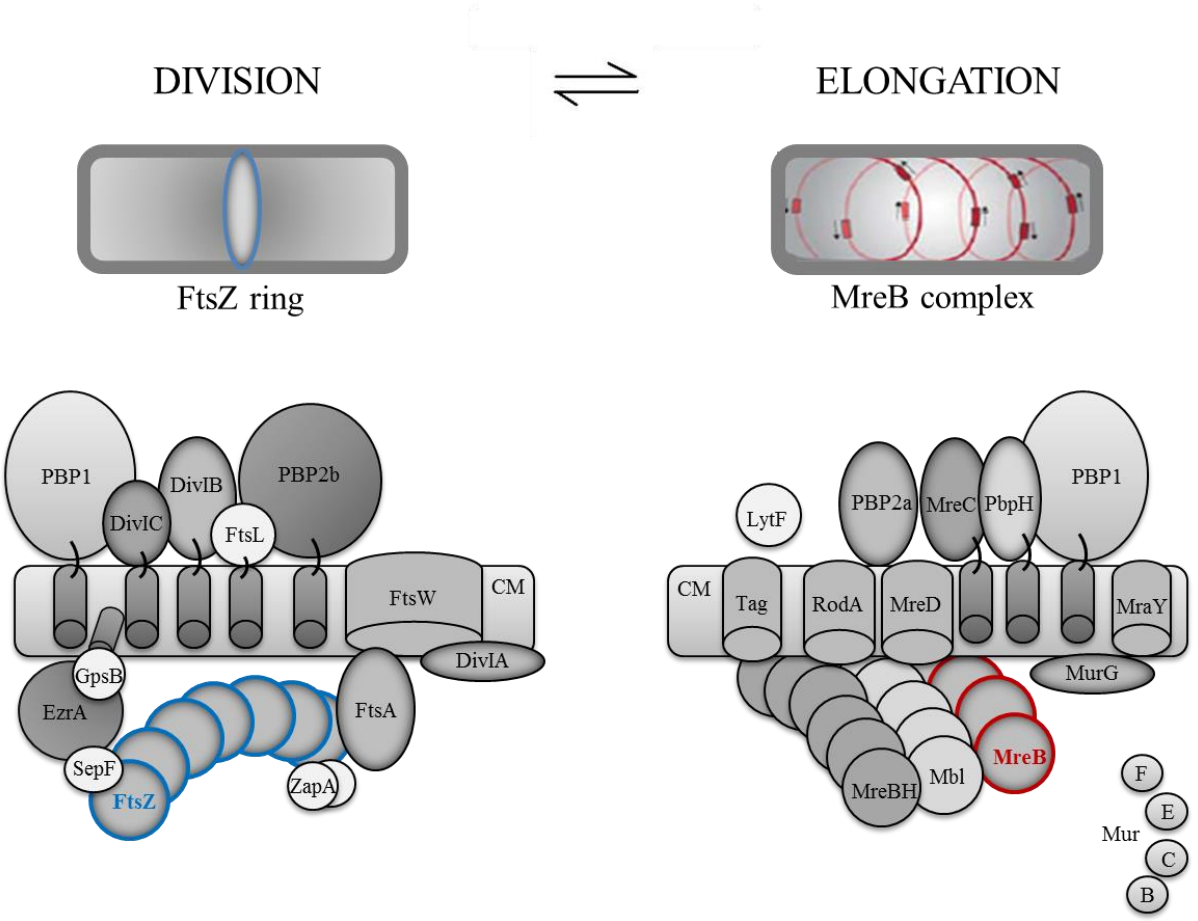

Figure 1.3. Peptidoglycan synthesis complexes during division and elongation in *B. subtilis*. FtsZ (blue) assembly of the divisome machinery at the septal ring (Left). MreB (red) assembly of the PG synthesis machinery for lateral wall (Right). Adapted from (20, 22, 25, 32, 97).

target for antibiotics and antimicrobial compounds. These compounds can either inactivate a biosynthetic enzyme, sequester a substrate, or actively degrade PG (Figure 1.2). There are many different compounds that attack the cell envelope, here focus will be given to only those that pertain to the later chapters.

1.2.1 Moenomycin

Moenomycins (MOE) are the only known group of antibiotics that directly inhibit bacterial peptidoglycan glycosytransferases (67), and they are produced by at least four different streptomycete strains (94). Based on their chemical composition, moenomycins are classified as phosphoglycolipids (85, 96). They directly inhibit peptidoglycan glycosyltransferases (PGTs) involved in the penultimate step of bacterial cell wall biosynthesis (86) by mimicking their substrate, lipid IV (Lipid II with an additional disaccharide) (Figure 1.4). Even though they are not used in humans due to their suboptimal pharmacokinetic properties, MOE have been successfully commercialized as animal growth promoters under the trademarks Flavomycin and Flavophospholipol (a natural mixture of structurally related phosphoglycolipids) (70).

There have been no reports of animal or human isolates resistant to MOE, although many *Enterococcus faecium* strains are reported to be naturally resistant (1). No significant natural cross-resistance has been revealed between MOE and other clinically useful classes of antibiotics and no plasmid-borne moenomycin resistance determinants have been detected (70). Even though its mode of action is well understood, there are currently no mechanisms to explain resistance to MOE. This is the main focus of chapter 4.

1.2.2 Peptidoglycan hydrolases.

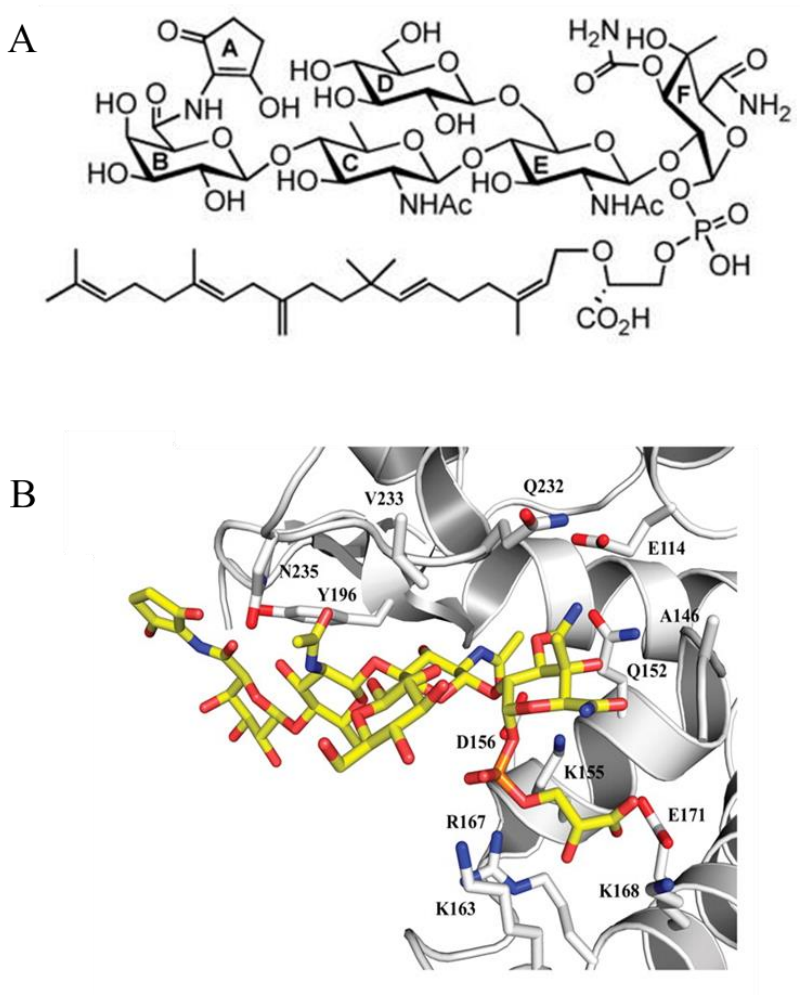

Figure 1.4. Moenomycin structure and its interaction with transglycosylase. A) Chemical structure of the glycolipid moenomycin (82). B) Crystal structure of moenomycin (yellow backbone) bound to the transglycosylase domain (white backbone) of PBP2 of *S. aureus* (55).

Bacterial peptidoglycan hydrolases have a large range of functions. They can be involved in growth, cell division, autolysis and even signaling. Furthermore, there are examples of hydrolases for every glycosidic bond in peptidoglycan (91). Some of these enzymes act on the bond between the glycan strand and the peptides, such as N-Acetylmuramyl-L-alanine amidases (44), while others act on the peptide sidechain like carboxy- and endopeptidases (35). There are also hydrolases that cleave within the glycan strand (glycosidases), such as N-acetylglucosaminidases, lysozymes and lytic transglycosylases (44).

Lysozymes hydrolyze the β-1,4 glycosidic bonds between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlucNAc) resulting in a product with the a terminal reducing MurNAc residue. In contrast, lytic transglycosylases cleave the glycosidic bond with a concominant transglycosylation reaction, resulting in the formation of the 1,6-anhydro ring at the MurNAc residue of the product (Figure 1.5) (91).

Aside from its muramidase activity, lysozyme also shows a cationic antimicrobial peptide activity (43), and hence, resistance to lysozyme can be achieved through either modification of the substrate (for example, O-acetylation) and/or change the overall net charge of the cell envelope (for example, D-alanylation) (51).

Lysozymes are ubiquitous since they can be produced by a broad range of organisms from phages, bacteria and fungi to vertebrates and invertebrates. A few bacterial lysozymes have been described, such as Pesticin from *Yersinia pestis* (92), two autolytic lysozymes from *Enterococcus faecium* (3, 49), cellosyl from *Streptomyces coelicolor* (73, 89) and the autolysin LytC from *Streptococcus pneumoniae* (33).

Different soil bacteria that share their niche with *B. subtilis* are known to produce and secrete peptidoglycan hydrolases. The genome of *S. coelicolor* is predicted to encode for 56

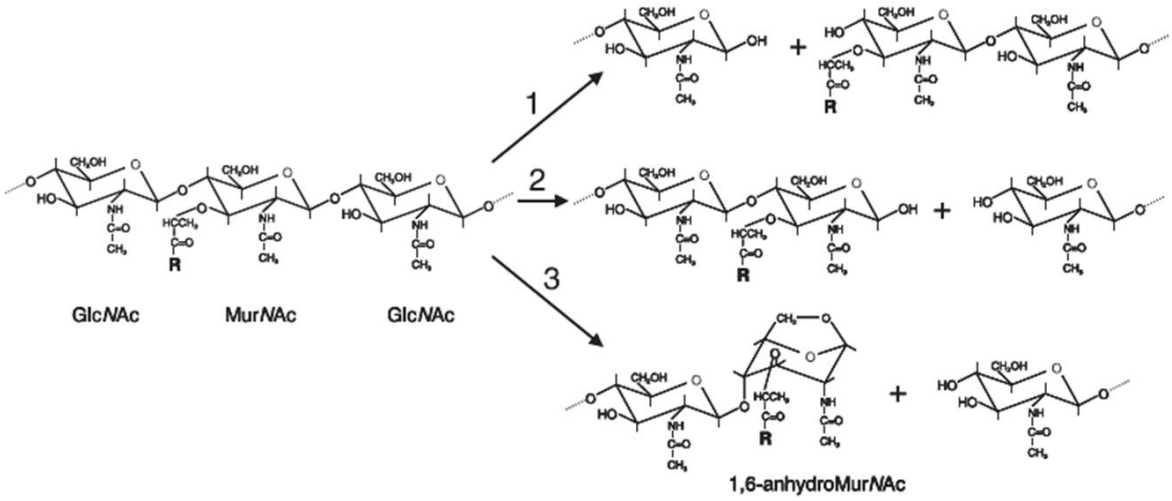

Figure 1.5. Enzymatic cleavage of peptidoglycan by **1,** N-Acetyl-glucosaminidases, **2,** lysozymes and **3,** lytic transglycosylases. R, peptide attached to the lactyl residue of MurNAc (91).

candidate cell wall hydrolases genes, some of these have been confirmed to be hydrolytic enzymes that are able to degrade purified cell walls (38). The predatory *M. xanthus* also produces several hydrolytic enzymes with different activities (81).

1.2.3 Signaling by antimicrobial compounds.

In addition to inhibiting cell growth and killing of competitors, it is now thought that microbial metabolites are involved in cell-signalling within a microbial populations as well as at the interspecies level (54, 75).

Free living soil bacteria such as myxococci, bacilli, and streptomycetes undergo dramatic developmental changes in response to environmental stimuli. When these bacteria encounter each other, the secondary metabolites released to the environment generate a developmental response in the surrounding neighbors (79). For example, sporulation of a lawn of *S. coelicolor* is disrupted by a growing colony of *B. subtilis* within the lawn (80). Interspecies chemical communication is of growing importance and relevance and it will be discussed briefly on chapter 2.

1.3 RNA polymerase, sigma factors and ECF sigma factors.

The action of antimicrobial compounds on the cell wall triggers a specific and highly regulated stress response. In Gram positive bacteria, this stress response is mediated by two component systems and extracytoplasmic sigma factors (47). Several factors ensure the specificity of this response, but for the purpose of this dissertation focus will be given to those at the transcriptional level, and more specifically, to those which pertain to ECF σ factors.

1.3.1 Transcriptional regulation.

Gene expression is a highly regulated process. A cell not only needs to express all the necessary proteins for housekeeping functions, but it also needs to adapt to different environments and, in some cases, differentiate into multiple cell types. Gene expression involves the transcription of DNA into RNA and the subsequent translation to protein. Since transcription is the first step in gene expression it is, in most cases, regulated at many levels (42). The process of transcription consists of initiation, processive elongation and termination (21).

Transcription initiation, the most heavily regulated stage of the transcription cycle, can be subdivided into promoter complex formation, abortive initiation, and promoter escape (7). Each step involves sequence specific DNA-protein interactions between the RNA polymerase (RNAP), the σ subunit and the promoter DNA. The promoter is defined as the region of DNA that interacts directly with RNAP during initiation of transcription. The recognized sequence of the promoter varies depending on the holoenzyme (RNAP core + σ). For σ^{70} class promoters (see below), the key recognition elements are usually located at -35 and -10 base pairs upstream of the transcription start point (68).

1.3.2 RNAP and σ factors

Even though core RNAP is competent for transcription, it is not capable of promoter-directed transcription initiation (8). For transcription initiation, core RNAP requires the specificity determining σ factor. The vast majority of σ factors belong to the σ^{70} family due to their relationship to the principal σ factor of *Escherichia coli*, σ^{70} (68). A second family of σ factors, the σ^{54} family, forms a RNAP holoenzyme that recognizes promoters with alternative recognition elements and needs additional sources of energy for transcription initiation (9). On

the basis of gene structure and function, the σ^{70} family can be divided into different groups (40, 53).

Group 1 consists of the essential primary σ factors responsible for the majority of transcription of housekeeping genes. Group 2 σ factors are closely related to the primary σ factors but are dispensable for bacterial growth. Group 3 σ factors are more distantly related to σ^{70} and usually activate a response to specific signals, such as developmental checkpoints. Group 4, the largest and most diverse group, contains the ECF subfamily which regulates stress responses related to the cell envelope. Finally a proposed Group 5 includes proteins that function as σ factors but have not been widely recognized due to their divergent sequence; this group includes regulators of toxin production (40).

Multiple sequence alignments of proteins of the σ^{70} family reveal four regions of evolutionary conservation, termed regions 1 to 4 (σ_1 to σ_4) (40, 53). Only σ_2 and σ_4 are well conserved in all members of the σ^{70} family (Figure 1.6). σ_2 can be subdivided into four sub-regions ($\sigma_{2.2}$- $\sigma_{2.4}$) involved in the binding of σ to the core RNAP, and the recognition and melting of the -10 element of the promoter DNA. The crystal structure of σ_2 has been solved for many σ factors (12, 52, 59) and it shows that σ_2 is composed of three α helices. The third helix includes conserved residues involved in DNA melting and recognition of the -10 element of the promoter. σ_4 can be subdivided in two sub-regions ($\sigma_{4.1}$- $\sigma_{4.2}$) involved in binding to the core RNAP, recognizing of -35 element of the promoter, and contacting activators. The crystal structure for σ_4 has also been solved. σ_4 consists of two pairs of α helices where the carboxy-terminal pair forms a helix-turn-helix motif that contacts the -35 element of the promoter (12, 88). The helix formed by $\sigma_{4.1}$ and $\sigma_{4.2}$ sits in the major groove of the promoter DNA and several amino acids directly contact the nucleotides (Figure 1.7 A and B) (50).

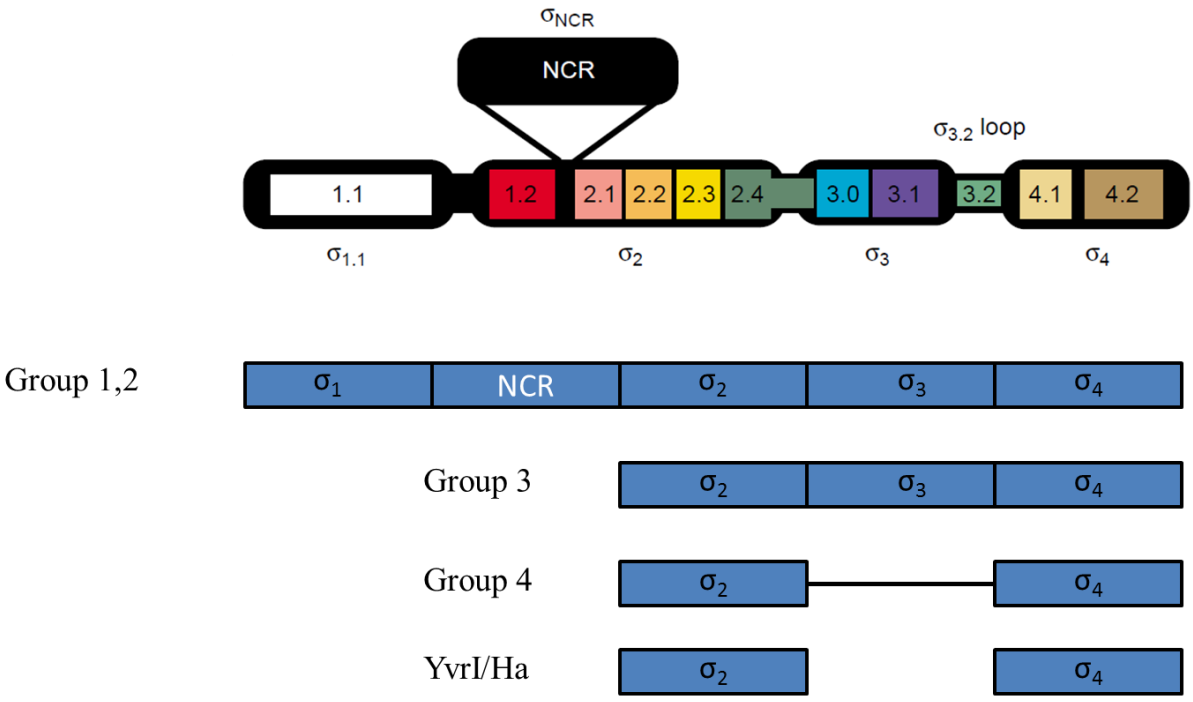

Figure 1.6. Conserved σ factor regions. Modified from (41, 58, 65)

The protein structure of RNAP holoenzyme bound to fork-junction promoter DNA has been solved for *Thermus aquaticus* (Taq) (65, 66). The structure shows that all of the sequence-specific contacts with the promoter are mediated by the σ subunit (Figure 1.8). This structure also shows two important aspects of promoter recognition: 1) there seems to be structural plasticity in the holoenzyme that allows repositioning of the β-flap and the bound σ_4 with respect to the DNA, and 2) the RNAP can bend the intervening DNA to correctly position the -10 and -35 elements with respect to each other.

Usually, bacteria contain one σ factor devoted to housekeeping functions and an array of alternative σ factors that can vary in number and function. In general, organisms with more varied lifestyles have more σ factors. These organisms may encounter many different environments and stresses that would require adjustments to their metabolism and physiology. Since all of these σ factors compete for RNAP, it is not surprising that this process is tightly regulated. The σ factor pool in the cell can be adjusted by 1) synthesizing new σ, 2) activating or degrading existing σ, or 3) changing competition parameters for RNAP (36).

1.3.3 ECF σ factors of Bacillus subtilis and their role in cell envelope stress response

The genome of *B. subtilis* encodes 7 ECF σ factors: σ^M, σ^W, σ^X, σ^V, σ^Y, σ^Z and σ^{ylaC} (39, 40) (Figure 1.9). The target genes (or regulon) for five of these have been identified, however σ^Z and σ^{ylaC} still remain largely unknown (41). Out of the five better studied, σ^Y is the most elusive: it appears to control a small regulon of less than a dozen genes with poorly defined functions (16).

The other four, σ^M, σ^W, σ^X and σ^V, all seem to play important roles in setting up a defense mechanism against antimicrobial compounds and stresses. σ^M regulates a large set of genes that include essential functions of cell division and envelope synthesis (28). It is induced by

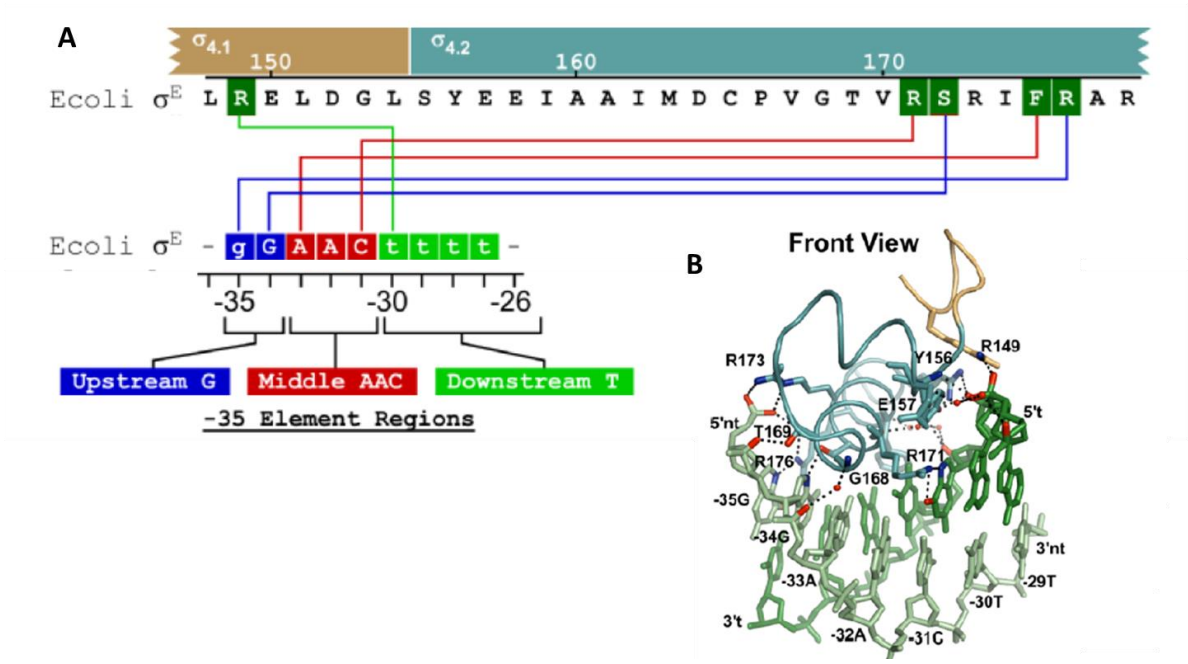

Figure 1.7. Structural basis of promoter -35 element recognition by σ_4 of σ^E. A) Correlation of σ_4 and -35 element sequences for *E. coli* σ^E. The top shows the sequence of the proposed -35 element DNA binding region. The residue positions that are important in -35 element DNA recognition in the Ec σ^E_4/-35 element DNA structure are highlighted green. The bottom shows the known -35 consensus sequence from σ^E. The three -35 element regions are highlighted with the upstream G region (blue), the middle AAC motif (red), and the downstream T rich region (green). Lines connecting the two sections indicate protein residue–DNA base interactions important for -35 element recognition in the Ec σ^E_4/DNA structure. B) Stereo view of the Ec σ^E_4/-35 element DNA complex. The protein is shown as an α-carbon backbone worm, with $\sigma^E_{4.1}$ colored yellow and $\sigma^E_{4.2}$ colored light blue. The DNA is color-coded in green (light for non-template and dark for template). Modified from (50).

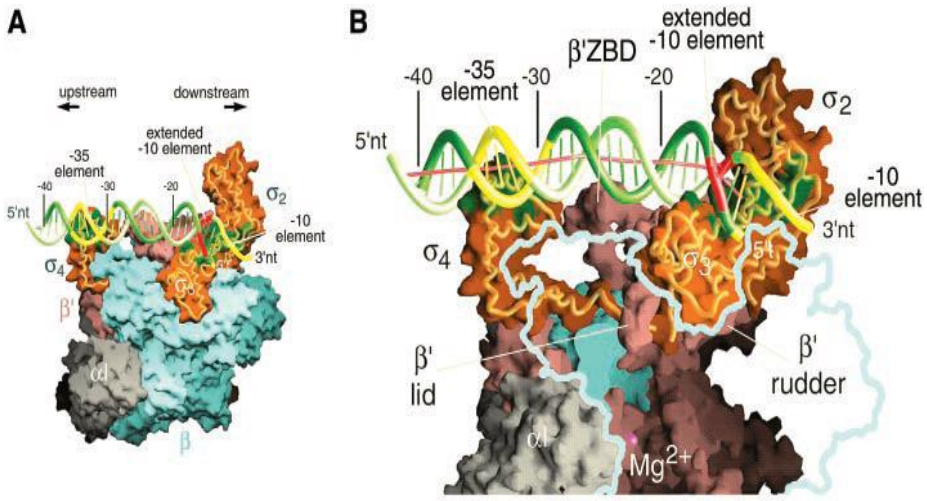

Figure 1.8. Taq RNAP holoenzyme/fork-junction DNA structure. (A) Overall view of the complex. (B) Magnified view showing only a part of the complex (66).

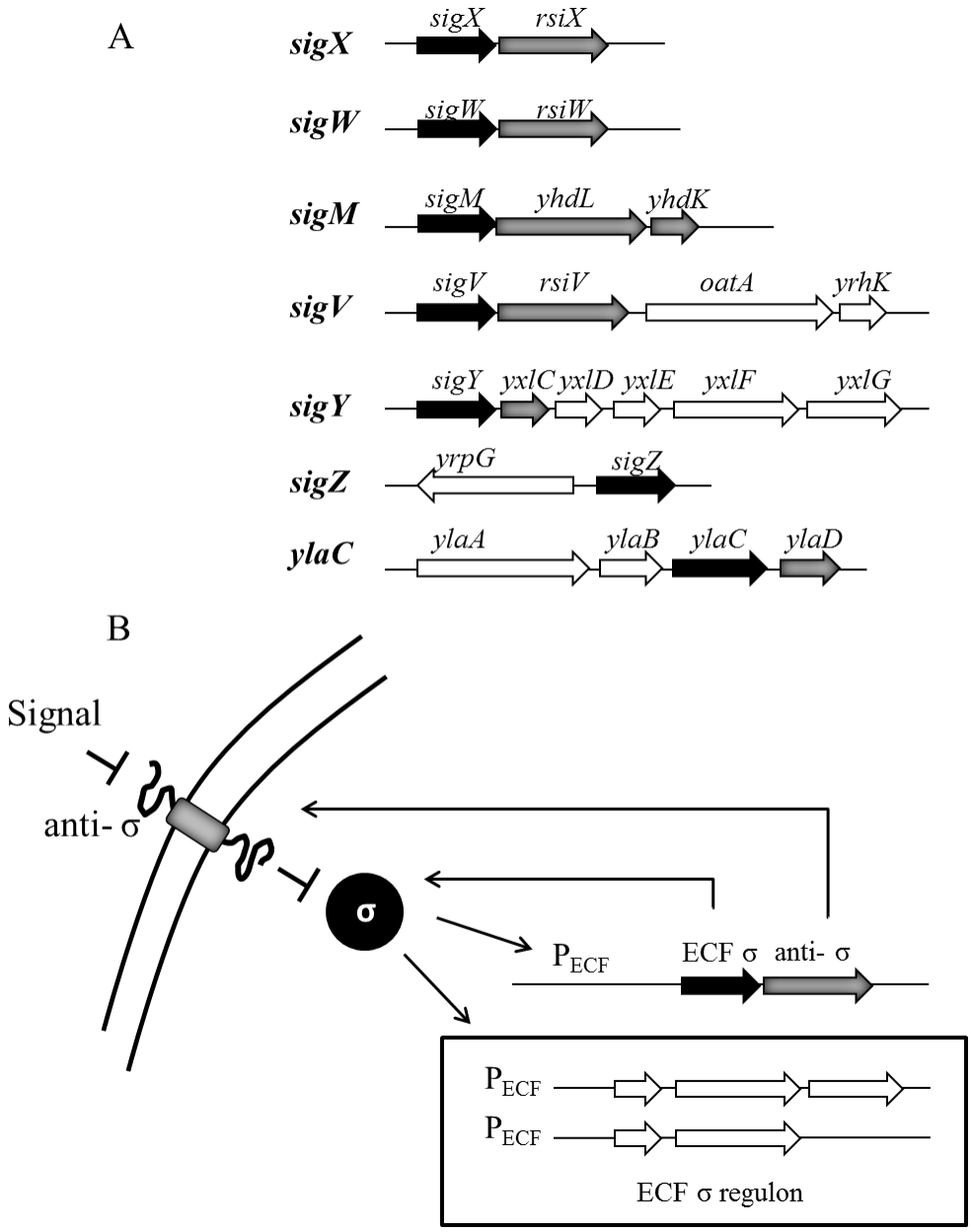

Figure 1.9. ECF σ factors operons and regulation in *B. subtilis*. A) Operon organization. ECF σ factor genes are depicted as black arrows and anti σ genes as grey arrows. Genes that are co-transcribed with the ECF σ factor operon are shown as white arrows. B) ECF σ factor positive autoregulation. ECF σ factor is inactivated and bound to the membrane by the anti-σ factor, until a signal triggers its release. The free ECF σ factor can now drive the transcription of other genes, often distributed around the chromosome, in response to that signal. Modified from (40).

bacitracin, vancomycin and moenomycin, amongst other stresses, and its activation confers resistance to both bacitracin and moenomycin. Bacitracin inhibits PG synthesis by binding to undecaprenyl pyrophosphate (UPP) and thereby preventing its recycling to the monophosphate form (78) (Figure 1.2). σ^M contributes to bacitracin resistance by upregulating the expression of *bcrC* (15, 61), a UPP phosphatase which competes with bacitracin for the UPP substrate (5). Moenomycin inhibits the transglycosylation step in PG synthesis (67). However, the mechanism by which σ^M confers moenomycin resistance is not clear, and is the main focus of chapter 4.

The σ^W regulon includes at least 60 genes that inactivate, sequester, or eliminate toxic compounds from the cell (39). σ^W is probably the best studied ECF σ factor of *B. subtilis*. It is induced by several cell wall acting antibiotics including fosfomycin, bacitracin and vancomycin (10, 17, 71), detergents (e.g. SDS and Triton-X) (17), and alkali stress (98). Activation of σ^W in turn provides resistance to some of its inducers, such as fosfomycin, by up-regulating the expression of *fosB*, a bacillithiol-S-transferase that inactivates fosfomycin (13, 31). A few other mechanisms of resistance provided by σ^W have been studied, but they are beyond the scope of this dissertation.

The σ^X regulon includes genes which serve to alter cell surface properties to provide protection against antimicrobial peptides (14). Two important operons, *dltABCDE* and *pssA-ybfM-psd*, are under σ^X regulation. The Dlt proteins incorporate positively charged D-alanine into TA (69). PssA and Psd catalyze the synthesis of the neutral cytoplasmic membrane lipid phosphatidylethanolamine (62). The incorporation of both positively charged TA and neutral lipids reduces the overall negative charge of the membrane providing protection against cationic antimicrobial peptides (14).

σ^V is perhaps the most recently studied ECF σ factor of *B. subtilis*. Originally thought to regulate only a small set of genes (99), σ^V didn't acquire relevance until studies in *E. feacalis* showed that in this organism, σ^V contributes to survival following heat, acid and ethanol treatment (4); and more recently, lysozyme (51). In *B. subtilis* σ^V regulates a set of ~30 genes and is highly induced by, and provides resistance to, lysozyme (Chapter 2) (37).

1.4 ECF σ factor regulation

The operon organization of the ECF σ factors of *B. subtilis* is variable. In general, the σ factor is co-transcribed with its anti-σ factor, which is usually a membrane protein that inactivates σ by sequestering it to the cytoplasmic membrane (Figure 1.8). In most cases, the ECF σ factors of *B. subtilis* positively regulate their own expression (40).

The ECF σ factors of *B. subtilis* recognize structurally similar promoter sequences, characterized by a highly conserved AAC motif in the -35 region and a CGT motif in the -10 region (40), which suggests a potential for regulatory overlap (Figure 1.10). It has been shown that the autoregulatory promoter sites for the *sigW* and the *sigX* genes are specifically recognized by their cognate σ factor; however, only one or two base pair changes in the -10 element can switch the target promoter from one σ to another (72). Previous studies in our laboratory have shown a significant overlap in the regulon of σ^W, σ^X, σ^M, and more recently σ^V, suggesting an overlap in their recognition specificities (37, 60). Due to this overlap, a given phenotype often involves several ECF σ factors (15, 56, 57, 60).

The concept of overlap in regulation between σ factors is not uncommon. In *E. coli* this phenomenon has been studied in some detail, particularly for σ^{70} and σ^S. The stress response σ^S

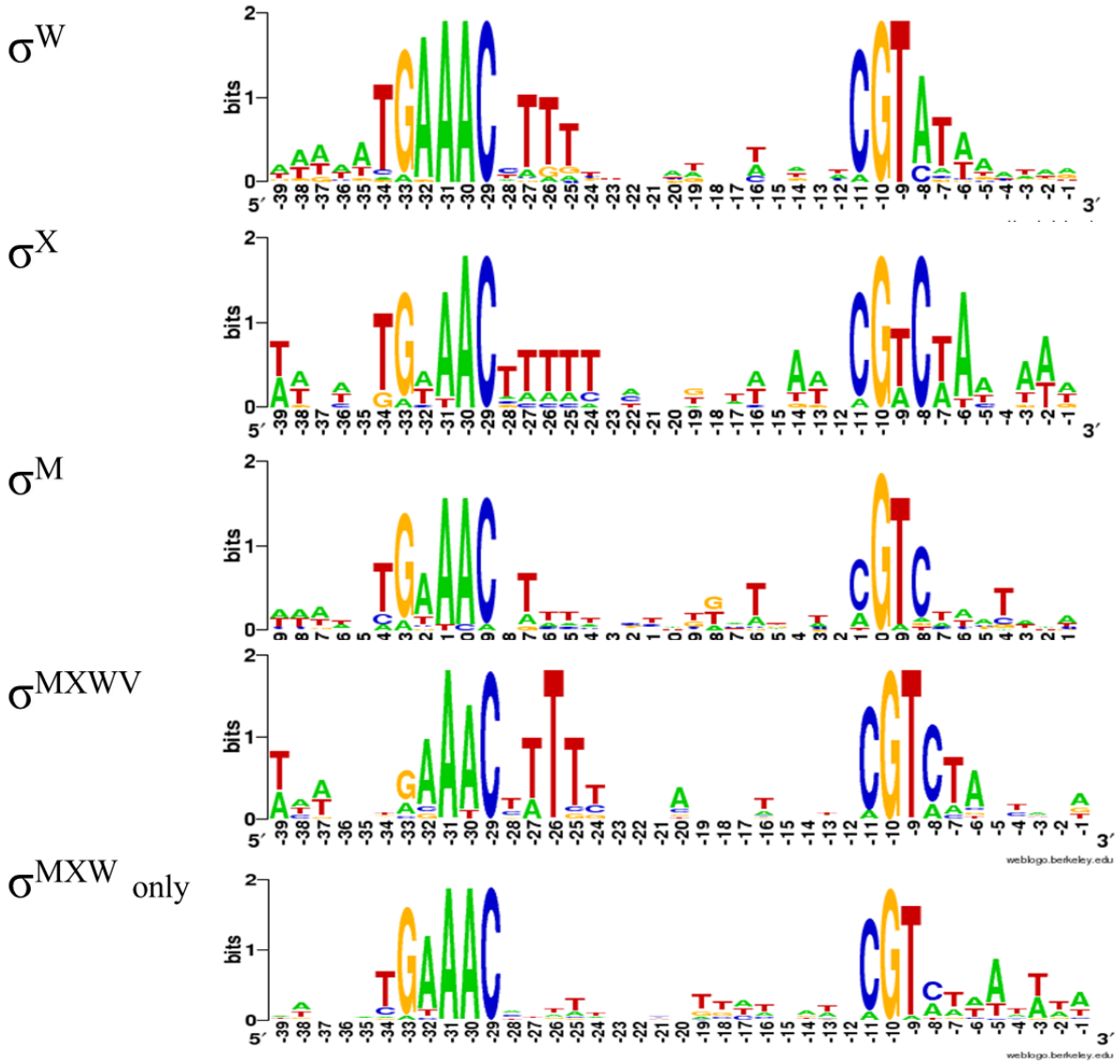

Figure 1.10. Promoter sequence consensus recognized by the ECF σ factors of *B. subtilis*. Alignments were generated using the promoter sequences recognized by each single or multiple σ factors and the consensus were created using the Weblogo serves (http://weblogo.berkeley.edu/). Adapted from (37, 60)

of *E. coli* recognizes almost identical promoter elements to the housekeeping σ^{70}. However, there have been reports of variable cis-acting promoter features and trans-acting protein factors that determine whether a promoter is recognized by RNAP containing σ^{S} or σ^{70} (84). A similar situation exists for σ^{32}/σ^{70}, suggesting that extensive functional overlap between σ factors is an important phenomenon (93).

There are several promoter structures that confer specificity for σ factor recognition. In chapter 3, we provide evidence for a novel one within the spacer region of ECF σ – recognized promoters.

1.5 Dissertation summary

This dissertation is aimed at taking further steps into the understanding of the role that ECF σ factors play in regulating the stress response triggered by cell envelope acting antimicrobials. The combination of transcriptional, physiological and biochemical approaches served to elucidate a few of these issues and to open up new interesting lines of research.

In chapter 2 a mutant strain lacking all seven ECF σ factors was used to ectopically induce the expression of σ^{V} to study the transcriptomic response ensued by the activation of this ECF σ factor. The regulon of σ^{V} revealed abundant overlap with the regulons of σ^{M}, σ^{X} and σ^{W}. Two of the operons regulated by σ^{V}, *dltABCDE* and *oatA*, proved to be indispensable in conferring σ^{V}-dependent lysozyme resistance in *B. subtilis*.

From the studies derived in chapter 2, it was observed that a stretch of Ts in the spacer region of the promoters regulated by σ^{MXWV} was highly conserved, and virtually absent from promoters only regulated by σ^{MXW}. In chapter 3 we hypothesized that the stretch of Ts is

important for specificity of recognition by ECF σ factors, and show *in vivo* and *in vitro* data that this is likely the case.

Finally, chapter 4 aims to elucidate the mechanism by which σ^M is responsible for moenomycin resistance in *B. subtilis*. We serendipitously came across a protein of previously unknown function, YpmB, which seems to have an important role in cell wall synthesis.

Overall, *B. subtilis* proves to be once again a great model for studying cell envelope stress response. The crucial function of the bacterial cell wall makes it the perfect target for antibiotics and PG degrading enzymes. The ECF σ factors of *B. subtilis* play a crucial role in regulating the setup of a defense mechanism in response to such stresses. Hence the study of such mechanisms is of pivotal importance in health and industry.

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CHAPTER 2

BACILLUS SUBTILIS SIGMA "V" CONFERS LYSOZYME RESISTANCE BY ACTIVATION OF TWO CELL WALL MODIFICATION PATHWAYS: PEPTIDOGLYCAN O-ACETYLATION AND D-ALANYLATION OF TEICHOIC ACIDS

The seven extracytoplasmic function (ECF) sigma (σ) factors of *Bacillus subtilis* are broadly implicated in resistance to antibiotics and other cell envelope stressors mediated, in part, by regulation of cell envelope synthesis and modification enzymes. We here define the regulon of σ^V as including at least 20 operons many of which are also regulated by σ^M, σ^X, or σ^W. The σ^V regulon is strongly and specifically induced by lysozyme and this induction is key to the intrinsic resistance of *B. subtilis* to lysozyme. Strains with null mutations in either *sigV* or in all seven ECF σ factor genes (Δ7ECF) have essentially equal increases in sensitivity to lysozyme. Induction of σ^V in the Δ7ECF background restores lysozyme resistance, whereas induction of σ^M, σ^X or σ^W does not. Lysozyme resistance results from the ability of σ^V to activate the transcription of two operons: the autoregulated *sigV-rsiV-oatA-yrhK* operon and *dltABCDE*. Genetic analyses reveal that *oatA* and *dlt* are largely redundant with respect to lysozyme sensitivity: single mutants are not affected in lysozyme sensitivity whereas a double *oatA dltA* mutant is as sensitive as a *sigV* null strain. Moreover, the triple *sigV oatA dltA* mutant is no more sensitive than the *oatA dltA* double mutant, indicating that there are no other σ^V-dependent genes necessary for lysozyme resistance. Thus, σ^V confers lysozyme resistance by activation of two cell wall modification pathways: O-acetylation of peptidoglycan catalyzed by OatA and D-alanylation of teichoic acids by DltABCDE.

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2.1 Introduction

Bacillus subtilis provides an important model system for the investigation of antibiotic resistance mechanisms in gram positive bacteria. As a soil-dwelling bacterium, *B. subtilis* inhabits a highly variable and competitive environment and, as a consequence, has evolved an arsenal of protective stress responses. Soil bacteria include many of the most prolific producers of antibiotics, including members of both the Firmicutes (including *Bacillus spp*.) and, most notably, the Actinobacteria. Antibiotics frequently target the bacterial cell envelope, including both the peptidoglycan cell wall and cell membrane. In response to low levels of antibiotics and other cell envelope active compounds, *B. subtilis* induces complex and multifaceted cell envelope stress responses (41).

Regulation of cell envelope stress responses in *B. subtilis* frequently involves one or more of seven extracytoplasmic function (ECF) sigma (σ) factors (σ^M, σ^W, σ^V, σ^X, σ^Y, σ^Z, σ^{YlaC}). The three most active in non-stressed cells, and the best characterized, are σ^M, σ^W and σ^X (36). σ^M regulates a large set of genes that encode essential functions for cell division and envelope synthesis, and its expression is induced by cell envelope active antibiotics, acid, heat, ethanol and superoxide stresses (27, 40). The σ^W regulon includes at least 60 genes that inactivate, sequester, or eliminate toxic compounds from the cell, and its expression is induced by a variety of cell envelope active compounds, detergents, and alkali stress (15, 19, 35, 43, 73). The σ^X regulon includes genes which serve to alter cell surface properties to provide protection against antimicrobial peptides (17) and is also induced by antibiotics that inhibit cell wall synthesis (22).

The ECF σ factors of *B. subtilis*, like those of other bacteria, are regulated at multiple levels (64). In general, each σ factor is co-transcribed with an adjacent gene encoding an anti-σ factor, which is usually a membrane protein that sequesters its cognate σ factor to the cytoplasmic membrane (74). In response to an inducing signal, the anti-σ factor is inactivated, often by proteolytic degradation (11, 34). The released σ factor then binds core RNA polymerase (RNAP) and directs the activation of specific promoter sites. In most, but not all, cases expression of ECF σ factors is positively autoregulated. Studies to date in *B. subtilis* suggest that each ECF σ factor (with the exception of σ^Z) activates its own expression (2), but does not activate expression of other ECF σ factors (36). In some cases, expression is also directed by an additional σ^A-dependent promoter. In contrast, in *Mycobacterium tuberculosis*, activation of one ECF σ factor can induce the expression of another, leading to a transcriptional cascade (60). The potential for transcriptional cascades, in which activation of an ECF σ factor induces expression of another transcription factor (or even another ECF σ factor), complicates efforts to define those targets that are transcribed directly as a result of σ factor reprogramming of RNAP.

Previous studies have revealed significant overlap in the regulons controlled by σ^M, σ^W and σ^X and, as a result, the stimulons induced by various cell envelope stresses often overlap extensively (41, 50). Deciphering the stimulons induced by cell envelope active compounds is complex due to both the induction of multiple stress-responsive regulators by a single stimulus, and substantial overlap between the target genes activated by each ECF σ factor. Regulon overlap in *B. subtilis* results largely from the fact that ECF σ factors recognize similar promoter sequences that share a highly conserved AAC motif in the -35 region and a CGt motif in the -10 region, but may differ in other discriminatory positions (36). In some cases, promoters are exclusively activated by only one ECF σ, whereas in other cases two or more ECF σ factors can

activate a single target promoter (18, 48). As a result of this regulon overlap, some phenotypes are only evident when two or more of the ECF σ factors are deleted (47, 49).

In contrast with σ^M, σ^W and σ^X, the roles of the other four ECF σ factors (σ^V, σ^Y, σ^Z and σ^{YlaC}) are still poorly understood. An initial study of σ^Y showed that this σ factor controls a small regulon and likely controls expression of a toxic bacteriocin and its cognate immunity gene (21). The regulons and functions of σ^Z and σ^{ylaC} have not been well defined. Two previous studies have sought to define the set of genes regulated by σ^V (2, 75). However, the prolonged incubation after induction of σ^V, the potential for cross-regulation as noted above, and the lack of a specific natural inducing signal, have prevented clear insights into the unique physiological role(s) of σ^V.

Here we show that the ECF σ factor σ^V plays a major role in resistance to lysozyme. The σ^V regulon is strongly and specifically induced by lysozyme and includes ~20 operons. Two of the σ^V -regulated operons are crucial for lysozyme resistance: the *dlt* operon and *oatA* which is transcribed as part as the *sigV* operon. We conclude that lysozyme resistance in *B. subtilis* is largely mediated by activation of two cell wall modification pathways: OatA-dependent peptidoglycan O-acetylation and D-alanylation of teichoic acids by DltABCDE.

2.2 Materials and methods

2.2.1. Strain construction and growth conditions.

All *B. subtilis* strains were constructed in the 168 background (Table 2.1). Unless otherwise stated bacteria were grown in liquid Luria-Bertani (LB) medium at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bactoagar (Difco). All cloning was done in *E. coli* DH5α using ampicillin (AMP, 100 μg/ml) for selection. Chromosomal DNA and plasmid DNA

Table 2.1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Construction or reference
B. subtilis		
168	*trpC2*	Lab strain
BSU2007	168 Δ*sigMWXYVZylaC*	(1)
HB-12010	BSU2007 *amyE*::P_{xylA}-*sigV*	pVG001 → BSU2007
HB-12020	BSU2007 *amyE*::P_{xylA}-*sigW*	pVG003 → BSU2007
HB-12035	BSU2007 *amyE*::P_{xylA}-*sigM*	pVG0013 → BSU2007
HB-12036	BSU2007 *amyE*::P_{xylA}-*sigX*	pVG004 → BSU2007
HB-12027	168 *sigV*::*kan*	LFH-PCR → 168
HB-12082	168 *amyE*::P_{xylA}-*sigV*	pVG001 → 168
HB-0048	CU1065 *dltA*::*spec*	(17)
HB-12093	168 *dltA*::*spec*	HB-0048 ChrDNA → 168
HB-12083	168 *oatA*::*tet*	LFH-PCR → 168
HB-12092	168 *oatA*::*tet dltA*::*spec*	HB-12083 ChrDNA → HB-12093
HB-12129	168 *sigV*::*kan oatA*::*tet dltA*::*spec*	HB-12027 ChrDNA → HB-12092
HB-10016	168 *sigM*::*tet*	(48)
HB-10102	168 *sigW*::*mls*	(48)
HB-10103	168 *sigX*::*kan*	(48)
Plasmids		
pVG-001	pSWEET-P_{xylA}-*sigV* (cm^R)	This work
pVG-003	pSWEET-P_{xylA}-*sigW* (cm^R)	This work
pVG-004	pSWEET-P_{xylA}-*sigX* (cm^R)	This work
pVG-0013	pSWEET-P_{xylA}-*sigM* (cm^R)	This work

transformations were performed as previously reported (32). The following antibiotics were used for selection at their respective concentrations: spectinomycin (SPC; 100 μg/mL) and macrolide-lincosoamide-streptogramin B (MLS; contains 1 μg/ml erythromycin and 25 μg/ml lincomycin).

2.2.2. Inducible expression of ECF σ factors in B. subtilis.

The pSWEET plasmid, which integrates into the *amyE* locus, was used to construct xylose-dependent expression strains (10). *sigV*, *sigM*, *sigW* and *sigX* were amplified from 168 chromosomal DNA using primers 4556/4557, 4970/4590, 4558/4559 and 4560/4561 (Table S2.1) respectively and cloned into pSWEET using PacI and BamHI sites to create pVG001, pVG003, pVG004 and pVG013, respectively (Table 2.1). Inducible expression from each construct was checked using reporter strains. The plasmids were transformed into a *B. subtilis* strain carrying in-frame deletions of all seven ECF σ factor genes (Δ7ECF) (1) with chloramphenicol (CAT, 5 μg/ml) selection to create strains HB12010, HB12020, HB12036 and HB12035, respectively (Table 2.1). The same strategy was used to integrate an ectopic copy of P_{xylA}-*sigV* into the wild-type strain 168 to generate strain HB12082.

2.2.3. Generation of mutant strains.

Long flanking homology PCR (LFH-PCR) was used to generate deletion mutations in which the designated coding region is largely replaced by an antibiotic cassette as previously described (49, 72). Strain 168 chromosomal DNA was used for PCR amplification of flanking fragments of each gene using primers 5148/5501 and 5502/5151 for *sigV* and 5156/5157 and 5158/5159 for *oatA* (Table S2.1).

The PCR products were joined to an antibiotic cassette using joining PCR with outside primers. The final LFH product was used to transform 168 with selection for kanamycin (KAN, 10 μg/ml) for *sigV*::*kan* and tetracycline (TET, 5 μg/ml) for *oatA*::*tet*.

2.2.4. Lysozyme sensitivity measurements.

Lysozyme sensitivity was determined using a disk diffusion assay performed as described previously (7, 49). Briefly, the wild-type strain 168 and various mutant strains were grown to mid-logarithmic phase (OD600 of 0.4) in LB medium at 37°C with aeration. A 100-µl aliquot of these cultures was mixed with 4 ml of 0.75% Müller-Hinton (MH) soft agar (kept at 50°C) and directly poured onto MH plates (containing 15 ml of 1.5% MH agar). The plates were then dried for 20 min in a laminar airflow hood. Filter paper disks containing 5 µl of 100 mg/ml lysozyme were then placed on the top of the agar, and the plates were incubated at 37°C overnight. The diameters of the inhibition zones (clear zones) were measured.

2.2.5. RNA extraction for transcriptome analyses.

A culture of HB12010 (Δ7ECF P_{xylA}-*sigV*) was grown in LB at 37°C with shaking to an OD600 of 0.4 then incubated for 20 minutes either with or without 2% xylose. A culture of 168 was grown similarly and treated either with or without 0.5 µg/ml lysozyme. Total RNA was isolated from three different biological replicates for each experiment with the RNeasy minikit following the manufacturer's instructions (Qiagen Sciences, MD). After DNase treatment with Turbo DNA-free (Ambion), RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop Tech. Inc., Wilmington, DE) and kept at -20°C. Microarray analyses. 20 µg of total RNA was used to make cDNA using the SuperScriptTM Plus Indirect cDNA Labeling System (Invitrogen; L1014-04). cDNA was labeled using Alexa Fluor® labeling and microarray analysis were performed as described previously (29). Six microarrays (biological triplicates with a dye-swap) were analyzed for both the σ^V regulon and lysozyme stimulon determinations. Images were processed and normalized using the GenePix Pro 4.0 software package which produces (red

and green [R and G]) fluorescence intensity pairs for each gene. Each expression value is represented by up to 12 separate measurements (duplicate spots on each of six arrays). Mean values and standard deviations were calculated with MS Excel. The normalized microarray datasets were filtered to remove those genes that were not expressed at levels significantly above background in either condition (sum of mean fluorescence intensity <20). In addition, the mean and standard deviation of the fluorescence intensities were computed for each gene, and those for which the standard deviation was greater than the mean value were ignored. The fold induction values were calculated using the average signal intensities from the three arrays in the different conditions. The microarray datasets are available in the NCBI GEO database under accession number GSE31563.

2.2.6. Quantitative RT-PCR.

For quantitative real time PCR (qRT-PCR) specific primers were designed using the *B. subtilis* genome sequence to amplify 100 bp products (Table S2.1). 2 μg of total RNA (isolated as described above for transcriptome analysis) was used to make cDNA using TaqMan® Reverse transcription reagents following the manufacturer's instructions (Applied Biosystems). The cDNA was used for qRT-PCR using iQTM SYBR® Green Supermix in an Applied Biosystems 7300 Real Time PCR System. Quantification of 23S RNA levels was used as an internal control. The foldchange was calculated using the difference in Ct for both conditions.

2.2.7. Determination of consensus promoter sequences.

The promoter consensus sequence alignment was performed using the Weblogo software (http://weblogo.berkeley.edu/). The σ^X regulon (11 promoters), σ^W regulon (30 promoters) and σ^M regulon (30 promoters) are based on published results (15, 17, 18, 27).

2.2.8 Spot-on-lawn assays.

Spot-on-lawn assays were performed as described (15). Briefly, lawn cells were grown to an OD600 of 0.4 in LB. A 100 ml aliquot of these cultures was mixed with 4 ml of 0.7% MS (Mannitol Soya) soft agar (kept at 50°C) and directly poured onto MS plates (containing 15 ml of 1.5% MS agar). Plates were dried for 20 min in a laminar flow hood. *S. coelicolor* M145 was grown and kept as spore preparations as previously described (42). 5 µL of the spore preparation was spotted on top of the agar. Plates were incubated at 28°C and observed after 2 and 5 days.

2.3. Results and discussion

2.3.1 Induction of σ^V in a Δ7ECF strain identifies direct targets of σ^V RNAP.

Previous studies have reported that artificial induction of σ^V induces dozens of genes (2, 75), suggesting that this ECF σ factor is likely important under some undefined conditions. However, only a small subset of genes were consistently detected in these studies which involved long incubations after σ^V induction (at least 2 hrs). Therefore, we sought to re-investigate the σ^V regulon under conditions that reduce indirect effects and preclude transcriptional cascades due to activation of other ECF σ factors.

To define the σ^V regulon we induced expression of σ^V in a strain devoid of all other ECF σ factors (Δ7ECF) (1, 47). We used DNA microarray hybridization to monitor transcriptional changes 20 min. after induction of σ^V to selectively detect direct effects and thereby define

promoters activated by σ^V RNAP. Analysis of the resulting transcriptome revealed the up-regulation of ~30 operons including many known from previous work to be also regulated by σ^M, σ^W and/or σ^X (Figure 1A and Tables S2.2 and 2.2). There was also weak up-regulation of some members of the σ^B-dependent general stress response (Table S2.2). These results indicate that σ^V can directly activate numerous promoter sites independent of any influence it may also have on the expression of other ECF σ factors.

The most dramatic effect of inducing σ^V was the strong up-regulation of the *sigV* operon itself, consistent with prior reports of positive autoregulation (2). The induction of s*igV* itself is not informative, since this gene was induced by xylose. However, we also observed very strong induction (>40-fold) of genes downstream of *sigV* including *rsiV* (encoding anti-σ^V), *oatA* (encoding a peptidoglycan O-acetyltransferase; (44)), and *yrhK* (unknown function). Since the strain background used for this study (Δ7ECF) carries an in-frame deletion of *sigV* this induction is likely indicative of the autoregulation that would occur in response to natural inducers.

Most of the remaining genes that responded strongly to the induction of σ^V are known members of the σ^M, σ^W and σ^X regulons. Since this experiment was done in a background carrying in-frame deletions of all three of these ECF σ factors, we conclude that this reflects an overlap in the promoter recognition properties of these ECF σ factors rather than a transcriptional cascade. The induced operons (Table 2.2) include *abh*, *ywaC*, *bcrC*, *dltABCDE*, *pbpX*, and *yqjL*. Abh is a paralog of AbrB and functions as a transition state regulator affecting antibiotic synthesis and resistance (23, 48), YwaC is a ppGpp synthase (48), BcrC functions as an inducible undecaprenyl-pyrophosphate phosphatase and thereby contributes to bacitracin resistance (9, 18), the *dlt* operon encodes enzymes for teichoic acid D-alanylation (53), PbpX is

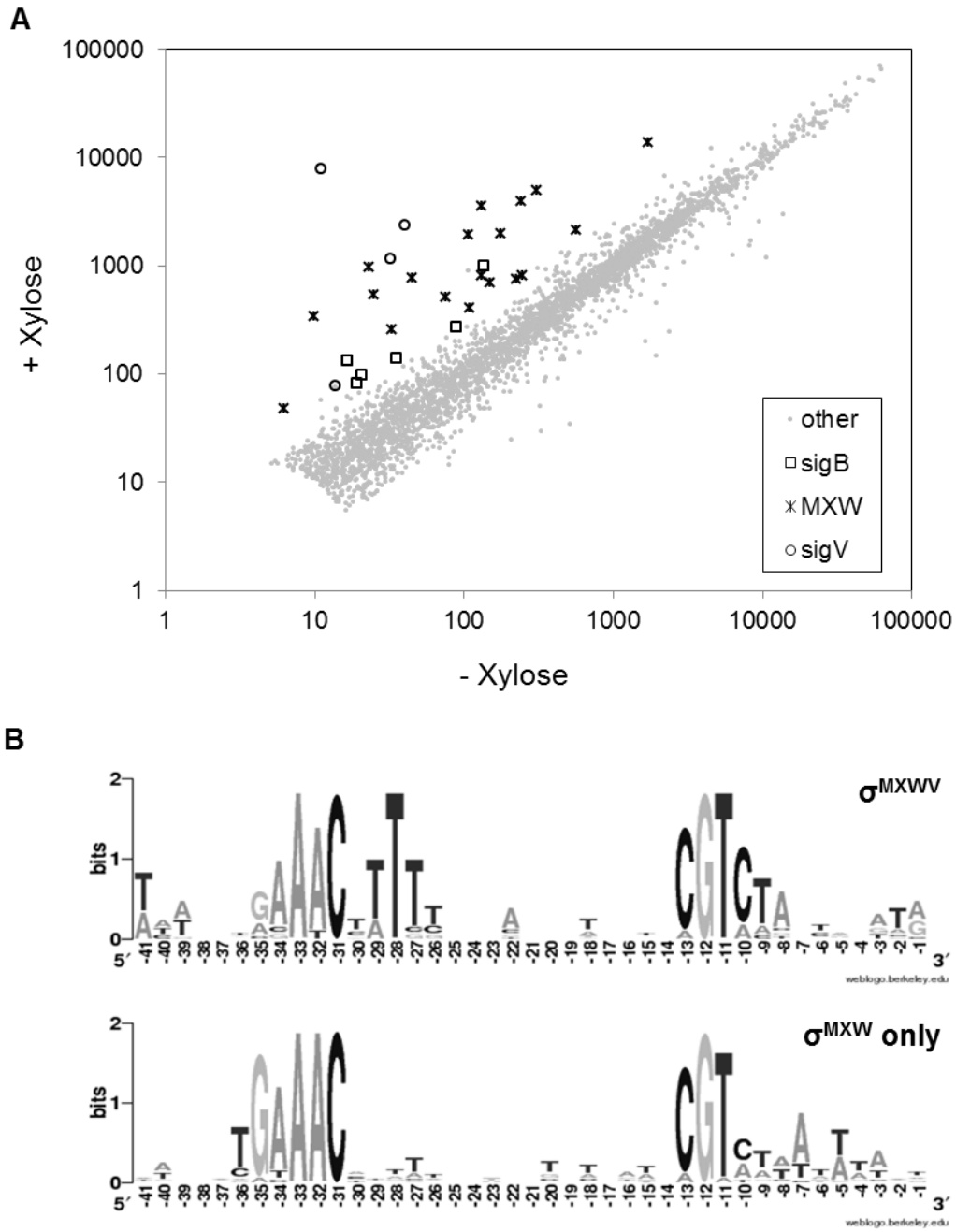


Figure 2.1. The σ^V regulon. A) The scatterplot represents the average expression levels of genes in induced (+ xylose) versus non-induced (- xylose) cultures of *B. subtilis* Δ7ECF P_{xylA}-*sigV*. The known regulons of σ^M, σ^X or σ^W (MXW), σ^B, and the genes belonging to the *sigV* operon are labeled. B) The promoter consensus sequence alignment was performed using the Weblogo software (http://weblogo.berkeley.edu/) and represents the promoters that are regulated by σ^M, σ^X or σ^W and also regulated by σ^V (σ^{MXWV}; top panel) and those promoters that are regulated by σ^M, σ^X or σ^W that are not regulated by σ^V (σ^{MXW}; bottom panel).

Table 2.2. The σ^V regulated genes and their functional role.

Category (operon)	Foldchange[a] +/- xyl	Foldchange[a] +/- lys	Regulator[b]	Function	Reference[c]
Regulation					
sigV rsiV	357	73	V	ECF σ and anti-σ factor	(63)
abh	26	2	MXW	Transition state regulator (AbrB paralogue)	(66)
ywaC	8	2	MWX	ppGpp synthase (putative)	(52)
Cell division and shape					
mafradCmreBCminCD	3	2	MW	Cell division and shape determination	(24)
Cell envelope					
bcrC	42	4	MX	undecaprenyl pyrophosphate phosphatase	(9)
ddl murF	3	2	MWX	Peptidoglycan biosynthesis	
dltABCDE	14	2	MX	D-alanylation of teichoic acids	(17)
pbpX	21	3	XW	penicillin binding protein	(17)
oatA yrhK	46	63	V	*oatA*: O-acetylation of peptidoglycan; *yrhK*: unknown	(7)
Detoxification					
yrhHIJ	26	3	MXW	cytochrome P450 regulation	(46)
yqjL	8	2	MW	Hydrolase, paraquat resistance	(20)
Miscellaneous					
mmgD	5	2	E	2-methylcytrate synthase	(14)
scoB	3	2	E	succinyl CoA:3-oxoacid CoA-transferase (subunitB)	
spoIIB	2	2		stage II sporulation regulation	(55)
yutH	2	2		spore coat-associated protein	(68)
Unknown					
yebC	7	3	M	putative integral inner membrane protein	
yocL	5	2	E	hypothetical protein	(28)
ycgR	3	2	M	putative permease	
ytvB	3	4		putative conserved membrane protein	
ydgA	2	2	K	conserved hypothetical protein	(61)
yvaFE	2	2		putative transcriptional regulator and metabolite-efflux transporter	
ytwF	2	2		putative sulfur transferase	
ycgQ	2	2	M	conserved hypothetical protein	(27)

a) Foldchange shown for operons represents the average of the foldchanges of each gene in the operon.
b) V refers to σ^V, M refers to σ^M, X refers to σ^X, W refers to σ^W, E refers to σ^E and K refers to σ^K.
c) For those where the reference is not listed the function annotation is based on GenoList (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList)

an unknown function low molecular weight penicillin-binding protein, and YqjL contributes to resistance to paraquat by an unknown mechanism (20).

These transcriptional profiling results indicate that σ^V activates both its own operon and a well-defined subset of the σ^M, σ^X, and σ^W regulons. Previously, we demonstrated that a key feature distinguishing σ^X and σ^W specific promoters is the sequence of the -10 consensus element: promoters with the sequence CGTA are generally recognized by σ^W, those with sequence CGAC are recognized by σ^X, and those with the sequence CGTC may be recognized by both (57). To begin to define possible promoter features that account for ability of σ^V to activate its specific subset of target operons, we created a consensus alignment of those promoters that belong to the σ^M, σ^X or σ^W regulons that were not activated by induction of σ^V (MXW) and compared this with the consensus of those that were also activated by σ^V (MXWV).

Interestingly, the consensus for the σ^{MXWV}-regulated genes contains a T-rich -30 to -26 region that is not conserved in the σ^{MXW}-only regulated genes (Figure 2.1B). We are currently testing the hypothesis that this spacer region sequence is important for promoter recognition by σ^V (Chapter 3).

2.3.2. σ^V is specifically induced by lysozyme.

One of the genes most strongly induced by σ^V is *oatA* (formerly *yrhL*) which is immediately downstream of *sigV-rsiV*. *B. subtilis oatA* encodes an ortholog of an *S. aureus* peptidoglycan O-acetyltransferase and has been shown genetically to affect levels of peptidoglycan O-acetylation (44). OatA provides lysozyme resistance in pathogenic Staphylococcus species (5, 7) and *Lactococcus lactis* (70). Furthermore, possible orthologs of σ^V were found to be induced by lysozyme exposure in *Enterococcus faecalis* (45, 46) and *Clostridium difficile* (38). Together,

these results suggest that *B. subtilis sigV*, and therefore the σ^V regulon, might be induced by lysozyme and thereby provide lysozyme resistance.

To test for induction by lysozyme we used reporter strains with *lacZ* fusions to the autoregulated promoters of *sigV*, *sigM*, *sigW*, and *sigX*. β-galactosidase measurements with and without lysozyme treatment show that the *sigV* promoter is strongly (~90-fold) and specifically induced by lysozyme (Figure 2.2 A). To verify and extend these results we performed qRT-PCR with RNA isolated from wild-type cells treated with different concentrations of lysozyme (Figure 2.2 B). As little as .01 μg/ml lysozyme strongly induced *sigV* activity (~10-fold) and, even at 1 μg/ml lysozyme, there was little if any observable lysis of cells during the 20 min. of treatment. This demonstrates that activation of σ^V is extremely sensitive to even mild digestion of the cell wall and is not correlated with cell lysis.

2.3.3 The lysozyme stress response is dominated by strong activation of the σ^V regulon.

We next sought to obtain a global view of the lysozyme stress response by monitoring the changes in the transcriptome induced by short treatment (20 min.) with sub-lethal levels of lysozyme known to be sufficient for full induction of the σ^V regulon. This allows us to compare the response elicited in wild-type cells upon naturally inducing σ^V with the more artificial situation of ectopically inducing σ^V in the Δ7ECF background. Remarkably, the lysozyme stimulon is dominated by the strong (>50-fold) induction of *sigV* and the immediately adjacent *rsiV, oatA*, and *yrhK* genes. Thus, not only does lysozyme selectively activate σ^V, there are no other cell envelope stress systems that appear to respond strongly to this level of lysozyme. Altogether, the lysozyme stimulon includes weak induction of as many as 76 operons (Figure 2.3

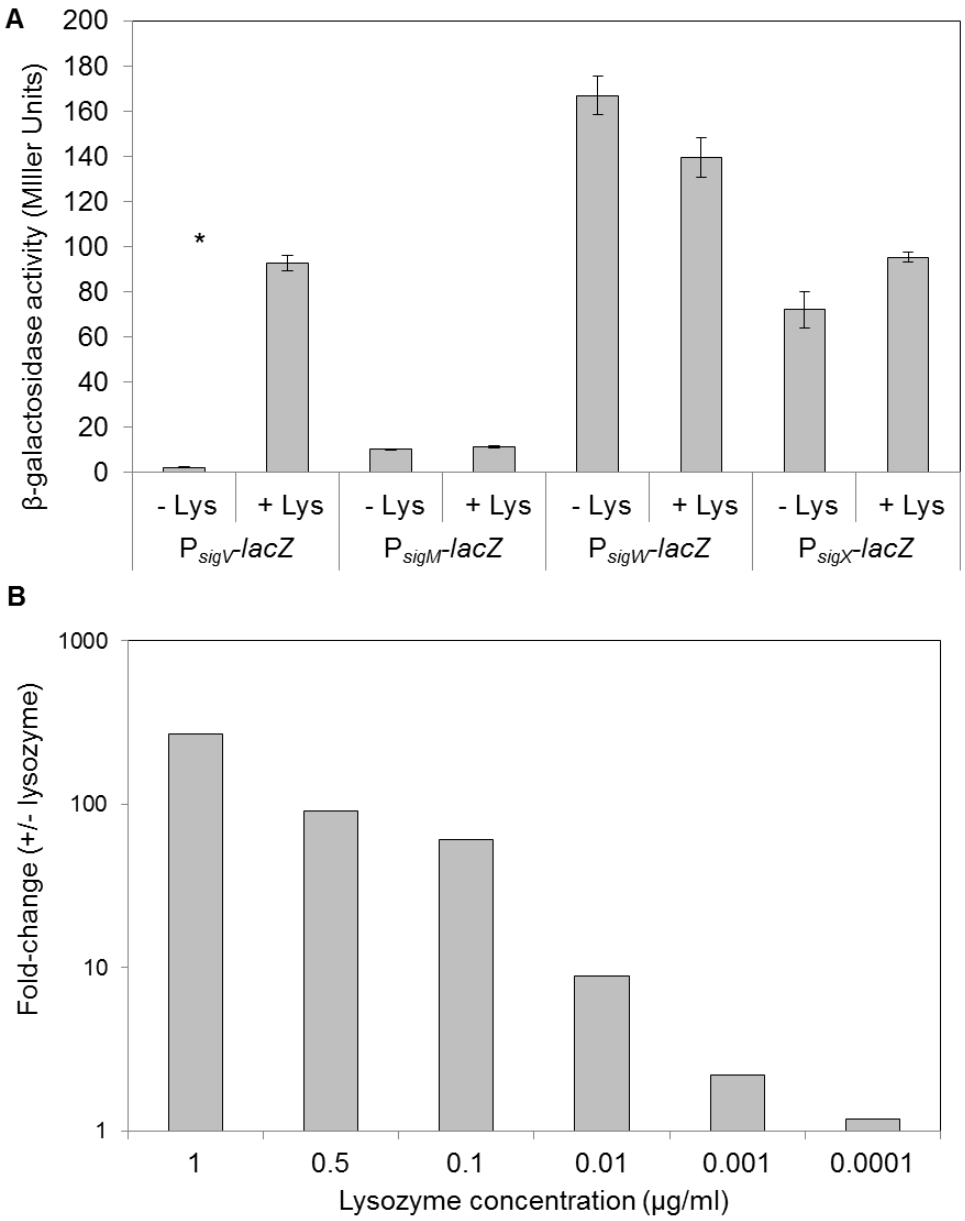


Figure 2.2. σ^V is strongly and specifically induced by lysozyme. A) β-galactosidase activity of P_{sigV}-*lacZ*, P_{sigM}-*lacZ*, P_{sigW}-*lacZ*, and P_{sigX}-*lacZ* with or without treatment with a sub-inhibitory concentration of lysozyme. Experiment was performed in 3 biological replicas and repeated at least three times. Bars represent mean values with error bars indicating the standard deviation. Student's *t*-tests were performed, and a statistically significant difference (*P*-value < 0.005) between the control (- Lys) and lysozyme treated cells (+Lys) is denoted with an asterisk (*). B) qRT-PCR of *sigV* expression under lysozyme induction. The 168 strain was grown to an OD600 of 0.4 and incubated for 20 min. with the addition of different concentrations of lysozyme. qRT-PCR was performed with primers specific for *sigV* and for 23S rRNA as a control. The bars show the fold-change of induction after treatment with lysozyme. The results shown are representative of experiments performed at least three times.

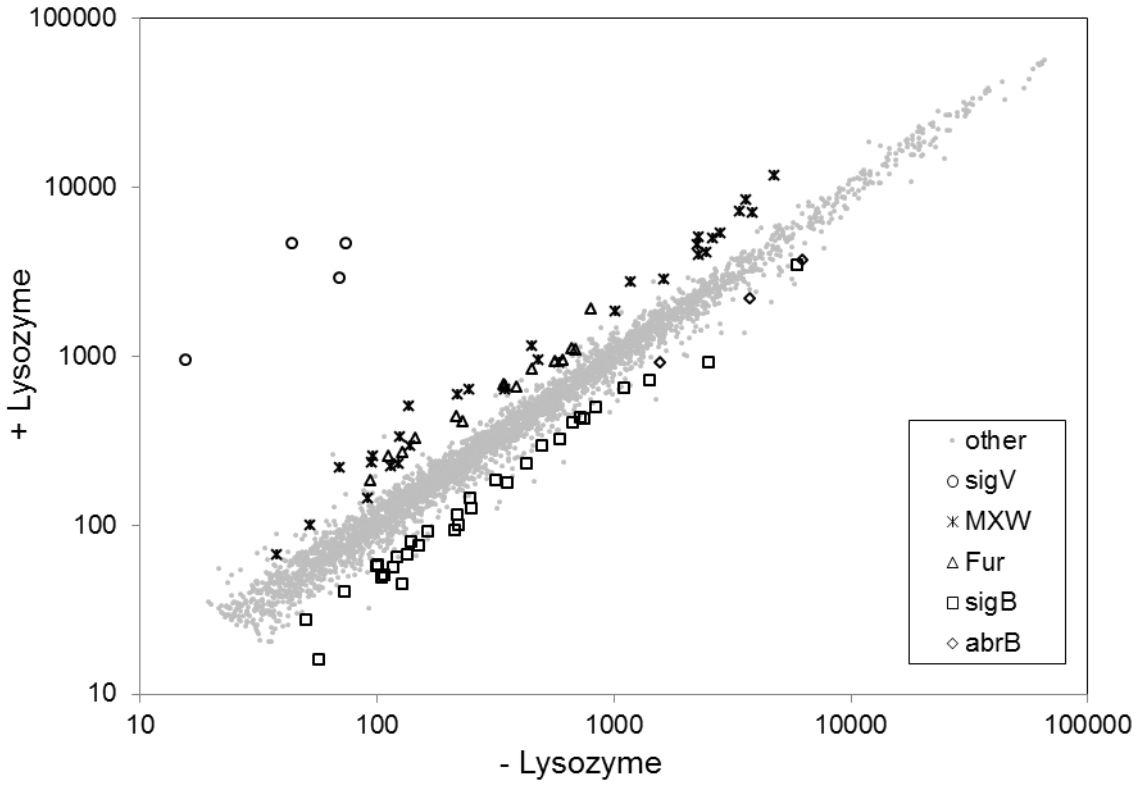


Figure 2.3. The lysozyme stimulon. The scatterplot represents the average expression levels of genes in induced (+ lysozyme) versus non-induced (- lysozyme) cultures of *B. subtilis* 168. Genes belonging to the regulons of σ^M, σ^X, or σ^W (MXW), σ^B, Fur, and AbrB, and the genes belonging to the *sigV* operon are labeled.

and Table S2.3), including several members of the Fur regulon (3). Other inducible genes have functions in cell wall biosynthesis, cell division, and antibiotic resistance (Tables 2.2 and S2.3). Overall, there is very good congruence between the σ^V regulon and the lysozyme stimulon (Table 2.2).

One notable difference between the σ^V regulon (Figure 2.1 A) and the lysozyme stimulon (Figure 2.3) is that in the former the induction of genes also potentially regulated by other ECF σ factors was generally much stronger. This likely reflects the fact that the σ^V regulon was determined in a strain background devoid of other ECF σ factors that might have otherwise contributed to the background expression of these genes. In other words, in wild-type cells the lysozyme-dependent induction of some genes is superimposed on their basal transcription. Of the seven ECF σ factors, at least two (σ^M and σ^X) are found associated with RNAP in non-stressed cells (25) and this likely contributes to basal gene expression.

2.3.4 oatA is cotranscribed with sigV and rsiV.

The transcriptional analyses above revealed a coordinate induction of *oatA* with the upstream *sigV* and *rsiV* genes, suggestive of a likely operon structure. However, a 110 bp gap separates *rsiV* from the downstream *oatA* gene and this region contains a predicted transcription terminator. To test the hypothesis that these genes are expressed as a single transcript, we performed qRT-PCR of the intergenic junctions between *sigV-rsiV*, *rsiV-oatA*, and *oatA-yrhK* (Figure 2.4). We detected an increase in expression of all intergenic junctions correlated with the induction of *sigV*. These results suggest that the predicted terminator is, at best, only partially efficient and the downstream genes can be expressed as part of a read-through transcript. Furthermore, there is no predicted σ^V-dependent promoter in the intergenic region between *rsiV*

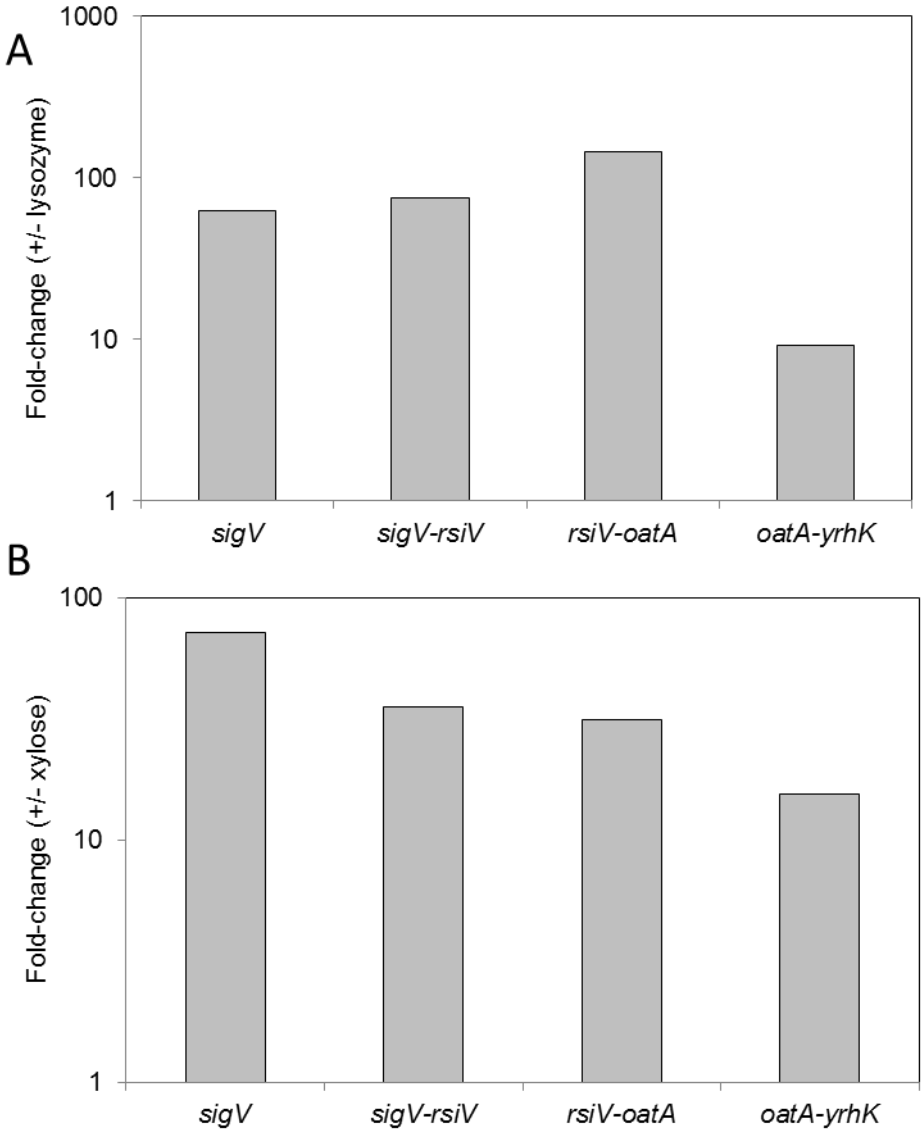


Figure 2.4. *oatA* is part of the *sigV* operon. qRT-PCR of intergenic junctions after induction of σ^V. A) The 168 strain was grown to an OD600 of 0.4 and incubated for 20 min. either with (+Lys) or without (-Lys) 0.5 μg/ml of lysozyme. B) The 168 P_{xylA}-*sigV* strain was grown to an OD600 of 0.4 and incubated for min either with (+Xyl) or without (-Xyl) the addition of 2% xylose. qRT-PCR was used to quantify the fold-change of each junction region after treatment. Results shown are representative of experiments repeated at least three times.

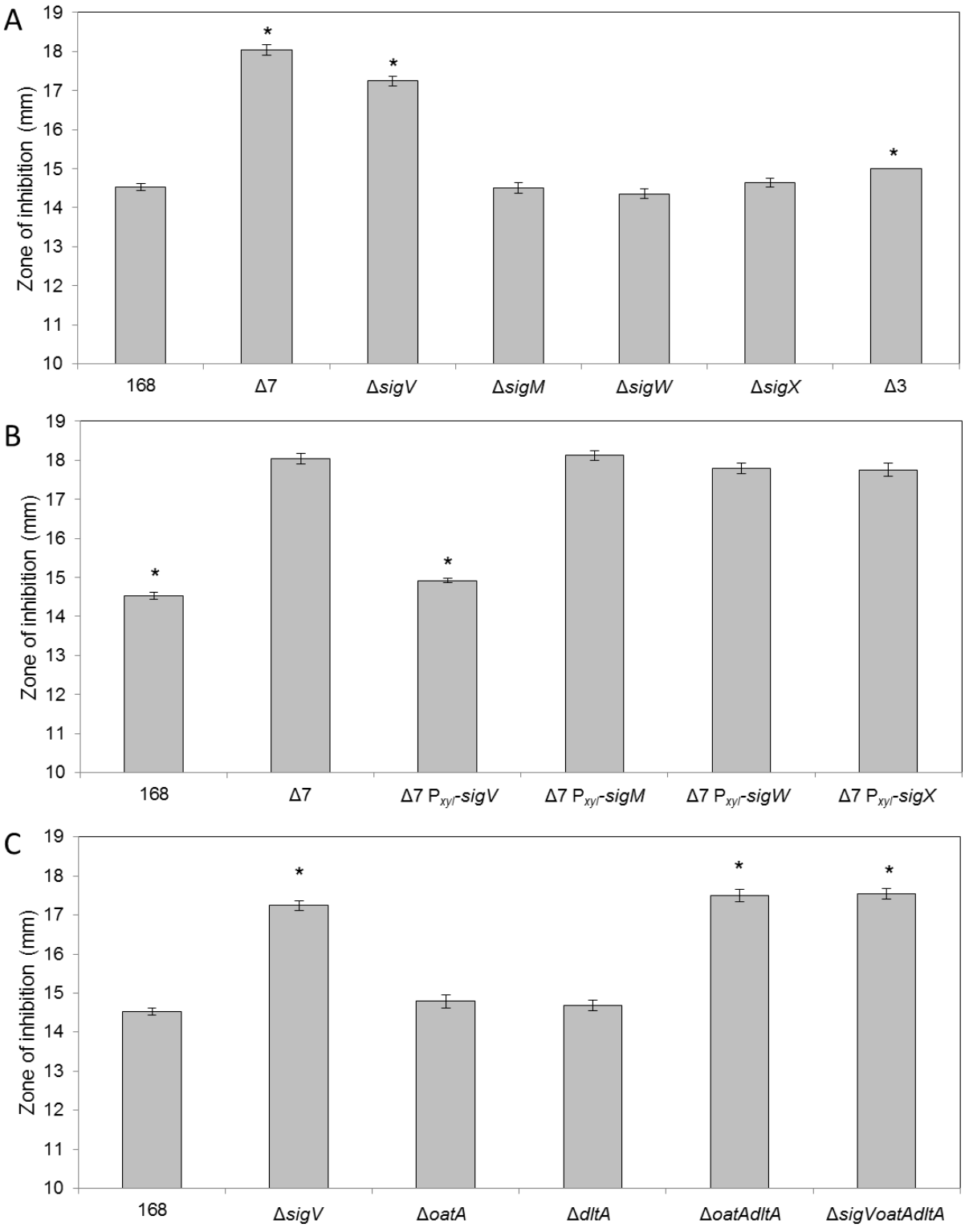
and *oatA*, and *lacZ* fusions made with different fragments upstream of *oatA* have no activity even when σ^V is induced (data not shown). Inspection of published tiling array data (59) also supports a likely four gene operon extending from *sigV* to *yrhK*. We therefore conclude that *oatA* is co-transcribed with *sigV, rsiV*, and *yrhK* and that the σ^V-dependent induction of *oatA* reflects the activity of the *sigV* autoregulatory promoter.

2.3.5. σ^V plays a central role in lysozyme resistance.

We assessed the role of σ^V in lysozyme resistance using a modified disk diffusion protocol (7). A *sigV* null mutant is nearly as sensitive as a strain missing all 7 ECF σ factors, implying that this single ECF σ factor is the major lysozyme resistance determinant (Figure 2.5 A). In preliminary studies using a strain with the *sigV* gene disrupted by a co-directional kan cassette, the role of σ^V in lysozyme resistance was partially masked by read-through transcription into *oatA* (data not shown). Therefore, we used an allelic replacement mutation in which the *sigV* gene was disrupted by a divergently oriented antibiotic cassette (Figure 2.5 A). Identical results were also seen when an in-frame deletion of *sigV* (from Δ7ECF) was used instead of an allelic replacement mutant (data not shown). σ^V had no apparent role in resistance to mutanolysin, which is known to cleave peptidoglycan irrespective of MurNAc O-acetylation (71), or to several cell wall active antibiotics (bacitracin, nisin, moenomycin, D-cycloserine, polymyxin-B, cefuroxime, fosfomycin, vancomycin, and ramoplanin) (data not shown).

In contrast to *sigV*, single mutations of the most active ECF σ factors (σ^M, σ^X and σ^W) did not affect lysozyme sensitivity. Even a triple *sigM sigX sigW* null mutant (Δ3) had only a modest increase in lysozyme sensitivity (Figure 2.5 A). Thus, these σ factors may play a small role in lysozyme resistance, but this is negligible in cells expressing σ^V. We next tested lysozyme

Figure 2.5. σ^V confers resistance to lysozyme through regulation of *oatA* and the *dlt* operon. Zone of inhibition experiments were used to quantify lysozyme sensitivity in *B. subtilis* strains. Strains were grown to an OD_{600} of 0.4 and an inoculum of this culture was used to make a lawn of cells on 0.75% MH agar. Disks containing lysozyme were placed on top of the lawn and the inhibition of growth was measured after incubation at 37°C for 16 h. Each bar represents the average zone of inhibition of a least three assays performed with three biological replicas of each strain. The zone of inhibition is expressed as the total diameter (± standard error) of the clear zone. A. Lysozyme sensitivity of wildtype and ECF σ factor mutants. B. Lysozyme sensitivity in wildtype, Δ7ECF, and ECF σ factor-inducible strains. Data shown is for cultures grown in inducing conditions (2% xylose). C. Comparison of lysozyme sensitivity in wildtype and *oatA* and *dltA* mutants. For all three panels, a statistically significant difference (*P*-value < 0.005) as determined by student's *t*-tests is denoted as an asterisk (*).


A
Zone of inhibition (mm)
19
18
17
16
15
14
13
12
11
10
*
*
*
168
Δ7
ΔsigV
ΔsigM
ΔsigW
ΔsigX
Δ3
B
Zone of inhibition (mm)
*
*
168
Δ7
Δ7 Pxyl-sigV
Δ7 Pxyl-sigM
Δ7 Pxyl-sigW
Δ7 Pxyl-sigX
C
Zone of inhibition (mm)
*
*
*
168
ΔsigV
ΔoatA
ΔdltA
ΔoatAdltA
ΔsigVoatAdltA

resistance in the Δ7ECF strain upon induction of various ECF σ factors (Figure 2.5 B). Only induction of σ^V restored lysozyme resistance whereas induction of σ^M, σ^X and σ^W had little if any effect. These results demonstrate that σ^V is both necessary and sufficient for the induction of lysozyme resistance determinants.

2.3.6 Lysozyme resistance is due to σ^V-dependent activation of OatA and Dlt.

Since induction of σ^V can activate the expression of 20 or more operons (Figure 2.1, Table 2.2), we next sought to identify which σ^V-regulated genes are important for lysozyme resistance. We focused our attention on two σ^V-activated functions previously implicated in lysozyme resistance in other organisms: *oatA* and the *dlt* operon (5, 7, 46). Although single mutations of either *oatA* or *dltA* did not affect lysozyme sensitivity, a double *oatA dltA* mutant was fully as sensitive as a *sigV* null mutant (Figure 2.5 C). We therefore suggest that up-regulation of either or both of these operons can account for the role of σ^V in lysozyme resistance. Support for this notion is provided by the finding that the mutation of *sigV* in an *oatA dltA* double mutant does not further increase sensitivity (Figure 2.5 C).

These results suggest that in *B. subtilis* lysozyme resistance is provided by σ^V through the up-regulation of *oatA* and the *dlt* operon. The regulation of the *dlt* operon has been studied in detail. The *dlt* operon is potentially activated by σ^D (54), σ^X (17) and σ^M (27). As noted above, *oatA* is cotranscribed with *sigV*. To corroborate and extend our microarray results, we performed qRT-PCR studies in cells where σ^V was induced either ectopically with xylose or by lysozyme treatment (Table 2.3). As expected, lysozyme treatment strongly induced *sigV* and *oatA* in the wild-type and *sigX* null mutant cells, but not in the *sigV* null mutant. Conversely, ectopic induction of *sigV* also strongly induced *oatA* and *dltA* expression (Table 2.3). Ectopic induction

of *sigX* induced *dltA*, consistent with the reported regulation of this operon by σ^X (17), but did not induce *oatA*.

As noted above, induction of σ^X is unable to restore lysozyme resistance to the Δ7ECF strain (Figure 2.5 B). However, induction of σ^X is clearly sufficient for the strong activation of the *dlt* operon (Table 2.3). This suggests that induction of the dlt operon is not sufficient to provide lysozyme resistance in a strain lacking the other six ECF σ factors. In apparent contrast to this result, OatA and Dlt are redundant in providing lysozyme resistance to wild-type cells (only a double *oatA dltA* mutant was as sensitive to lysozyme as a *sigV* mutant; Figure 2.5 C). Therefore, in an *oatA* mutant, *dlt* appears to be the only σ^V-dependent operon required to provide resistance. However, in the Δ7 strain, which is significantly altered in its physiology (47), the artificial induction of Dlt (upon activation of σ^X) is not sufficient for lysozyme resistance. These results can be reconciled if other ECF σ factor-dependent genes also make contributions to lysozyme resistance, including perhaps *pbpX* (39).

2.3.6. Diverse mechanisms of lysozyme resistance.

Lysozyme hydrolyses the β-1,4 glycosidic bond between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlucNAc) (71). In addition to its muramidase activity, lysozyme also has a cationic antimicrobial peptide activity (37, 51).

There have been several different mechanisms reported for lysozyme resistance in Gram positive bacteria. Most commonly, resistance is achieved by either modification of the peptidoglycan substrate by MurNAc O-acetylation (7, 13) or by changes in the overall net charge of the cell envelope by D-alanylation (37). In *Staphylococcus aureus*, OatA-dependent O-acetylation and D-alanylation of teichoic acids function synergistically to provide full lysozyme

Table 2.3. qRT-PCR quantitation of *sigV, oatA,* and *dltA* expression

	Transcript (fold-induction)		
Strain / condition	*sigV*	*oatA*	*dltA*
168 +/- lysozyme	50.5	25.7	2.6
Δ*sigV* +/- lysozyme	n.a.	1.3	2.0
Δ*sigX* +/- lysozyme	82.4	19.6	5.8
Δ7 P_{xylA}-*sigV* +/- xylose	n.a.	5.6	5.8
Δ7 P_{xylA}-*sigX* +/- xylose	1.0	0.7	37.4

n.a: not applicable, since these induction values are not meaningful since the gene is deleted (row 2) or artificially induced from P_{xylA} (row 4).

resistance (5). In this organism, the GraRS two component system plays a key role in lysozyme resistance by activating expression of the dlt operon (37). Resistance to lysozyme in *Enterococcus faecalis* is provided by both *oatA* and *dltA*, and σ^V plays an additional, undefined role: a *sigV dltA oatA* triple mutant is more sensitive to lysozyme than either a *sigV* or *dltA oatA* mutant (46). In this organism, the ECF σ^V factor acts as a general stress response σ (4), and is not required for the expression of *oatA* or *dltA* (34). *Lactococcus lactis* also protects its peptidoglycan by O-acetylation and, in this case, *oatA* transcription is regulated by SpxB (70). In *L. monocytogenes*, lysozyme resistance is achieved by both O-acetylation and N-deacetylation (58). In *B. anthracis*, lysozyme resistance is mediated by both O-acetylation, catalyzed by two distinct enzymes, and N-de-acetylation (44), but the regulation of these resistance determinants is not yet characterized.

Here we define both the genetic determinants and the regulatory pathways that control intrinsic lysozyme resistance in *B. subtilis*. In comparison to highly lysozyme resistant pathogens, such as *S. aureus*, *B. subtilis* is relatively sensitive to lysozyme. Nevertheless, this organism clearly maintains an inducible resistance system controlled by σ^V. *B. subtilis* was shown previously to contain a functional OatA homolog: ~35% of MurNAc residues were O-acetylated in wild-type cells and this was reduced 2.5-fold in an *oatA* null mutant (44). Based on the results here, it seems likely that the level of MurNAc modification increases significantly in cells exposed to even low levels of lysozyme, thereby providing an adaptive mechanism for lysozyme resistance. *B. subtilis* also extensively modifies teichoic acids by D-alanylation (53) which is mediated by the products of the *dlt* operon (50). The *dlt* operon has been shown to be regulated by σ^D (54), σ^X (17), σ^M (27) and, as shown here, σ^V. Our results indicate that in *B. subtilis*, σ^V is the major ECF σ factor responsible for lysozyme resistance: a *sigV* mutant is

nearly as sensitive as the Δ7ECF mutant (Figure 2.5 A). When induced, σ^V activates expression of both *oatA*, encoded as part of the autoregulated *sigV* operon, and the *dltA* operon (Tables 2.2 and 2.3). These two cell wall modification pathways provide redundant mechanisms of lysozyme resistance, as also noted in *S. aureus* (37).

While this work was in preparation, very similar results were obtained by Ellermeier and coworkers (39). They also note that *sigV* is cotranscribed with *oatA* and that induction of this operon by lysozyme contributes to lysozyme resistance. They further show that the *dlt* operon and *pbpX* also contribute to lysozyme resistance. While the results of these two studies are generally in good agreement, we find that a *sigV* null mutant is nearly as sensitive as the Δ7ECF strain whereas Ho et al. report that a *sigV* null mutation has only a modest effect (2-fold) on lysozyme resistance which is greatly enhanced in strains additionally defective in *sigX* and/or *sigM*. Our results also differ with respect to the importance of the *dlt* operon for lysozyme resistance. Whereas we see no significant effect of a *dlt* null mutation on lysozyme resistance (Figure 2.5 C), Ho et al. report that a *dlt* null mutation has a greater effect than a *sigV* null. The reasons for these differences are presently unclear.

Although hen egg white lysozyme is commonly used for testing lysozyme resistance, it is a surrogate for the physiological stresses likely to be encountered in the environment. In human mucosal secretions, lysozyme can be present at levels of up to 5 mg/ml which thereby provides an important component of innate immunity (26). Indeed, peptidoglycan O-acetylation and lysozyme resistance correlate with pathogenicity in *S. aureus* (6), *E. faecalis* (46), and likely in other human pathogens.

The role of lysozymes and lysozyme resistance mechanisms has not been as well studied in soil bacteria. However, soil bacteria are known to produce and in some cases secrete

peptidoglycan degrading enzymes. *Myxococcus xanthus*, which feeds on other soil bacteria, secretes several lytic enzymes (8) including at least one with lysozyme-like activity (31). *Streptomyces coelicolor* also secretes several muramidases (30). *Bacillus spp.* also produce a number of autolytic and peptidoglycan degrading enzymes (62). We are currently studying the possibility that σ^V provides resistance to peptidoglycan degrading enzymes produced by other soil bacteria.

2.4. Recent findings

In hopes of continuing these studies, and to test if σ^V is involved in resistance to PG hydrolases from other organisms, we set up a series of interspecies interaction experiments with known PG degrading enzymes producers that inhabit the soil.

The sporulating actinomycete *S. coelicolor* is known to produce and secrete several PG degrading enzymes (30), and its interaction with *B. subtilis* has been studied (65). In collaboration with the Elliot lab (McMaster University), we obtained a wild-type *S. coelicolor* strain as well as several mutants that either lack or over-produce these hydrolases. Compared to wild-type, there was no resistance/sensitivity phenotype observed for either a *sigV* mutant or an *rsiV* mutant. However, the *sigV* mutant lawn creates a response of increased release of pigment by the *S. coelicolor* strain, whereas in the *rsiV* mutant, the pigment production seems to be abolished in *S. coelicolor* (Figure 2.6). We believe this pigment to be actinorhodin, the most abundant antibiotic produced by *S. coelicolor* (16), and in fact, overproduction of actinorhodin in response to competition with other soil bacteria has been reported already (56). However, whether σ^V is involved in this process for *B. subtilis* remains unclear.

M. xanthus is also known to produce several degrading enzymes (31, 67). In

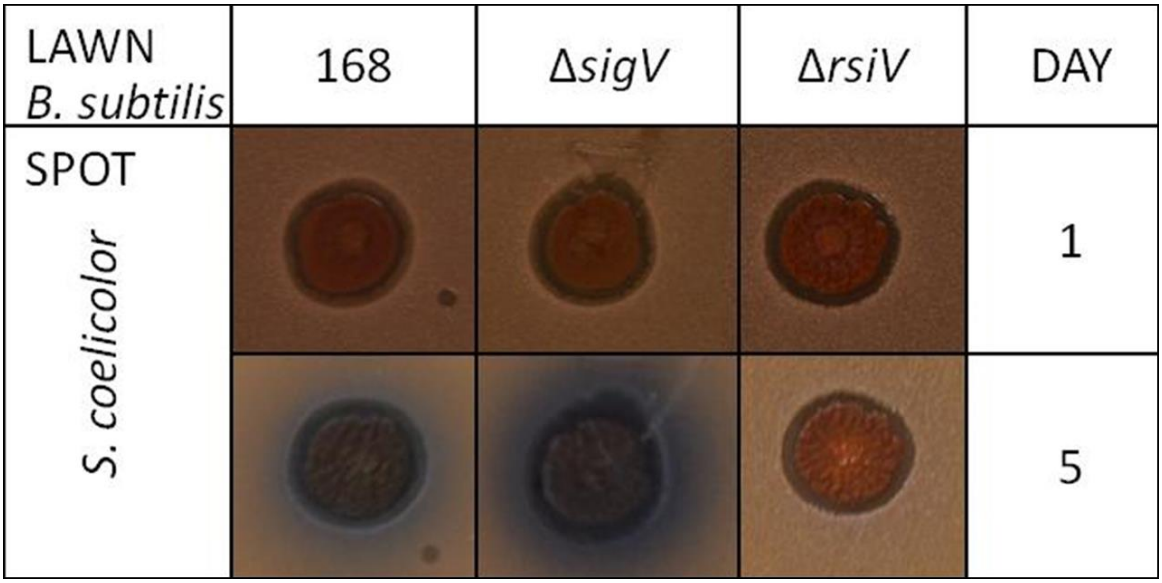


Figure 2.6. Spot on lawn assays depicting the sensitivity of the wildtype, Δ*sigV* and Δ*rsiV B. subtilis* lawn strains to spots of *S. coelicolor* spore preparations. No significant difference in inhibition is observed, however, and increased pigment production can be observed for the Δ*rsiV* lawn.

collaboration with the Kirby lab (University of Iowa) we obtained a wild-type *M. xanthus* strain to test against *B. subtilis*. We performed the experiments in a similar way than with *S. coelicolor* but were not able to obtain a phenotype (data not shown). These experiments were challenged by the slow growth of *M. xanthus* and the difficulties to grow these two organisms together. Hence we don't discard the idea that we could have possibly found interactions, were the experiments done differently.

Autolysins are well known cell wall degrading enzymes produced by many species of Bacillus, including *B. subtilis* (62). These autolysins can be purified rapidly using LiCl (12). We tested supernatants, LiCl extracts as well as concentrated cultures of different Bacillus spp, but unfortunately could not find any phenotype of the *sigV* mutant or the *rsiV* mutants when compared to wild-type (data not shown).

Furthermore, there are different groups currently working on σ^V and its regulation. Hastie, *et. al*. have found that the mechanism for σ^V activation in *B. subtilis* is controlled by Regulated Intramembrane Proteolysis (RIP) and that it requires the Site-2 protease RasP (33). Additionally, Varahan et. al, report that in *E. faecalis*, Eep, a membrane-bound zinc metalloprotease, is involved in induction of σ^V under lysozyme treatment by affecting the stability of RsiV (69).

It seems unlikely that the σ^V role in *B. subtilis* is exclusively to provide lysozyme resistance, however more experiments might be needed to test if σ^V is involved in other processes as well.

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2.6. Supplementary information

Table S2.1 Primers used in this study.

Primer	Description	Sequence
4556	sigV-fwd	CGCTTAATTAAATCCTAGGTAACAGCCTACG
4557	sigV-rev	CGAGGATCCTATATTCTTCTCTTAATTGC
4558	sigW-fwd	CGCTTAATTAACGAAGCTCGTATACATACAG
4559	sigW-rev	CGAGGATCCCACAATTTGTTCAGGACAGC
4560	sigX-fwd	CGCTTAATTAACTTTTCAAGCTATTCATACG
4561	sigX-rev	CGAGGATCCTAAATATCCTGAGGCGAACG
4970	sigM-fwd	CGCTTAATTAACGTGTATAACATAGAGGGGA
4590	sigM-rev	CGAGGATCCGCTTCTCGAGTTCTTCCTCA
5148	sigV::kan-up-fwd	CAGGCGGCAGAACAAGGCGTTATTG
5501	sigV::kan-UP-rev	CGAGCGCCTACGAGGAATTTGTATCGGCCAACCTGTAGAAATCTTG
5502	sigV::kan-DO-fwd	CCTATCACCTCAAATGGTTCGCTGACGCGCCTATACAGAGCATT
5151	sigV::kan-do-rev	CGGTTCTGCATCTTCGTCAGTGAGC
5050	RT-dltA fwd	ATCTCCTATCCTTGTGTACGGCCAC
5051	RT-dltA rev	GCAATTCTGCTCCAGAGCTTTCGAT
5352	RT-oatA-fwd	TGGCTTGGAACGAGGTCTTACGGAA
5353	RT-oatA-rev	TGCTGATGACAGATGTCGTTTTCCA
4368	RT-23S-fwd	AAAGGCACAAGGGAGCTTGACTGCGAGA
4369	RT-23S-rev	ATGAGCCGACATCGAGGTGCCAAACCT
5193	RT-sigV-rsiV fwd	CGAATACCGTCAAAACGCGCCTATA
5194	RT-sigV-rsiV rev	GCTGATGTCGGCCACATAACGATTC
5195	RT-rsiV-oatA fwd	GCGTCATTTCAAACTTGCTCGTGGG
5196	RT-rsiV-oatA rev	GGCCATCAAGTCCAGGAATGTATCG
5197	RT-oatA-yrhK fwd	TTGACTGCTCTTATTGTTCAGGCGA
5198	RT-oatA-yrhK rev	TTTGGATGTCATGTTCTTCATTTCCTTTCA
5156	oatA::tet-up-fwd	ACGAGACGGTCACAGACACGCCAGA
5157	oatA::tet-up-rev	GAGAACAACCTGCACCATTGCAAGATAGGTGATAAGTGATAACTG
5158	oatA::tet-do-fwd	GGGATCAACTTTGGGAGAGAGTTCCCTGATGGTGTTCACTTGGT
5159	oatA::tet-do-rev	CGCGCCGGCCTTATTATTCATATCG

Table S2.2. Genes induced ≥3-fold by xylose in strain Δ7 P_{xylA}-*sigV*.

Gene	Fold-change	Regulator	Gene	Foldchange	Regulator
sigV	708.5	V	*yvaG*	7.3	B
xylB	623.4	XYL	*yebC*	6.5	M
xylA	140.7	XYL	*radC*	6.0	M
xynP	68.8	XYL	*yrhM*	5.7	V
yrhL	58.6	V	*mmgE*	5.6	
xynB	46.0	XYL	*ydaE*	5.6	B
ywoA	41.6	M, X, W	*yhxD*	5.3	
yrhK	35.4	V	*ydaD*	5.2	B
yrhI	34.0	M,W	*mmgD*	5.1	
spo0M	30.9	W	*bofC*	4.7	B
abh	26.2	M,X	*maf*	4.6	M
pbpX	21.1	M,X	*yjgC*	4.3	B
dltC	17.6	M,X	*yqhA*	4.2	B
yrhJ	16.8	M,W	*yfhF*	3.9	B
xylR	15.7	XYL	*cydB*	3.7	W
dltD	15.7	M,X	*ynaI*	3.7	
dltB	15.6	M,X	*yqeZ*	3.6	W
ysnF	14.1	B	*yfjS*	3.4	
yjgD	11.9	B	*paiB*	3.4	
cydD	11.7	B	*yjlB*	3.3	
dltE	10.9	M,X	*ycdH*	3.3	
ytbD	8.2		*yveL*	3.3	
ytbE	7.9	B	*yqfB*	3.2	W
dltA	7.8	M,X	*yqfA*	3.2	W
ywaC	7.7	M,W	*mtlD*	3.1	
yqjL	7.5	M	*yfkD*	3.0	B

V refers to σ^V, M refers to σ^M, X refers to σ^X, W refers to σ^W, B refers to σ^B. XYL refers to xylose metabolism.

Table S2.3. Genes induced ≥1.5-fold by lysozyme in strain 168.

Gene	Fold-change	Regulator	Gene	Fold-change	Regulator
yrhM	104.7	V	*lytE*	1.8	
yrhL	63.1	V	*murF*	1.8	M
yrhK	61.6	V	*yoqL*	1.8	
sigV	41.3	V	*mreC*	1.8	M
ytvB	4.0		*yxiL*	1.7	
ywoA	3.6	MX	*yopO*	1.7	
ypjC	3.1		*yuiI*	1.7	Fur
yrhI	3.1	MX	*ykuD*	1.7	M
pbpX	2.6	X	*yvaE*	1.7	
radC	2.6	M	*yfnI*	1.7	M
yozO	2.6	W	*ydgA*	1.7	
ywcB	2.5		*abrB*	1.7	
yebC	2.5	M	*minD*	1.7	M
spoIIIAG	2.5	E	*yxiJ*	1.7	
maf	2.5	M	*yxiG*	1.7	
ycgR	2.4	M	*ydhN*	1.7	
dltB	2.4	MX	*yxjF*	1.7	
yybK	2.3		*yfmG*	1.7	
ywbN	2.3	Fur	*yxiF*	1.7	
dltD	2.3	MX	*yxzC*	1.7	
ddl	2.3	M	*yqfD*	1.6	E
yfiY	2.2	Fur	*dhbB*	1.6	Fur
yjbC	2.2	W	*ytvI*	1.6	
ywbO	2.2	Fur	*ywbM*	1.6	Fur
ymaG	2.2		*deaD*	1.6	
yjfC	2.2		*yhdG*	1.6	TnrA/GlnRA
ywaC	2.1	M	*minC*	1.6	M
yopM	2.1		*yfiM*	1.6	
mmgD	2.1		*ywhH*	1.6	
dltC	2.0	MX	*yybI*	1.6	
yczG	2.0		*yxiH*	1.6	
yxeB	2.0	Fur	*proJ*	1.6	
ywtC	2.0		*fhuC*	1.6	Fur
spoIIB	2.0		*yvaF*	1.6	
dltE	2.0	MX	*yxzG*	1.6	
ykuO	2.0	Fur	*yqfT*	1.6	
fhuG	1.9	Fur	*rocB*	1.6	
gerAB	1.9		*scoB*	1.6	
yopX	1.9		*ylbJ*	1.6	E

Table S2.3. Continued.

Gene	Fold-change	Regulator	Gene	Fold-change	Regulator
yocL	1.9		*yopV*	1.6	
ywbL	1.9	Fur	*yoeB*	1.6	
ytnA	1.9	M	*braB*	1.5	TnrA/GlnRA
ycgQ	1.9	M	*ywcA*	1.5	
dhbE	1.9	Fur	*yxiI*	1.5	
dltA	1.9	MX	*mreB*	1.5	M
wapA	1.9		*yclN*	1.5	Fur
mreD	1.9	M	*yoaG*	1.5	W
ydaH	1.8	M	*dhbF*	1.5	Fur
yxiM	1.8		*yefC*	1.5	
maeN	1.8		*yccC*	1.5	TnrA/GlnRA
yqjL	1.8	M	*yxdL*	1.5	
abh	1.8	MXW	*spoIVCA*	1.5	
dhbC	1.8	Fur			
ydeD	1.8				

V refers to σ^V, M refers to σ^M, X refers to σ^X, W refers to σ^W, E refers to σ^E.

CHAPTER 3

THE -30/-26 STRETCH OF "T"S: A NEW PROMOTER ELEMENT THAT CONFERS SPECIFICITY FOR THE EXTRACYTOPLASMIC FUNCTION SIGMA FACTORS OF *BACILLUS SUBTILIS* WITH OVERLAPPING REGULONS

The extracytoplasmic function (ECF) sigma (σ) factors of *B. subtilis* regulate a number of functions important for cell survival under different stresses. Out of the 7 ECF σ factors of *B. subtilis*, three have been studied in quite some detail (σ^M, σ^W, σ^X). More recently, the regulon and induction signal of a fourth σ (σ^V) has been found. Interestingly, these four σ factors show overlap in the genes that they regulate which complicates the study of their functions. There have been several promoter structures proposed to provide specificity for σ factor recognition; here we describe a novel one. In a previous study, we had shown that the promoters that are able to be recognized by σ^{MXW} and σ^V have a conserved stretch of Ts right downstream of their -35 element. We hypothesized that this stretch of Ts could be important for σ^V recognition, and thus serve as a discriminator for the rest of the ECF σ factors of *B. subtilis* with overlapping regulons. Here, we provide *in vivo* data that shows that this element is, in fact, important for promoter recognition by σ^V, and that disrupting the stretch of Ts has a detrimental effect on transcription driven by this on σ factor. Additionally, disrupting the stretch of Ts has a positive effect on transcription dependent on σ^M in three of the four promoters tested, suggesting that this novel promoter element could serve as a discriminator between σ^V and σ^M in *B. subtilis*.

The majority of this work was performed by Veronica Guariglia-Oropeza, with the exception of the P_{murG} mutagenesis which was performed by Albert Chen as part of his undergraduate project.

3.1. Introduction

Gene expression is a highly regulated process. A cell not only needs to express all the necessary proteins for housekeeping functions, but it also needs to adapt to different environments and, in some cases, differentiate into multiple cell types. Gene expression involves the transcription of DNA into RNA and the subsequent translation to protein. Since transcription is the first step in gene expression it is, in most cases, regulated at many levels (19), often involving sequence specific DNA-protein interactions between the RNA polymerase (RNAP), the σ subunit and the promoter DNA. The recognized sequence of the promoter varies depending on the holoenzyme (RNAP core + σ). For σ^{70} class promoters, the key recognition elements are usually located at -35 and -10 base pairs upstream of the transcription start point (32).

σ factors are believed to recognize clearly distinguishable promoter DNA determinants to activate a different set of genes, known as their regulons, however, in many cases two or more σ factors recognize the same core promoter elements, and the specificity of the response is achieved by variable combinations of *cis*-acting promoter features, and *trans*-acting protein factors (41).

One of the best studied cases of overlap in σ factor promoter recognition occurs in *E. coli* between the house keeping σ factor, σ^{70}, and the alternative σ factor, σ^{S}; both of which recognize very similar core promoter elements. Within the promoter, several different specificity elements have been described (Figure 3.1). UP elements consists of A+T-rich sequences located upstream

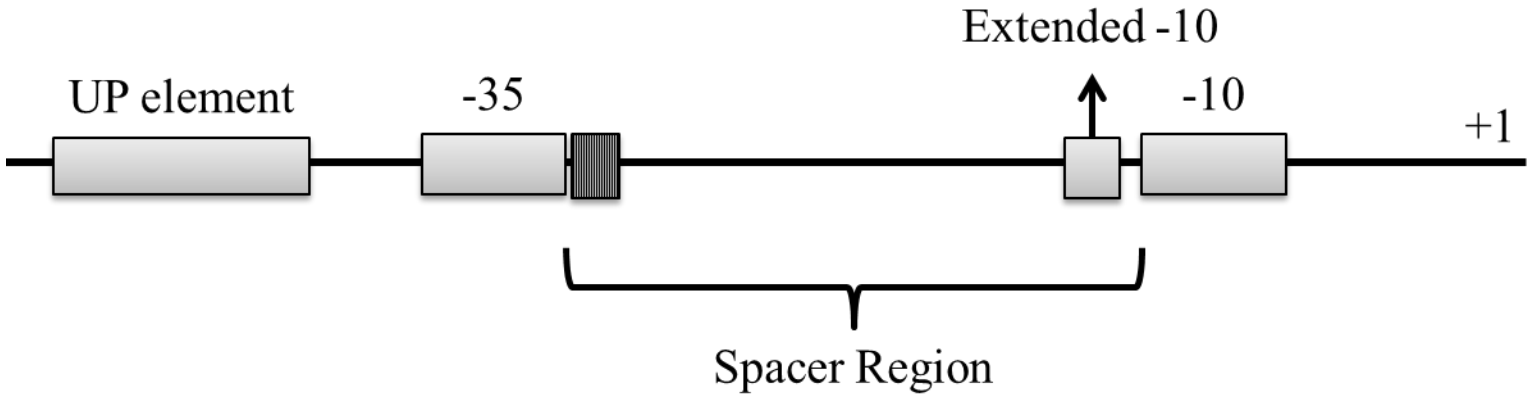

Figure 3.1. Promoter elements that provide σ factor specificity. Graphic representation of the different promoter determinants involved in specificity that have been described to date. The UP element, -35 and -10 elements, and the extended -10 element are depicted as gray boxes. The spacer region and the +1 transcriptional start site are also labeled. The proposed -30/-26 stretch of Ts is shown as a striped box.

of the -35 element of many promoters (11, 36, 37). In *E. coli*, a distal UP-element site has been shown to be beneficial for σ^S recognition, whereas a proximal UP-element favors σ^{70} selectivity. Additionally, σ^S is able to recognize less conserved -35 elements (42), however how σ^S utilizes the -35 element remains unclear. The spacer region has long been proposed to have a role in sigma factor specificity (2). It has been shown that σ^S is able to tolerate sub-optimal spacer lengths, whereas σ^{70} strongly prefers promoters with 17 bp spacing between the -10 and -35 elements (42). Additionally, it's been speculated that the spacer can influence the trajectory or flexibility of DNA as it enters the RNAP channel and that region 1.1 of σ^{70} monitors channel entry (21). Interestingly, a -13 C directly upstream of the -10 element (within the spacer region) has been shown to be a hallmark of σ^S-dependent promoters and conversely, counter selected in σ^{70}-dependent promoters (12, 25, 30, 43). And finally, an A/T rich discriminator region downstream of the -10 promoter element is common in σ^S-dependent promoters, suggesting a possible promoter melting defect of this alternative σ factor (25). In fact, promoter melting deficiency has been established as a trademark of alternative σ factors, since many have non conserved amino acids at the positions implicated in promoter melting, and a balance between melting and recognition seems to be important for promoter recognition specificity (24). Most likely, a combination of several of these factors ultimately determines the specificity of each promoter.

The genome of *B. subtilis* encodes 7 ECF σ factors: σ^M, σ^W, σ^X, σ^V, σ^Y, σ^Z and σ^{ylaC} (15, 16). The regulons for five of these have been identified (Reviewed in (18)). σ^M regulates a large set of genes that include essential functions of cell division and envelope synthesis (9). The σ^W regulon includes at least 60 genes that inactivate, sequester, or eliminate toxic compounds from the cell (15). The σ^X regulon includes genes which serve to alter cell surface properties to

provide protection against antimicrobial peptides (5). σ^V regulates a set of ~30 genes and is involved in resistance to lysozyme (13). σ^Y appears to control a small regulon of less than a dozen genes with poorly defined functions (8). The regulons and functions of σ^Z and σ^{ylaC} have not been yet determined.

Functional redundancy and regulatory overlap among the ECF σ factors of *B. subtilis* has been thoroughly documented (6, 10, 13, 27, 34), and in several cases, the associated phenotypes to lacking a σ factor can only be obtained when mutating two or more of them (26, 28). Sequence comparisons studies indicate that the promoters recognized by σ^X and σ^W share similar -35 elements but are distinguished by different base preference at two key positions in the -10 element (22). A later study showed that changes to the -10 element are sufficient to switch a promoter from the σ^X to the σ^W regulon and vice versa (35).

Even though overlap in regulation has been consistently shown among the ECF σ factors of *B. subtilis*, additional promoter specificity determinants have not been reported. Here we propose a novel promoter element, the -30/-26 stretch of Ts, within the spacer region (Figure 3.1) which is involved in promoter recognition by the most active ECF σ factors of this organism.

3.2. Materials and methods

3.2.1. Strains and growth conditions.

All *B. subtilis* strains were constructed in the 168 background (Table S3.1). Unless otherwise stated bacteria were grown in liquid Luria-Bertani (LB) medium at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bactoagar (Difco). All cloning was done in *E. coli* DH5α using ampicillin (AMP, 100 μg/ml) for selection. Chromosomal DNA and plasmid DNA transformations were performed as previously reported (14). The following antibiotics were used

for selection at their respective concentrations: chloramphenicol (CAT, 10 μg/mL), macrolide-lincosoamide-streptogramin B (MLS; contains 1 μg/ml erythromycin and 25 μg/ml lincomycin), and neomycin (NEO; 10 μg/ml).

3.2.2. Promoter site directed mutagenesis

The stretch of Ts for each promoter was mutated using overlap extension PCR (20). Briefly, for each promoter a set of primers were designed, two universal and flanking, and three pairs of mutagenic overlapping primers to introduce the TTTTT → AAAAA, TTTTT → TAAAT, or TTTTT → TTATT (Table S3.3). Each mutagenic primer is used in pair with a flanking primer to generate two fragments that will have overlapping ends. These two fragments are used in a second PCR "fusion" reaction using only the flanking primers, and the resulting fusion product is amplified by further PCR.

3.2.3. Transcriptional fusions.

Promoter regions were amplified from *B. subtilis* chromosomal DNA using a forward primer (~100 bp upstream of the -35 consensus) with restriction site HindIII and a reverse primer (typically ~50 bp downstream of the start codon) with restriction site BamHI (Table S3.3). The resulting fragments were digested with HindIII and BamHI and cloned into pJPM122 (39) and verified by DNA sequencing (Table S3.2). Promoter fusions were introduced into the SPβ prophage by a double-crossover event, in which each pJPM122 derivative was linearized with ScaI and transformed into *B. subtilis* strain ZB307A with selection for neomycin resistance. The SPβ lysates were prepared by heat induction and used to transduce the wild-type 168, the Δ7ECF, and the inducible ECF σ factor strains HB12010, HB12020, HB12035 and HB12036.

3.2.4 β-galactosidase assays

Strains carrying promoter–*lacZ* fusions were grown to an OD_{600} of 0.4 in LB, induced with the addition of 2% xylose, and samples were collected after 20 min incubation. Non induced controls were incubated for 20 min without additions. β-Galactosidase assays were performed as previously described (29).

3.2.5 Protein purification

Core RNAP was purified from 50 g cell paste of *B. subtilis* as previously described (17). Briefly, after lysis, RNAP is precipitated using 5% Polymin P fractionation and then eluted from the pellet using TGED buffer pH 8 + 1M NH_4Cl. From the 1M eluate, proteins were precipitated by adding and equal volume of saturated (100%) ammonium sulfate. The pellet was then desalted by dialysis on TGED buffer. RNAP was further purified through three FPLC chromatography steps: Heparin column, MonoQ column and a final size exclusion step with Superdex-75 column (Amersham biosciences). Finally RNAP was dialyzed into storage buffer (TGED, 50% Glycerol, 100mM NaCl).

The *sigV* gene was PCR amplified from *B. subtilis* chromosomal DNA with primers 4626/4627 designed to engineer an NdeI site upstream and a BamHI site downstream of the *sigV* gene (Table S3.3). The PCR product was cloned into pET11a (Novagen) via the NdeI and BamHI sites to generate pVG010 (Table S3.2). The sequence of *sigV* in pVG010 was verified by DNA sequencing (Cornell DNA sequencing facility). The resultant plasmid was used to transform BL21/DE3(pLys) cells to create strain HE-12023 (Table S3.1). Cells were grown to mid-logarithmic phase at 37°C in 1 L of LB medium and 100 mg ml-1 of ampicillin. σ^V expression was induced with 1 mM IPTG for 3 h at 37°C. Cells were collected by centrifugation,

resuspended in 20 ml of disruption buffer [50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1 mM dithiothreitol (DTT), 1 mM b-mercaptoethanol, 233 mM NaCl, 10% (v/v) glycerol] and lysed by sonication. The inclusion bodies were recovered by centrifugation and washed twice with 10 ml TEDG buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1 mM DTT, 10% glycerol] containing 0.5% (v/v) Triton X-100 and then dissolved in 10 ml of the same buffer plus 1% (v/v) Sarkosyl.

After centrifugation to remove the insoluble fraction, the supernatant was gradually diluted to 100 ml with TEDG-0.01% Triton X-100, to allow renaturation. Following renaturation, σ^V was purified through two FPLC chromatography steps, a MonoQ column and a Superdex-75 column (Amersham biosciences). Finally the sample was dialysed into TEDG-0.1 M NaCl-0.01% (v/v) Triton X-100–50% (v/v) glycerol and stored at -80°C. σ^M was purified in a similar fashion using strain HE-4600 and following a previously described protocol (9).

3.2.5 In-vitro transcription

Run-off *in vitro* transcription was performed as previously described (35) using the universal flanking primers to amplify the P_{dltA} promoter variants to use as templates. Typical transcription reaction mixtures (20 µl) contained 0.36 pmol of core RNAP, 4.5 pmol of σ, 4.2 pmol of δ, and 0.04 pmol of template DNA in transcription buffer (20 mM Tris-HCl [pH 8.0], 10 mM MgCl2, 50 mM KCl, 0.5 mM DTT, 0.1 mg of bovine serum albumin/ml, 5% [vol/vol] glycerol, and the RNase inhibitor RNasin from Promega at 0.8 U/reaction), to which were added nucleoside triphosphate mixtures containing 10 nmol of ATP, GTP, and CTP, 1 nmol of UTP, and 0.6 pmol of [a-32P]UTP (3,000 Ci/mmol).

Core RNAP, σ, and δ were mixed on ice for 15 min to form RNAP holoenzyme before the addition of template DNA and incubation at 37°C for 10 min to allow promoter binding. Nucleoside triphosphates were added, and transcription was allowed to proceed for 10 min at 37°C. Reactions were terminated by the addition of 80 µl of stop solution (2.5 M NH4 acetate, 10 mM EDTA, and 0.1 mg of glycogen/ml), extracted with phenol-chloroform, and precipitated with ethanol. The pellets were dissolved in 8 µl of loading buffer (20 mg of xylene cyanol FF/ml, 20 mg of bromophenol blue/ml, and 60 mg of urea/ml in 13 Tris-borate-EDTA buffer) and subjected to 8 M urea–6% polyacrylamide gel electrophoresis. Reaction products were visualized by using a Molecular Dynamics PhosphorImager system and ImageQuant software.

3.3. Results and discussion

Out of the seven ECF σ factors of *B. subtilis*, σ^M, σ^W, σ^X and σ^V are the best understood. A transcriptional study on the induction of σ^V in the absence of all of the other ECF σ factors has shown that the set of genes regulated by σ^V predominantly overlaps with the regulons of σ^M, σ^W and σ^X. Furthermore, a consensus built with an alignment of the promoters of genes that belong to the σ^M, σ^X or σ^W regulons and are also regulated by σ^V shows a conserved stretch of Ts that is absent in a consensus sequence of promoters of the genes that belong to the σ^M, σ^X or σ^W regulons but are not regulated by σ^V (Figure 3.2). To test if this stretch of Ts has a role in σ specificity, we undertook a mutational analysis of five different promoters, four of them containing the stretch of Ts and regulated by several ECF σ factors, and one which lacks the stretch of Ts and is regulated by one σ factor (Table 3.1).

3.3.1 In vivo analysis – Stretch of Ts deleted

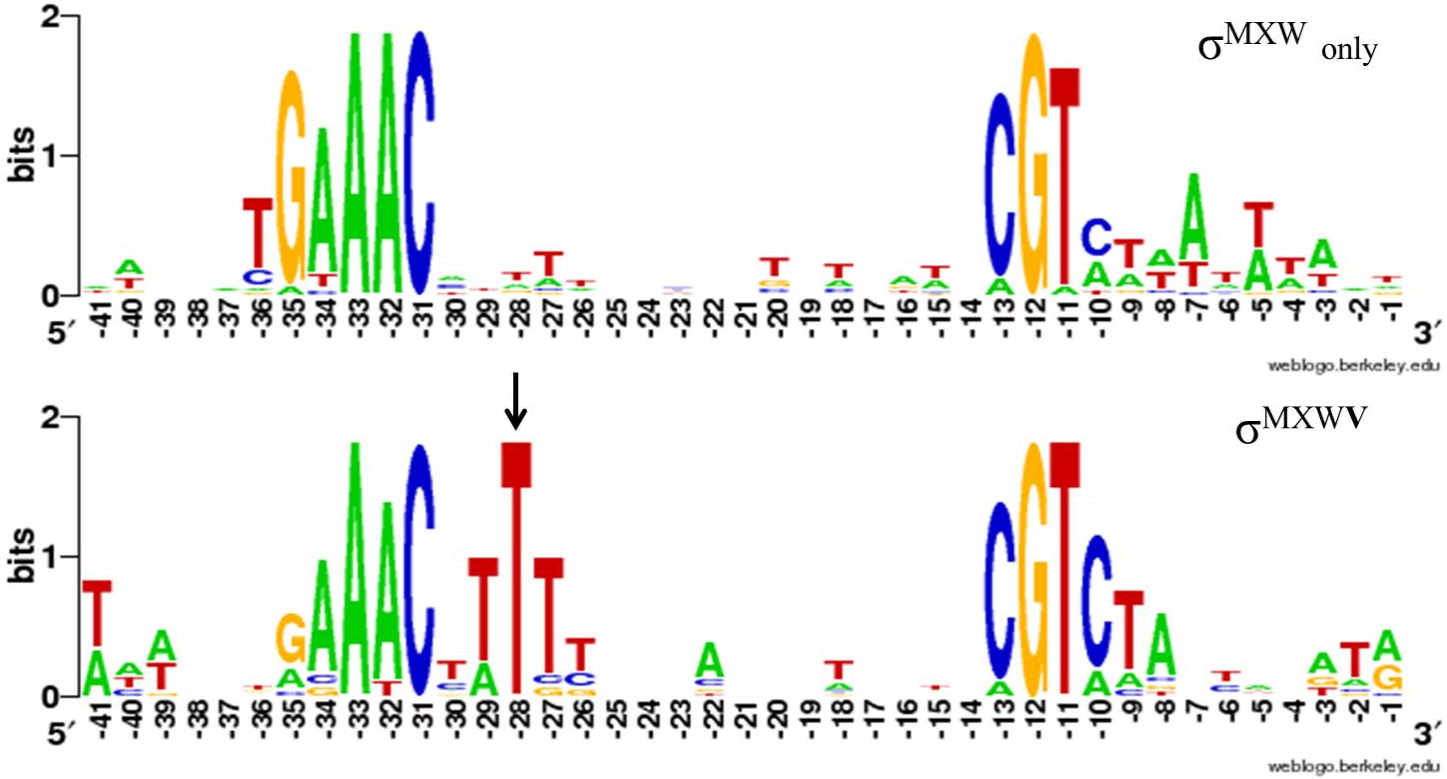

Figure 3.2. The -30/-26 stretch of Ts is conserved in σ^V -regulated promoters. The promoter consensus sequence alignments were obtained using the Weblogo software (http://weblogo.berkeley.edu/) and represent the promoters that are regulated by σ^M, σ^X or σ^W that are not regulated by σ^V (σ^{MXW}; top panel) and those that are regulated by σ^M, σ^X or σ^W and also regulated by σ^V (σ^{MXWV}; bottom panel). Promoter sequences were obtained from several studies (4, 5, 7, 9, 13, 23) The highly conserved -28 T is labeled with an arrow. Modified from (13).

Table 3.1. ECF σ promoters with overlapping regulation.

Promoter	Sequence	Regulator
$P_{dltABCDE}$	aaaaaTGAAACttttt**gagc**-atctgatCGTCaaataatcA	X, M, V
P_{bcrC}	ttattTGAAACtttt**catgagtaagatt**AGTCtactaaAta	M, X, V
P_{pbpX}	tttttGACAACttttttt**agggctttatt**CGTCtaacaaaac	X, M, V
P_{abh}	aagcgGGAAACttttt**caaagtttcatt**CGTCtaCGATaTA	X, M, W, V
P_{murG}	ttacgGGAAAC**ccgagagcctctgaagt**CGTCtcaataaaGac	M

The region to be mutated is highlighted in gray. Regulator refers to the ECF σ factors of *B. subtilis* in order of their contribution to promoter activity.

The four promoters chosen are all regulated by σ^V as well as by other ECF σ factors (Table 3.1). All of these promoters have a stretch of T's immediately downstream of the -35 element. We used overlap extension PCR (20) to mutate the T's to A's sequentially (from TTTTT to AAAAA, TAAAT, or TTATT). We then cloned the mutated fragments into the vector pJPM122 to create transcriptional *lacZ* fusions which were then integrated into the SPβ phage (39). These phages were used to transduce the fusions into the desired strains of *B. subtilis*.

Our laboratory has developed a set of strains where induction of each ECF σ factor can be achieved ectopically and under xylose control in the absence of all other ECF σ factors. (1, 13). In this way, we can study the activity of the mutated promoters driven by only one σ factor at a time, to decipher the contribution of the stretch of Ts, or lack thereof, on transcription activity driven from each promoter. We used the SPβ lysates to transduce the wild-type and mutant promoter fusions into the inducible strains Δ7sigV, Δ7sigM, Δ7sigW and Δ7sigX. As controls, we introduced the promoter variants into the wild-type strain, 168, to assess the normal activity of the wild-type promoter and variants in the presence of all ECF σ factors, as well as in the Δ7ECF as a negative control. Cells were grown and induced with xylose and β-galactosidase activity was measured under inducing and non-inducing conditions using a liquid assay (29). The activity driven from each mutated promoter was compared to that of the wild-type promoter.

The first ECF σ promoter tested was that upstream of *dltA*, the first gene in the *dltABCDE* operon. This operon codes for the proteins involved in D-alanylation of teichoic acids, one cell wall modification pathway that changes the overall net charge (33) and has been shown to be involved in resistance to cationic antimicrobial peptides and lysozyme (5, 13). Transcription of this promoter is predominantly regulated by σ^X (5), however activity dependent on σ^M (9) and σ^V (13) has also been reported.

Our results show that even mutating the highly conserved -28 T severely affects the activity of this promoter (Figure 3.3). In the wild-type strain 168 (where all ECF σ factors are present, albeit most likely at different concentrations), mutating the -28 T (from TTTTT to TTATT) decreases the activity of the promoter around 50%. This is also observed for σ^X and σ^V. Interestingly, the activity of this promoter dependent on only σ^M is almost doubled, suggesting the continuous stretch of five Ts, which is interrupted by mutating the -28 T, could be important for sigma factor specificity between σ^M and σ^X and σ^V. Further mutating the Ts to TAAAT or AAAAA reduces the activity dependent on all ECF σ factors tested, suggesting the promoter structure has been severely compromised.

The next two promoters tested, P_{bcrC} and P_{pbpX} showed a very similar pattern to that of P_{dltA}. The *bcrC* gene (formerly *ywoA*) encodes for an undecaprenyl pyrophosphate phosphatase and has been shown to be important for bacitracin resistance (3). Regulation of this gene is primarily dependent on σ^M, with an additional important role of σ^X (6). When σ^M and σ^X are absent, *bcrC* can also be transcribed via σ^V (13). Mutating the stretch of Ts in this promoter also disrupts its activity (Figure 3.4).

Mutating the -28 T decreases the activity of this promoter over 50% in both the wild-type background and the σ^V induced background, showing again the importance of this region. The fact that the activity of the wild-type is reduced to 50%, even when activity driven by σ^M is unaffected, suggests that another ECF σ factor (possibly σ^X) is important under the conditions tested. Unfortunately, the data obtained in the inducible σ^X was inconsistent (data not shown), so this hypothesis could not be tested.

The gene *pbpX* encodes for the penicillin-binding endopeptidase X, a low molecular weight PBP thought to localize with a FtsZ-like pattern during sporulation (38). Transcription of

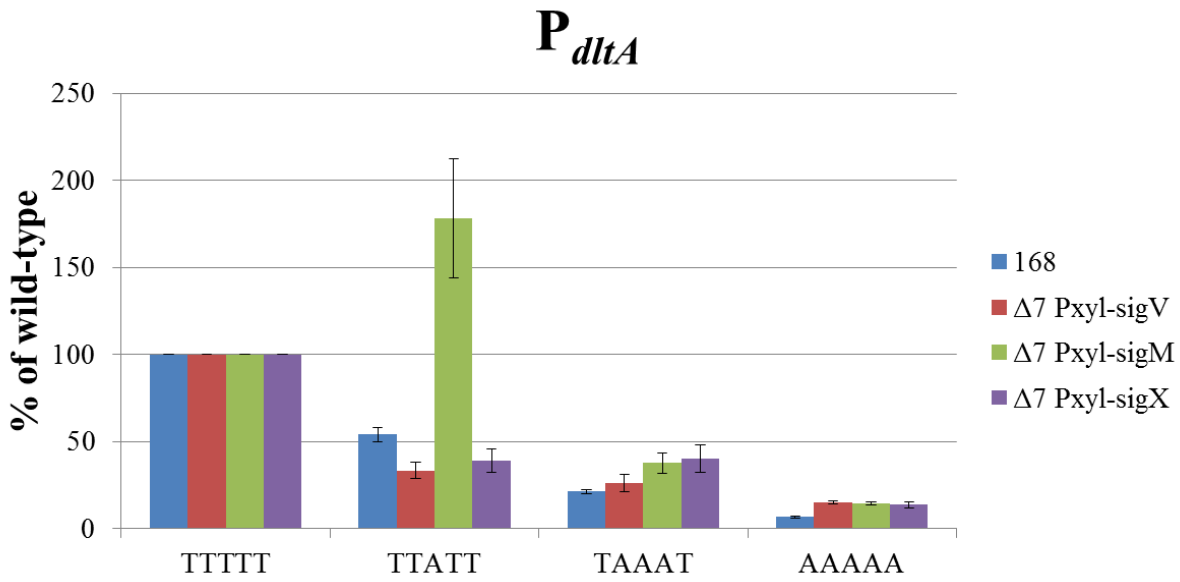

Figure 3.3. β-galactosidase activity driven from the P_{dltA} promoter and its mutant variants. Data corresponds to the induced (+ xylose) β-galactosidase liquid assays. The activity of the wild-type (TTTTT) promoter was been set to 100% and the mutant variant activities are expressed as the % of wild-type. Experiments were performed at least three times in duplicates; error bars depict the standard error.

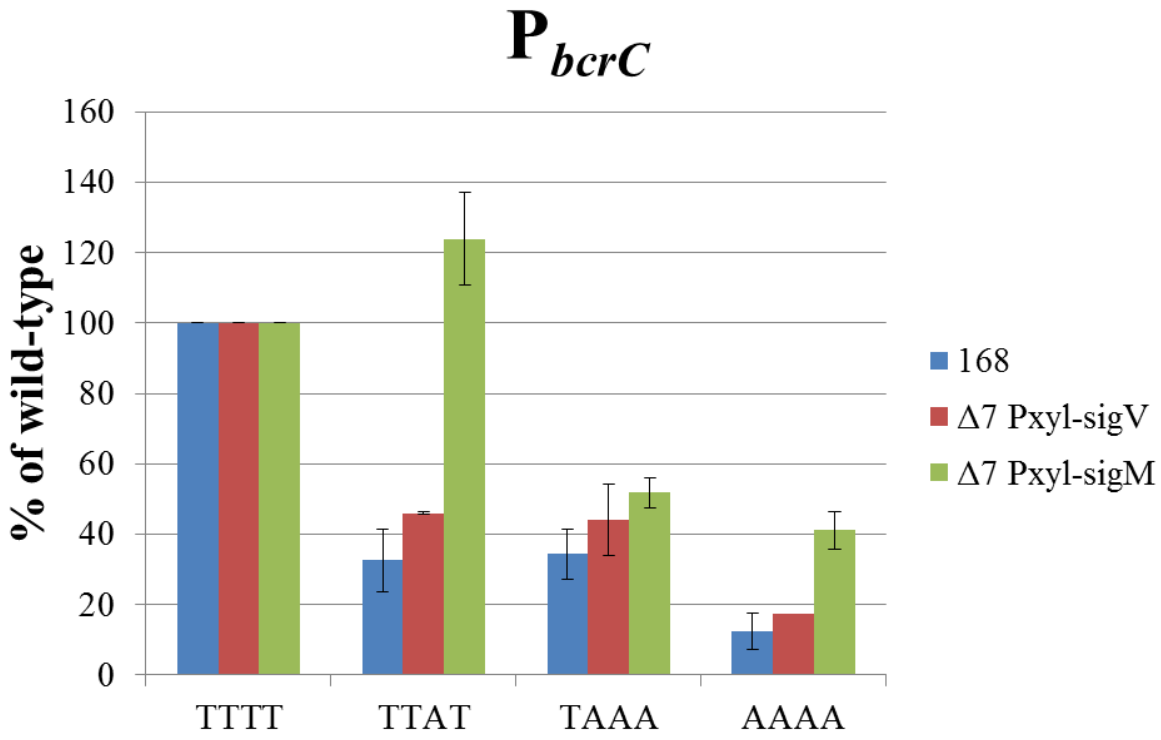

Figure 3.4. β-galactosidase activity driven from the P_{bcrC} promoter and its mutant variants. Data corresponds to the induced (+ xylose) β-galactosidase liquid assays. The activity of the wild-type (TTTT) promoter was been set to 100% and the mutant variant activities are expressed as the % of wild-type. Experiments were performed at least three times in duplicates; error bars depict the standard error.

pbpX is driven predominantly by σ^X (5), however activity from σ^M and σ^V have also been reported (9, 13). Just as for P_{dltA} and P_{bcrC}, mutating the -28 T on the spacer region of the promoter affects activity driven by the different ECF σ factors of *B. subtilis* involved in regulation of the transcription of this promoter (Figure 3.5). The activity of this promoter in both the wild-type and the σ^X induced background is reduced 50% on the mutated variant compared to the wild-type promoter, going in accordance with σ^X being the main ECF σ factor responsible for transcription of *pbpX*. Interestingly, mutation of -28 T severely decreases activity driven by σ^V, signifying again the importance of the stretch of Ts in σ^V specificity. In the same way as with P_{dltA}, mutating the -28 T increases activity of P_{pbpX} driven by σ^M in added evidence that this novel promoter element could be important for distinguishing recognition of ECF σ factors.

Lastly, we tested the ECF σ promoter upstream of *abh*. The *abh* gene encodes for a transcriptional regulator paralogous to AbrB, and as such, plays part in the complex interconnected system of regulatory functions that controls gene expression during the transition from active growth to stationary phase (40). The transcription of *abh* is predominantly driven by σ^X, a *sigX* mutant has decreased activity of a P_{abh}-*gfp* promoter fusion (31). However, there is evidence for the other three most active ECF σ factors of *B. subtilis*, σ^W, σ^M, and σ^V, to also be able to recognize this promoter (9, 13, 27, 31, 40).

The mutagenesis of the *abh* promoter further reinforces our finding that the stretch of Ts is crucial for activity dependent on σ^V, since mutating the -28 T on P_{abh} reduces activity in this background just as with the other three promoters tested (Figure 3.6). Interestingly, for the *abh* promoter, mutating the -28 T does not increase, but reduces, activity in the σ^M induced background, in contrast with what was observed for the other three promoters. The reasoning for this effect is still unclear, however it is possible that in the regulation of activity of this promoter

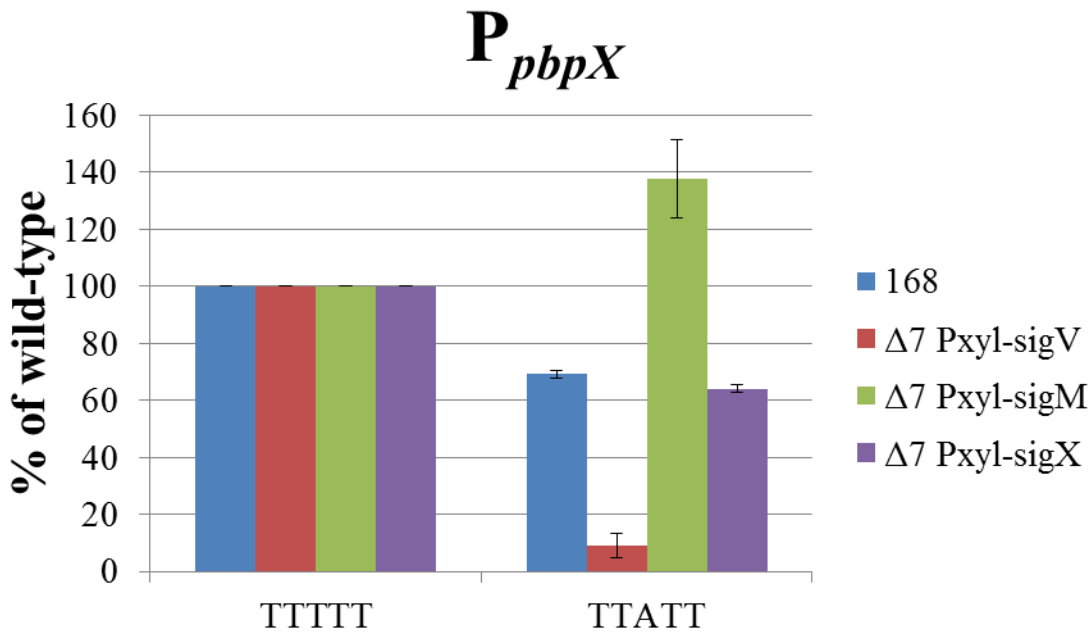

Figure 3.5. β-galactosidase activity driven from the P_{pbpX} promoter and its mutant variant. Data corresponds to the induced (+ xylose) β-galactosidase liquid assays. The activity of the wild-type (TTTTT) promoter was been set to 100% and the mutant variant activities are expressed as the % of wild-type. Experiments were performed at least three times in duplicates; error bars depict the standard error.

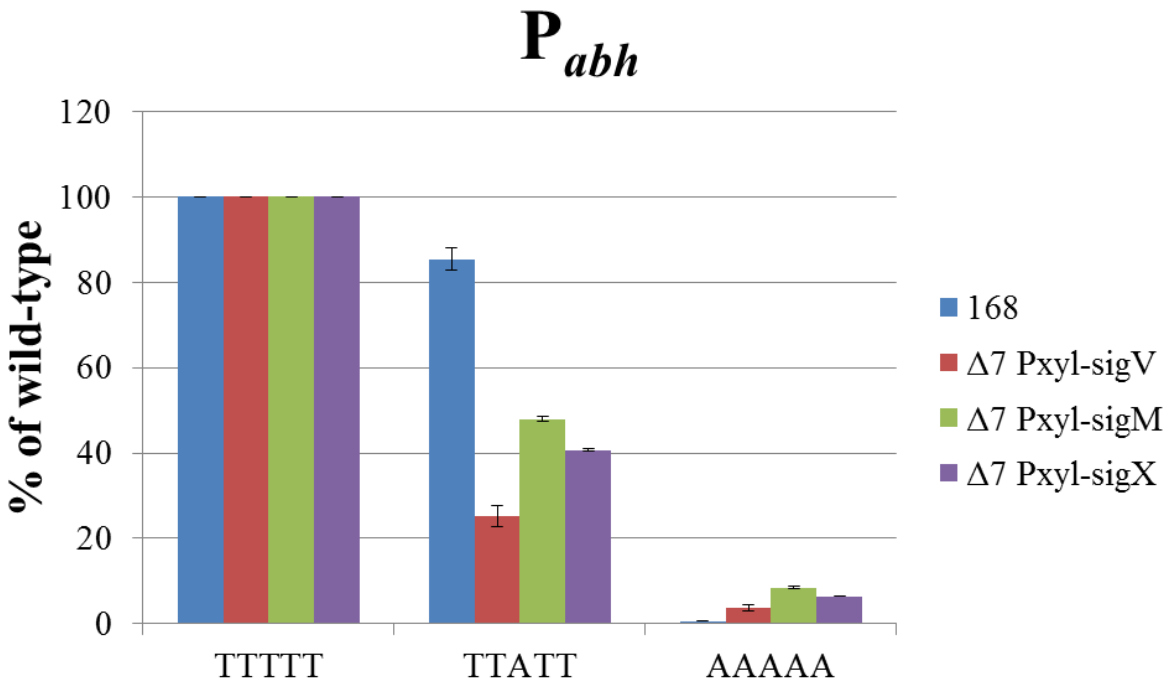

Figure 3.6. β-galactosidase activity driven from the P_{abh} promoter and its mutant variants. Data corresponds to the induced (+ xylose) β-galactosidase liquid assays. The activity of the wild-type (TTTTT) promoter was been set to 100% and the mutant variant activities are expressed as the % of wild-type. Experiments were performed at least three times in duplicates; error bars depict the standard error.

the stretch of Ts could also be important for σ^M recognition. Furthermore, even though the activity of the mutated P_{abh} is decreased for all three σ factors tested, the activity of this promoter is not as affected in the wild-type background, suggesting a combination of activities of σ^M, σ^X and σ^V or possibly other factors present in the wild-type strain are driving the activity from this promoter. Even though it had been reported that expression of *abh* was in part dependent on σ^W (31), under the conditions tested, we did not observe any P_{abh} promoter activity in the σ^W induced background (data not shown). The reasoning for this discrepancy is unclear, but could be due to the different strains and reporter fusions used.

3.3.2 In vivo analysis – Stretch of Ts added

To complement our analysis, we tested a fifth promoter, P_{murG}, which lacks a stretch of Ts and its transcription is dependent on σ^M only (Table 3.1). This promoter is inside the open reading frame of *murG*, the first gene in the essential peptidoglycan synthesis and division operon *murGmurB divIBylxXW sbp* (9). To this promoter we added a -30/-26 stretch of Ts to replace the CCGAG sequence in this position (Table 3.1) and tested for its activity in the ECF σ factor inducible strains (Figure 3.7). To our surprise, adding a stretch of Ts to this promoter increased its basal activity in the wild-type background, possibly suggesting that it can now be recognized better by some of the ECF σ factors. In fact, the activity of this promoter driven by σ^V has been increased 10-fold (Table 3.2), further confirming out hypothesis that the stretch of Ts is crucial for σ^V recognition.

Even though mutating the stretch of Ts seemed to, in some cases, decrease activity under σ^X induction, for P_{murG}, adding a stretch of Ts does not help the recognition of this promoter by this σ factor. Furthermore, the activity of *murG* dependent on σ^M was also increased

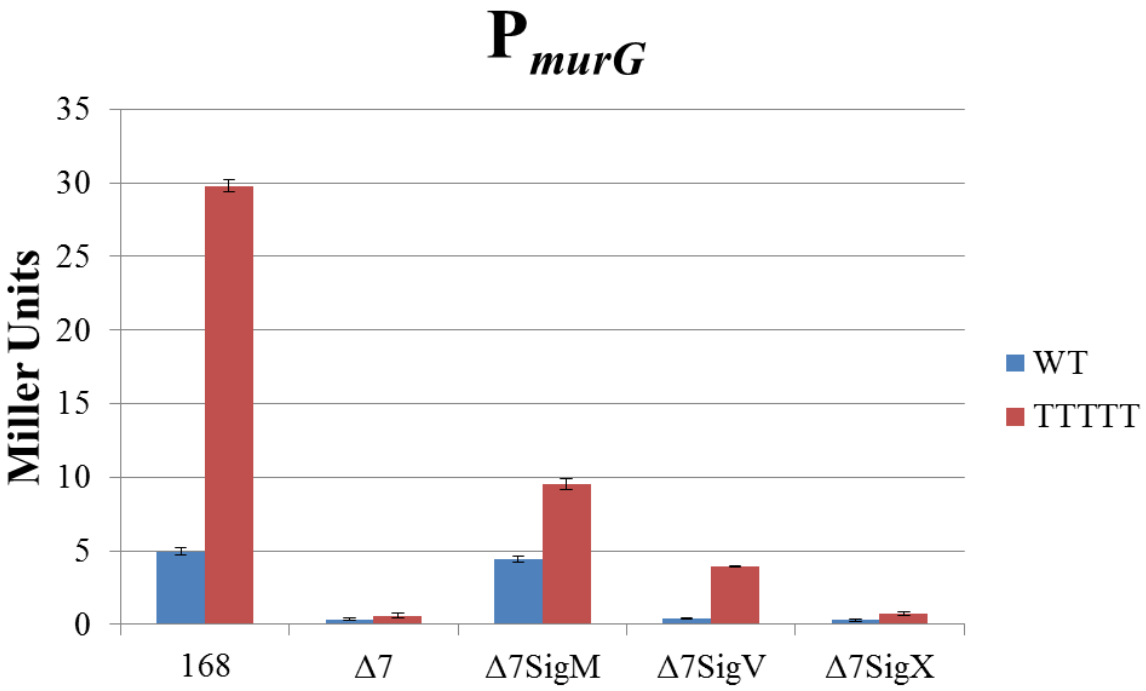

Figure 3.7. β-galactosidase activity driven from the P_{murG} promoter and its mutant variant. Data corresponds to the induced (+ xylose) β-galactosidase liquid assays. Experiments were performed at least three times in duplicates; error bars depict the standard error.

Table 3.2. β-galactosidase activity driven from the P_{murG} promoter and its mutant variant.

	Miller Units (AVE)	STE	Foldchange (TTTTT/WT)
168 WT	4.95	0.26	6.02
168 TTTTT	29.79	0.41	
Δ7 WT	0.34	0.09	1.72
Δ7 TTTTT	0.58	0.15	
Δ7SigM WT	4.45	0.19	2.14
Δ7SigM TTTTT	9.52	0.38	
Δ7SigV WT	0.40	0.04	9.73
Δ7SigV TTTTT	3.92	0.04	
Δ7SigX WT	0.32	0.09	2.30
Δ7SigX TTTTT	0.73	0.13	

(albeit only 2 fold), suggesting the addition facilitates somehow the recognition by σ^M as well. This fact seems puzzling given the fact that for three of the four promoters tested, mutating the -28 T, and hence disrupting the stretch of Ts, seemed to increase or not affect activity in the σ^M induced background. Whether the stretch of Ts plays a role in σ^X or σ^M specificity remains unclear, but it is clear that for some promoters it can affect its recognition.

3.4. Conclusions and further remarks

Overall, our *in vivo* data supports our hypothesis that the -30/-26 stretch of Ts constitutes a new promoter element that is important for σ^V recognition and specificity. However, how it affects recognition by other σ factors, and if it requires other *cis*- or *trans*- acting factors (as many overlapping σs do) remains unclear.

In an attempt to corroborate our *in vivo* results *in vitro*, we purified RNAP, σ^V, and σ^M to test with PCR amplicons of P_{dltA} and its mutant variants in run off *in vitro* transcription experiments (Figure 3.8 A and B). We were able to observe a transcript when σ^M was present and which was decreased after all Ts were mutated. However, we could not compare this with σ^V given that our protein preparation seemed to be inactive. We tried several concentrations of the σ factor and three different batches of protein but we could not get σ^V activity. It is possible that the inclusion body protocol is inadequate for σ^V purification, or that σ^V requires of additional factors for activity. In any case, further experiments will be needed to test this.

We find it puzzling that a single base change within the stretch of Ts could have such a drastic effect in promoter recognition, and although we don't discard the possibility that base specific contact between σ and DNA could occur in this region, we presume that the role of the stretch of Ts has more to do with the DNA topology or conformation that this region offers.

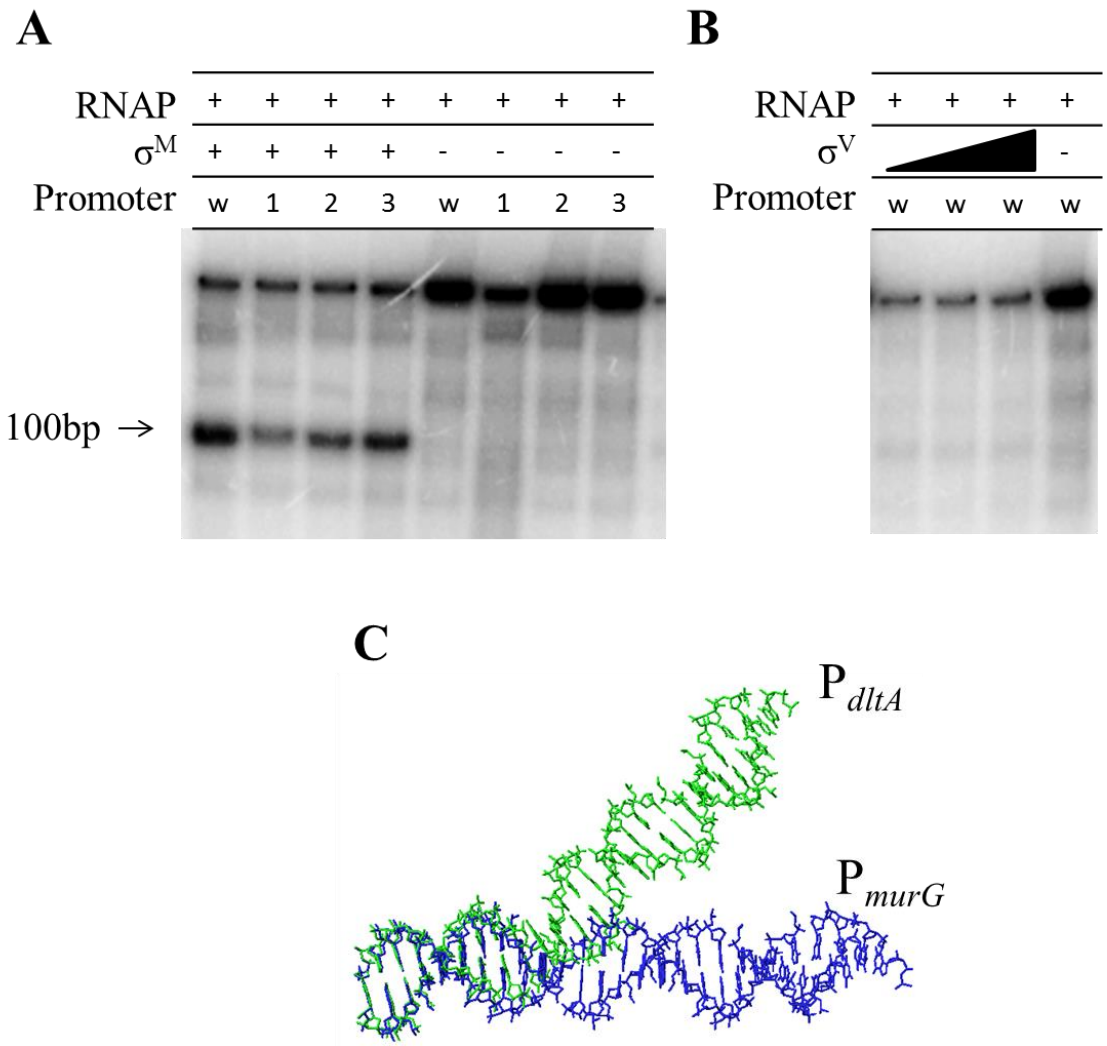

Figure 3.8 P_{dltA} stretch of Ts *in vitro* and *in silico* experiments. A) Run-off *in vitro* transcription studies with P_{dltA} as a template and purified σ^M W= TTTTT, 1= AAAAA, 2= TAAAT and 3= TTATT. B) Run off *in vitro* transcription studies with P_{dltA} as a template and purified σ^V W= TTTTT. C) Model representation of stretch of Ts-induced bending of P_{dltA} compared with P_{murG}. DNA bending prediction was constructed using Model.it.

Indeed, our preliminary data modeling the DNA structure of P_{dltA} (with stretch of Ts) and comparing it with P_{murG} (with no stretch of Ts) shows that the *dltA* promoter possess and overall bend that could facilitate sigma interaction (Figure 3.8 C).

In summary, the stretch of Ts constitutes a novel promoter element that is crucial for σ^V recognition and that has variable effects for the other ECF σ factors of *B. subtilis*. We foresee that the continuation of these studies, with an appropriate biochemical counterpart, will provide insight into the role of this novel specificity determinant.

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3.5. Supplementary information.

Table S3.1. Strains used in this study.

Strain	Genotype	Construction or reference
E. coli		
DH5α	supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17	Lab strain
BL21/DE3	BL21 with λ DE3 pLys	Lab strain
HE-12023	BL21 DE3 pLys pVG010	This study
HE-4600	BL21/DE3 pLys pWE01	(9)
B. subtilis		
168	trpC2	Lab strain
BSU2007	168 ΔsigMWXYVZylaC	(1)
HB-12010	BSU2007 amyE::PxylA-sigV	(13)
HB-12020	BSU2007 amyE::PxylA-sigW	(13)
HB-12035	BSU2007 amyE::PxylA-sigM	(13)
HB-12036	BSU2007 amyE::PxylA-sigX	(13)
ZB307A	W168 SPβc2βΔ2::Tn917::pBSK10Δ6 (MLSR)	Lab strain
HB-12050	ZB307A with SPβ PdltA(AAAAA)-lacZ	pVG016 → ZB307A
HB-12051	ZB307A with SPβ PdltA(TAAAT)-lacZ	pVG017 → ZB307A
HB-12052	ZB307A with SPβ PdltA(TTATT)-lacZ	pVG018 → ZB307A
HB-12053	ZB307A with SPβ PdltA(WT)-lacZ	pVG019 → ZB307A
HB-12057	168 SPβ PdltA(AAAAA)-lacZ	SPβ HB-12050 → 168
HB-12058	168 SPβ PdltA(TAAAT)-lacZ	SPβ HB-12051 → 168
HB-12059	168 SPβ PdltA(TTATT)-lacZ	SPβ HB-12052 → 168
HB-12060	168 SPβ PdltA(WT)-lacZ	SPβ HB-12053 → 168
HB-12061	Δ7 SPβ PdltA(AAAAA)-lacZ	SPβ HB-12050 → BSU2007
HB-12062	Δ7 SPβ PdltA(TAAAT)-lacZ	SPβ HB-12051 → BSU2007
HB-12063	Δ7 SPβ PdltA(TTATT)-lacZ	SPβ HB-12052 → BSU2007
HB-12064	Δ7 SPβ PdltA(WT)-lacZ	SPβ HB-12053 → BSU2007
HB-12065	Δ7sigV SPβ PdltA(AAAAA)-lacZ	SPβ HB-12050 → HB-12010
HB-12066	Δ7sigV SPβ PdltA(TAAAT)-lacZ	SPβ HB-12051 → HB-12010
HB-12067	Δ7sigV SPβ PdltA(TTATT)-lacZ	SPβ HB-12052 → HB-12010
HB-12068	Δ7sigV SPβ PdltA(WT)-lacZ	SPβ HB-12053 → HB-12010
HB-12069	Δ7sigM SPβ PdltA(AAAAA)-lacZ	SPβ HB-12050 → HB-12035
HB-12070	Δ7sigM SPβ PdltA(TAAAT)-lacZ	SPβ HB-12051 → HB-12035
HB-12071	Δ7sigM SPβ PdltA(TTATT)-lacZ	SPβ HB-12052 → HB-12035
HB-12072	Δ7sigM SPβ PdltA(WT)-lacZ	SPβ HB-12053 → HB-12035
HB-12073	Δ7sigWSPβ PdltA(AAAAA)-lacZ	SPβ HB-12050 → HB-12020
HB-12074	Δ7sigW SPβ PdltA(TAAAT)-lacZ	SPβ HB-12051 → HB-12020
HB-12075	Δ7sigW SPβ PdltA(TTATT)-lacZ	SPβ HB-12052 → HB-12020
HB-12076	Δ7sigW SPβ PdltA(WT)-lacZ	SPβ HB-12053 → HB-12020

Table S3.1. (continued)

HB-12077	Δ7sigX SPβ PdltA(AAAAA)-lacZ	SPβ HB-12050 → HB-12036
HB-12078	Δ7sigX SPβ PdltA(TAAAT)-lacZ	SPβ HB-12051 → HB-12036
HB-12079	Δ7sigX SPβ PdltA(TTATT)-lacZ	SPβ HB-12052 → HB-12036
HB-12080	Δ7sigX SPβ PdltA(WT)-lacZ	SPβ HB-12053 → HB-12036
HB-12141	ZB307A with SPβ PbcrC(WT)-lacZ	pVG015 → ZB307A
HB-12142	ZB307A with SPβ PbcrC(AAAA)-lacZ	pVG030 → ZB307A
HB-12143	ZB307A with SPβ PbcrC(TAAA)-lacZ	pVG014 → ZB307A
HB-12144	ZB307A with SPβ PbcrC(TTAT)-lacZ	pVG031 → ZB307A
HB-12145	ZB307A with SPβ empty	pJPM122 → ZB307A
HB-12146	168 with SPβ PbcrC(WT)-lacZ	SPβ HB-12141 → 168
HB-12147	168 with SPβ PbcrC(AAAA)-lacZ	SPβ HB-12142 → 168
HB-12148	168 with SPβ PbcrC(TAAA)-lacZ	SPβ HB-12143 → 168
HB-12149	168 with SPβ PbcrC(TTAT)-lacZ	SPβ HB-12144 → 168
HB-12150	168 with SPβ empty	SPβ HB-12145 → 168
HB-12152	Δ7sigV with SPβ PbcrC(WT)-lacZ	SPβ HB-12141 → HB-12010
HB-12153	Δ7sigV with SPβ PbcrC(AAAA)-lacZ	SPβ HB-12142 → HB-12010
HB-12154	Δ7sigV with SPβ PbcrC(TAAA)-lacZ	SPβ HB-12143 → HB-12010
HB-12155	Δ7sigV with SPβ PbcrC(TTAT)-lacZ	SPβ HB-12144 → HB-12010
HB-12156	Δ7sigV with SPβ empty	SPβ HB-12145 → HB-12010
HB-12157	Δ7sigM with SPβ PbcrC(WT)-lacZ	SPβ HB-12141 → HB-12035
HB-12158	Δ7sigM with SPβ PbcrC(AAAA)-lacZ	SPβ HB-12142 → HB-12035
HB-12159	Δ7sigM with SPβ PbcrC(TAAA)-lacZ	SPβ HB-12143 → HB-12035
HB-12160	Δ7sigM with SPβ PbcrC(TTAT)-lacZ	SPβ HB-12144 → HB-12035
HB-12161	Δ7sigM with SPβ empty	SPβ HB-12145 → HB-12035
HB-12162	Δ7sigX with SPβ PbcrC(WT)-lacZ	SPβ HB-12141 → HB-12036
HB-12163	Δ7sigX with SPβ PbcrC(AAAA)-lacZ	SPβ HB-12142 → HB-12036
HB-12164	Δ7sigX with SPβ PbcrC(TAAA)-lacZ	SPβ HB-12143 → HB-12036
HB-12165	Δ7sigX with SPβ PbcrC(TTAT)-lacZ	SPβ HB-12144 → HB-12036
HB-12166	Δ7sigX with SPβ empty	SPβ HB-12145 → HB-12036
HB-12167	Δ7sigW with SPβ PbcrC(WT)-lacZ	SPβ HB-12141 → HB-12020
HB-12168	Δ7sigW with SPβ PbcrC(AAAA)-lacZ	SPβ HB-12142 → HB-12020
HB-12169	Δ7sigW with SPβ PbcrC(TAAA)-lacZ	SPβ HB-12143 → HB-12020
HB-12170	Δ7sigW with SPβ PbcrC(TTAT)-lacZ	SPβ HB-12144 → HB-12020
HB-12171	Δ7sigW with SPβ empty	SPβ HB-12145 → HB-12020
HB-12172	ZB307A with SPβ PpbpX(WT)-lacZ	pVG033 → ZB307A
HB-12173	ZB307A with SPβ PpbpX(AAAAA)-lacZ	pVG038 → ZB307A
HB-12193	ZB307A with SPβ PpbpX(TTATT)-lacZ	pVG032 → ZB307A
HB-12174	168 with SPβ PpbpX(WT)-lacZ	SPβ HB-12172 → 168
HB-12198	168 with SPβ PpbpX(TTATT)-lacZ	SPβ HB-12193 → 168
HB-12176	Δ7sigV with SPβ PpbpX(WT)-lacZ	SPβ HB-12172 → HB-12010
HB-12177	Δ7sigV with SPβ PpbpX(TTATT)-lacZ	SPβ HB-12193 → HB-12010

Table S3.1. (continued)

HB-12178	Δ7sigM with SPβ PpbpX(WT)-lacZ	SPβ HB-12172 → HB-12035
HB-12179	Δ7sigM with SPβ PpbpX(TTATT)-lacZ	SPβ HB-12193 → HB-12035
HB-12180	Δ7sigX with SPβ PpbpX(WT)-lacZ	SPβ HB-12172 → HB-12036
HB-12202	Δ7sigX with SPβ PpbpX(TTATT)-lacZ	SPβ HB-12193 → HB-12036
HB-12182	Δ7sigW with SPβ PpbpX(WT)-lacZ	SPβ HB-12172 → HB-12020
HB-12215	Δ7sigW with SPβ PpbpX(TTATT)-lacZ	SPβ HB-12193 → HB-12020
HB-12192	ZB307A with SPβ Pabh(WT)-lacZ	pVG037 → ZB307A
HB-12189	ZB307A with SPβ Pabh(AAAAA)-lacZ	pVG034 → ZB307A
HB-12190	ZB307A with SPβ Pabh(TAAAT)-lacZ	pVG035 → ZB307A
HB-12191	ZB307A with SPβ Pabh(TTATT)-lacZ	pVG036 → ZB307A
HB-12194	168 SPβ Pabh(AAAAA)-lacZ	SPβ HB-12189 → 168
HB-12195	168 SPβ Pabh(TAAAT)-lacZ	SPβ HB-12190 → 168
HB-12196	168 SPβ Pabh(TTATT)-lacZ	SPβ HB-12191 → 168
HB-12197	168 SPβ Pabh(WT)-lacZ	SPβ HB-12192 → 168
HB-12217	Δ7sigV SPβ Pabh(AAAAA)-lacZ	SPβ HB-12189 → HB-12010
HB-12218	Δ7sigV SPβ Pabh(TTATT)-lacZ	SPβ HB-12191 → HB-12010
HB-12216	Δ7sigV SPβ Pabh(WT)-lacZ	SPβ HB-12192 → HB-12010
HB-12210	Δ7sigM SPβ Pabh(AAAAA)-lacZ	SPβ HB-12189 → HB-12035
HB-12211	Δ7sigM SPβ Pabh(TTATT)-lacZ	SPβ HB-12191 → HB-12035
HB-12209	Δ7sigM SPβ Pabh(WT)-lacZ	SPβ HB-12192 → HB-12035
HB-12199	Δ7sigX SPβ Pabh(AAAAA)-lacZ	SPβ HB-12189 → HB-12036
HB-12200	Δ7sigX SPβ Pabh(TTATT)-lacZ	SPβ HB-12191 → HB-12036
HB-12201	Δ7sigX SPβ Pabh(WT)-lacZ	SPβ HB-12192 → HB-12036
HB-12213	Δ7sigW SPβ Pabh(AAAAA)-lacZ	SPβ HB-12189 → HB-12020
HB-12214	Δ7sigW SPβ Pabh(TTATT)-lacZ	SPβ HB-12191 → HB-12020
HB-12212	Δ7sigW SPβ Pabh(WT)-lacZ	SPβ HB-12192 → HB-12020
HB-12204	ZB307A with SPβ PmurG(WT)-lacZ	pVG040→ ZB307A
HB-12205	ZB307A with SPβ PmurG(TTTTT)-lacZ	pVG041 → ZB307A
HB-12207	168 SPβ PmurG(WT)-lacZ	SPβ HB-12204 → 168
HB-12208	168 SPβ PmurG(TTTTT)-lacZ	SPβ HB-12205 → 168
HB-12220	Δ7 SPβ PmurG(WT)-lacZ	SPβ HB-12204 → BSU2007
HB-12221	Δ7 SPβ PmurG(TTTTT)-lacZ	SPβ HB-12205 → BSU2007
HB-12222	Δ7sigM SPβ PmurG(WT)-lacZ	SPβ HB-12204 → HB-12035
HB-12223	Δ7sigM SPβ PmurG(TTTTT)-lacZ	SPβ HB-12205 → HB-12035
HB-12224	Δ7sigV SPβ PmurG(WT)-lacZ	SPβ HB-12204 → HB-12010
HB-12225	Δ7sigV SPβ PmurG(TTTTT)-lacZ	SPβ HB-12205 → HB-12010
HB-12226	Δ7sigX SPβ PmurG(WT)-lacZ	SPβ HB-12204 → HB-12036
HB-12227	Δ7sigX SPβ PmurG(TTTTT)-lacZ	SPβ HB-12205 → HB-12036

Table S3.2. Plasmids used in this study.

Plasmid	Description	Reference
pVG010	pET11a sigV 4626/4627	This study
pVG014	pJPM122 PbcrC (TAAA) 5073/5074 (5073/5078 + 5074/5077)	This study
pVG015	pJPM122 PbcrC (WT) 5073/5074	This study
pVG016	pJPM122 PdltA (AAAAA) 5081/5083 (5081/5085 + 5083/5084)	This study
pVG017	pJPM122 PdltA (TAAAT) 5081/5083 (5081/5087 + 5083/5086)	This study
pVG018	pJPM122 PdltA (TTATT) 5081/5083 (5081/5089 + 5083/5088)	This study
pVG019	pJPM122 PdltA (WT) 5081/5083	This study
pVG030	pJPM122 PbcrC (TTAT) 5073/5074 (5073/5076 + 5074/5075)	This study
pVG031	pJPM122 PbcrC (AAAA) 5073/5074 (5073/5080 + 5074/5079)	This study
pVG032	pJPM122 PpbpX (AAAAA) 5603/5604 (5603/5610 + 5609/5604)	This study
pVG033	pJPM122 PpbpX (WT) 5603/5604	This study
pVG034	pJPM122 Pabh (AAAAA) 5611/5612 (5611/5614 + 5613/5612)	This study
pVG035	pJPM122 Pabh (TAAAT) 5611/5612 (5611/5616 + 5615/5612)	This study
pVG036	pJPM122 Pabh (TTATT) 5611/5612 (5611/5618 + 5617/5612)	This study
pVG037	pJPM122 Pabh (WT) 5611/5612	This study
pVG038	pJPM122 PpbpX (TTATT) 5603/5604 (5603/5608 + 5607/5604)	This study
pVG040	pJPM122 PmurG (WT) 5644/5645	This study
pVG041	pJPM122 PmurG(TTTTT) 5644/5645 (5644/5647 + 5646/5645)	This study

Table S3.3. Primers used in this study.

Primer	Description	Sequence
4626	sigV pET11a fwd	CACCATATGAAGAAAAAACAAACAACAAAAGCGTTG
4627	sigV pET11a rev	ATAGGATCCTCTTATCCATTAAGAAAGAT
5073	PbcrC_UNI_Fwd	CGCAAGCTTCTGAAGCACTTTAATATCGG
5074	PbcrC_UNI_Rev	ATAGGATCCGTGTTCCCAAACAGCCAGAT
5075	PbcrC.1_Fwd	TCTATTTTTATTTGAAACAAAACATGAGTAAGATTAGTCT
5076	PbcrC.1_Rev	AGACTAATCTTACTCATGTTTTGTTTCAAATAAAAATAGA
5077	PbcrC.2_Fwd	TCTATTTTTATTTGAAACTAAACATGAGTAAGATTAGTCT
5078	PbcrC.2_Rev	AGACTAATCTTACTCATGTTTAGTTTCAAATAAAAATAGA
5079	PbcrC.3_Fwd	TCTATTTTTATTTGAAACTTATCATGAGTAAGATTAGTCT
5080	PbcrC.3_Rev	AGACTAATCTTACTCATGATAAGTTTCAAATAAAAATAGA
5081	PdltA_UNI.1_Fwd	CGCAAGCTTCAAAAACATACGCCGATATA
5083	PdltA_UNI_Rev	ATAGGATCCGAACCGGTATTCGCGGTGTG
5084	PdltA.1_Fwd	AAAAATGAAACAAAAAGAGCATCTGATCGT
5085	PdltA.1_Rev	ACGATCAGATGCTCTTTTTGTTTCATTTTT
5086	PdltA.2_Fwd	AAAAATGAAACTAAATGAGCATCTGATCGT
5087	PdltA.2_Rev	ACGATCAGATGCTCATTTAGTTTCATTTTT
5088	PdltA.3_Fwd	AAAAATGAAACTTATTGAGCATCTGATCGT
5089	PdltA.3_Rev	ACGATCAGATGCTCAATAAGTTTCATTTTT
5603	pbpX-UNI-Fwd	AATGATAAGCTTGGCTGAGTGAAAAACTCAGC
5604	pbpX-UNI-Rev	CAGGGATCCTCTTTTATTTAGTTTTCTCCG
5605	pbpX-mut.1-Fwd	ATTGCTTTTTTTGACAACAAAAAAAGGGCTTTATTCGTCTAA
5606	pbpX-mut.1-Rev	TTAGACGAATAAAGCCCTTTTTTTGTTGTCAAAAAAAGCAAT
5607	pbpX-mut.2-Fwd	ATTGCTTTTTTTGACAACTAAATTAGGGCTTTATTCGTCTAA
5608	pbpX-mut.2-Rev	TTAGACGAATAAAGCCCTAATTTAGTTGTCAAAAAAAGCAAT
5609	pbpX-mut.3-Fwd	ATTGCTTTTTTTGACAACTTATTTAGGGCTTTATTCGTCTAA
5610	pbpX-mut.3-Rev	TTAGACGAATAAAGCCCTAAATAAGTTGTCAAAAAAAGCAAT
5611	abh-UNI-Fwd	TCAAGGAAGCTTGTAACAGAAGTAATAC
5612	abh-UNI-Rev	GCAGGATCCAATGCCCGTCTCAACTC
5613	abh-mut.1-Fwd	TTATAGAAAGCGGGAAACAAAAACAAAGTTTCATTCGTCTA
5614	abh-mut.1-Rev	TAGACGAATGAAACTTTGTTTTTGTTTCCCGCTTTCTATAA
5615	abh-mut.2-Fwd	TTATAGAAAGCGGGAAACTAAATCAAAGTTTCATTCGTCTA
5616	abh-mut.2-Rev	TAGACGAATGAAACTTTGATTTAGTTTCCCGCTTTCTATAA
5617	abh-mut.3-Fwd	TTATAGAAAGCGGGAAACTTATTCAAAGTTTCATTCGTCTA
5618	abh-mut.3-Rev	TAGACGAATGAAACTTTGAATAAGTTTCCCGCTTTCTATAA
5644	UNI-PmurG-Fwd	GCGAAGCTTGGAATTCCGACTATTGTCCACGAAC
5645	UNI-PmurG-Rev	GCGAAGCTTGGAATTCCGACTATTGTCCACGAAC
5646	PmurG-mut.1-fwd	TGTATTTACGGGAAACTTTTTAGCCTCTGAAGTCGTCTC
5647	PmurG-mut.1-rev	GAGACGACTTCAGAGGCTAAAAAGTTTCCCGTAAATACA

CHAPTER 4

YPMB AND MOENOMYCIN RESISTANCE IN *BACILLUS SUBTILIS*

Moenomycin is a phospho-glycolipid antibiotic produced by various strains of Streptomyces. It targets the transglycosylation step of cell wall synthesis in Gram-positive bacteria. In *B. subtilis*, moenomycin selectively induces the extracytoplasmic function (ECF) sigma (σ) factor σ^M regulon. σ^M regulates genes that are involved in cell wall synthesis, division, and cell shape determination. A *sigM* deletion is much more sensitive to moenomycin than any other ECF σ mutation. Here we show that, in a mutant background lacking all seven ECF σ factors, induction of σ^M completely restores moenomycin resistance, whereas induction of σ^W, σ^X, or σ^V does not. Due to the considerable amount of overlap between the ECF σ factors of *B. subtilis*, we took a genetic strategy to search for the key moenomycin resistance determinant as genes uniquely controlled by σ^M. Furthermore, we looked at the genes that are positively co-regulated with σ^M and found two transcripts in the *ypmAB* region. We provide preliminary data that suggests that YpmB is a component of the cell wall biosynthesis complex and that it is possibly involved in the coordination steps of peptidoglycan synthesis between division and elongation. We have found that *ypmB* mutants are (in addition to moenomycin resistant) morphologically altered, sensitive to β-lactams, and unable to grow on defined media. This latter phenotype provided a selection for suppressors, some of which also restore the other Δ*ypmB* phenotypes. Intriguingly, many suppressors also have aberrant cell morphology, suggestive of cell wall synthesis defects. These suppressor strains were targeted for whole-genome re-sequencing, revealing several cell envelope- related pathways were affected. Overall, our data suggests that, although possibly not

directly involved in moenomycin resistance in *B. subtilis*, *ypmB* may play an important role in cell wall synthesis.

4.1 Introduction

The bacterial cell wall is a complex three-dimensional structure that protects the cell from its environment while ensuring its shape (26). The synthesis of its main component, peptidoglycan (PG), as well as its breakdown and assembly, are crucial processes during cell growth and division, and as such require high level coordination of multi-protein complexes (9). Rod-shaped bacteria, like *B. subtilis*, alternate their machineries between division and elongation in a process that is spatially and temporally regulated. Several lines of evidence suggest that the actin like protein, MreB, plays a central role in this regulation (47). Furthermore, the synthesis and maturation of PG is scaffolded on MreB during cell elongation, and coupled with the tubulin-like protein FtsZ at mid-cell during division.

Although seemingly crucial for survival, cell size control is still a poorly understood aspect of the cell cycle (45). However, it has been shown that during rapid growth in rich media, *B. subtilis* cells are approximately twice the length of cells grown in nutrient poor conditions (36). Additionally, growth-rate dependent increases in cell size have been shown to be correlated with increases in DNA content generated by multifork replication (37). It is now believed that increasing cell size during rapid growth may be a means of ensuring that division is coordinated with segregation of the fully replicated chromosome.

Moenomycin is a glycolipid antibiotic that inhibits an important step in cell wall synthesis, transglycosylation (32). Transglycosylation is performed by high molecular weight penicillin binding proteins (HMW PBP) which not only carry out this step, but also

transpeptidation. Moenomycin mimics lipid IV, the substrate of transglycosylation, therefore inhibiting the activity of transglycosylases (41). Mechanisms of resistance to moenomycin have not been described yet.

The genome of *B. subtilis* encodes 7 ECF σ factors, σ^M, σ^X, σ^W, σ^V, σ^Y, σ^Z and σ^{YlaC}. Of these, the physiological roles of σ^M, σ^W, σ^X, and more recently σ^V, have been well characterized, and their target regulons have been defined (10, 12, 18, 19). Both expression and activity of these ECF σ factors are often stimulated by cell wall-active antibiotics**.**

Here we show that resistance to moenomycin depends critically on σ^M, and attempt to determine the σ^M-dependent genes responsible for resistance. During our studies, we encountered *ypmB*, although not directly regulated by σ^M, seems to play an important role in cell wall synthesis and possibly interacts with several known σ^M-regulated cell wall synthesis components.

4.2 Materials and methods

4.2.1 Strain construction and growth conditions

All *B. subtilis* strains were constructed in the 168 background (Table 4.1). Unless otherwise stated bacteria were grown in liquid Luria-Bertani (LB) medium at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bactoagar (Difco). All cloning was done in *E. coli* DH5α using ampicillin (AMP, 100 μg/ml) for selection. Chromosomal DNA and plasmid DNA transformations were performed as previously reported (16). The following antibiotics were used for selection at their respective final concentrations: spectinomycin (SPC; 100 μg/mL), chloramphenicol (CAT, 10 μg/mL), macrolide-lincosoamide-streptogramin B (MLS; contains 1 μg/ml erythromycin and 25 μg/ml lincomycin), and kanamycin (KAN; 10 μg/ml).

Table 4.1. Strains, plasmids and primers used in this study.

Strain, plasmid or primer	Genotype of description	Reference or sequence
B. subtilis		
168	trpC2	Lab strain
BSU2007	168 Δ*sigMWXYVZylaC*	(3)
HB-12259	168 *ypmB*::mls	LFH-PCR → 168
HB-12279	168 *ypmA*::mls	LFH-PCR → 168
HB-12280	168 *ypmAB*::mls	LFH-PCR → 168
HB-12261	168 Pm1-*lacZ*	pVG046 → 168
HB-12282	168 Pm2-*lacZ*	pVG048 → 168
HB-12286	Δ*ypmB* Pm-*lacZ*	SPβ HB-0069 → HB-12259
HB-12054	168 Pm-*lacZ*	SPβ HB-0069 → 168
HB-0069	CU1065 SPβ-Pm-*lacZ*	(6)
HB-12010	BSU2007 *amyE*::P_{xylA}-*sigV*	(12)
HB-12020	BSU2007 *amyE*::P_{xylA}-*sigW*	(12)
HB-12035	BSU2007 *amyE*::P_{xylA}-*sigM*	(12)
HB-12036	BSU2007 *amyE*::P_{xylA}-*sigX*	(12)
HB-12032	168 *ponA*::*kan*	LFH-PCR → 168
HB-12033	168 *ypbG*::*kan*	LFH-PCR → 168
HB-13210	168 *yfnI*::*spc*	Anthony Kingston, unpublished
HB-10353	168 *disA*::*spc*	(23)
HB-12289	168 *ydaH*::*spc*	LFH-PCR → 168
Plasmids		
pVG046	pDG1663-Pm1(5823/5821) Amp^R	This work
pVG047	pPL82-*ypmAB*(5905/5907) Amp^R	This work
pVG048	pDG1663-Pm2(5920/55921) Amp^R	This work

Table 4.1 (continued)

Primers		
5821	Pm1-BamHI-Rev	CGCGGATCCGCTGCAAATGTGTCAGCATGGAACA
5823	Pm1-EcoRI-Fwd	CGCGAATTCGATGAGACAGCTATTCCAGCGAAAC
5829	ypmB::mls-up-fwd	TTCAGCTCTTGGCACAAGGGATTAC
5830	ypmB::mls-up-rev	GAGGGTTGCCAGAGTTAAAGGATCATGCTTGCCGAGACAAGAAGTACTG
5831	ypmB::mls-do-fwd	CGATTATGTCTTTTGCGCAGTCGGCGTGACGTACTTAGACAAAGAAGGGC
5832	ypmB::mls-do-rev	TCATGTTCAAGGCACACTTCACCGA
5864	ypmAB::mls-up-rev	GAGGGTTGCCAGAGTTAAAGGATCCAAGGCCGAACATCAGATTGTCTCT
5905	ypmAUP-fwd-bglII	GAGAGATCTGTGATACTAGTATGGCGTGTCCTGA
5907	ypmABDO-rev-sphI	GAGGCATGCTGTGATTGCCAGTGTGGTTGATGGT
5908	ypmA::mls-UP-fwd	GCCCGTTATATCGAACTGATGGCAA
5909	ypmA::mls-UP-rev	GAGGGTTGCCAGAGTTAAAGGATCCAGTGTACGATTCAATGTGTCCACA
5910	ypmA::mls-DO-fwd	CGATTATGTCTTTTGCGCAGTCGGCAGAGACAATCTGATGTTCGGCCTTG
5911	ypmA::mls-DO-rev	GTGTGGTTGATGGTGTTAATGCGGA
5920	Pm2-EcoR1-fwd	CGCGAATTCATCGAACGCACAGCGGCATCAATAA
5921	Pm2-BamHI-rev	CGCGGATCCTCTCGCCCATGTTGATCAAGTCGAA
5766	ponA::kan-up-fwd	GCACGTTCATTCACTTCCGTCATGA
5767	ponA::kan-up-rev	CCTATCACCTCAAATGGTTCGCTGTGAAGGACTCGATTTGCTGTTCGCT
5768	ponA::kan-do-fwd	CGAGCGCCTACGAGGAATTTGTATCGATACGTCGGATGGTGATTCGAACTC
5769	ponA::kan-do-rev	AAGGTTCCCAAAGAAGATTGGTCCG
5770	ypbG::mls-up-fwd	GAGCCAGTTCAAAATGTGCCGAATC
5771	ypbG::mls-up-rev	GAGGGTTGCCAGAGTTAAAGGATCCGCAACAGTTAGTACACCGGCAATT
5772	ypbG::mls-do-fwd	CGATTATGTCTTTTGCGCAGTCGGCAAAGTGCCTCTTCGTTTAGGTGCTG
5773	ypbG::mls-do-rev	GTATGTGACCCTAGATCTTCAAGGC
5806	ydaH::spec-up-fwd	GTCGAAGTTGCCGGAAAAGCAAAGA
5807	ydaH::spec-up-rev	CGTTACGTTATTAGCGAGCCAGTCGTCGCATAGGCTAAGGTTTCTATCG
5808	ydaH::spec-do-fwd	CAATAAACCCTTGCCCTCGCTACGCCTCTGTCACAATGGTCACATCAAG
5809	ydaH::spec-do-rev	CAGCGTGTATTGCTTGATGATGCCA

4.2.2 Generation of mutant strains.

Long flanking homology PCR (LFH-PCR) was used to generate deletion mutations in which the designated coding region was largely replaced by an antibiotic cassette as previously described (24, 43). Strain 168 chromosomal DNA was used for PCR amplification of flanking fragments of each gene using primers 5829/5830 and 5831/5832 for *ypmB*, 5908/5909 and 5910/5911 for *ypmA*, and 5908/5909 and 5831/5832 for *ypmAB* (Table 4.1).

The PCR products were joined to an antibiotic cassette using joining PCR with outside primers. The final LFH product was used to transform 168 with selection for MLS in all three cases (1 μg/ml erythromycin and 25 μg/ml lincomycin) to generate strains *ypmA*::*mls*, *ypmB*::*mls* and *ypmAB*::*mls*. The same strategy was used to create *ponA*::*kan*, *ypbG*::*kan* and *ydaH*::*spc* but selecting with KAN (kanamycin 10 μg/ml) or SPC (spectinomycin 100 μg/mL) respectively, and using the primers listed on Table 4.1.

4.2.3 Plasmid construction

Ectopic expression of *ypmB* at *amyE*, under the control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P_{spac}(hy), was attempted using plasmid backbone pPL82 (34). However, we were unable to obtain colonies suggesting that overexpression (even to un-induced levels due to "leakiness" of the promoter) is detrimental for growth at least in *E. coli*. Instead, we were able to clone *ypmAB* using primers 5905/5907 to create pVG047. This plasmid was then linearized and transformed to 168 and to Δ*ypmB* where it was shown to complement the mutant phenotype (data not shown).

To create HB-12261 (P_M1–*lacZ*), a DNA fragment containing Pm1 was PCR-amplified with primers 5823 and 5821 and cloned into pDG1663 (13). The resulting plasmid (pVG046)

was linearized by digestion with ScaI and integrated into the *thrC* locus. To create HB12282 (P_M2–*lacZ*), the same protocol was used except that the DNA fragment was synthesized using primers 5920 and 5921 and the resulting plasmid was pVG048.

4.2.4 Disk diffusion experiments

Disk diffusion assays were performed as described previously (24). Briefly, strains were grown in LB medium to an optical density at 600 nm (OD600) of 0.4. A 100-µl aliquot of these cultures was mixed with 4 ml of 0.7% LB soft agar (kept at 50°C) and directly poured onto LB agar plates (containing 15 ml of 1.5% LB agar). After 30 min at room temperature (to allow the soft agar to solidify), the plates were dried for 20 min in a laminar airflow hood. Filter paper disks containing either 50 µg of cefuroxime (CXM) or cephalosporin C (CEF-C) were placed on top of the agar, and the plates were incubated at 37°C overnight. The diameters of the inhibition zones (clear zones) were measured.

4.2.5 Bioscreen experiments

For liquid growth antibiotic susceptibility tests, fresh single colonies were first grown in LB broth to an OD600 of 0.4, diluted 1:100 in LB broth, and 200 µl of the diluted culture was dispensed in Bioscreen 100-well microtiter plate. Growth was measured spectrophotometrically (OD600) using a Bioscreen incubator (Growth Curves USA, Piscataway, NJ) at 37°C with vigorous shaking. The absorbance was recorded every 30 minutes for 24 hours.

4.2.6 Light Microscopy

For phase contrast microscopy, fresh colonies were grown on LB at 37 °C with shaking until the desired OD. 10 µl of cells were then mounted on Poly-L lysine coated microscope slides. Microscopy was performed using a Nikon eclipse e600 microscope. Images were acquired using an Infinity2 microscope digital camera.

4.2.7 β-galactosidase assays

For σ^M-predicted promoter studies, strains carrying promoter–lacZ fusions were grown to an OD_{600} of 0.4 in LB and then treated with 4 different concentrations of vancomycin, a known inducer of σ^M (10) and samples were collected after 30 minutes. β-Galactosidase assays were performed as described (27).

For σ^M induction experiments, the wild-type and *ypmB* mutant carrying a *lacZ* fusion to the autoregulatory *sigM* promoter were grown on LB without induction, and samples were taken at different OD_{600}. The assays were then performed as above.

4.2.8 Microarray analysis

Both wild-type and the *ypmB* mutant were grown in LB at 37°C with shaking to an OD_{600} of 0.4. Total RNA was isolated from two biological replicates using a RNeasy minikit following the manufacturer's instructions (Qiagen Sciences, MD). After DNase treatment with Turbo DNA-free (Ambion), RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop Tech. Inc., Wilmington, DE) and kept at -20°C. 20 µg of total RNA was used to make cDNA using the SuperScriptTM Plus Indirect cDNA Labeling System (Invitrogen; L1014-04). cDNA was labeled using Alexa Fluor® labeling and microarray analysis were performed as described previously (14). Four microarrays (biological duplicates with a dye-swap) were

analyzed. Images were processed and normalized using the GenePix Pro 4.0 software package which produces (red and green [R and G]) fluorescence intensity pairs for each gene. Each expression value is represented by up to 8 separate measurements (duplicate spots on each of four arrays). Mean values and standard deviations were calculated with MS Excel. The normalized microarray datasets were filtered to remove those genes that were not expressed at levels significantly above background in either condition (sum of mean fluorescence intensity <20). In addition, the mean and standard deviation of the fluorescence intensities were computed for each gene, and those for which the standard deviation was greater than the mean value were ignored. The fold induction values were calculated using the average signal intensities from the three arrays in the different conditions.

4.2.9 Suppressor selection

A total of ten suppressor mutants were isolated from DSM (Difco Sporulation Media) plates (on three different days), where the *ypmB* mutant failed to grow. Each suppressor mutant was re-streaked onto new DSM plates and checked for the presence of the *mls* cassette (marker for the mutation). Once checked, the suppressor mutants were labeled PU1-PU10 and characterized based on the Δ*ypmB* phenotype.

4.2.10 Whole genome sequencing

Chromosomal DNA was isolated from the *ypmB* mutant and four of the suppressors grown in LB medium to an OD_{600} of 0.4 by using the Qiagen DNeasy blood and tissue kit. The quantity and purity of DNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and DNA was sequenced and analyzed by the Cornell University Life

Sciences Core Laboratories Center using Illumina DNA sequencing. The sequence data were assembled with CLC genomics workbench (CLCBio) using the reference sequence under GenBank accession number ABQK00000000 (38). The sequencing coverage averaged >50X for each strain.

4.3 Results and discussion

4.3.1 Moenomycin resistance is conferred by σ^M in B. subtilis

We had previously showed that in *B. subtilis*, moenomycin can induce expression of σ^M and its regulon, and that a *sigM* mutant was highly resistant to moenomycin (10, 24, 40). Due to the rising evidence of overlap in regulation of ECF σ factors, we wanted to test if σ^M overexpression alone was enough to restore wild-type levels of resistance in a strain lacking all seven ECF σ factors.

Minimal Inhibitory Concentration measurements using moenomycin are impractical because higher concentrations of this antibiotic lead to an increased length in lag phase but do not prevent growth. After 24 h the wild-type strain is able to grow even after addition of 100 μg/mL of moenomycin, the Δ7ECF strain however, can only grow up to 0.5 μg/mL of this antibiotic (Figure 4.1 top). Ectopic overexpression of σ^M not only restores wild-type levels of resistance, but even decreases the lag phase observed for the higher concentrations, suggesting this strain has an increased level of resistance compared to wild-type (Figure 4.1 bottom). Interestingly, overexpression of σ^V, whose regulon overlaps considerably with that of σ^M, does not lead to this increase of resistance, and neither does overexpression of σ^X or σ^W (data not shown). This suggests that the gene/s responsible for moenomycin resistance in *B. subtilis* must be exclusively σ^M -regulated.

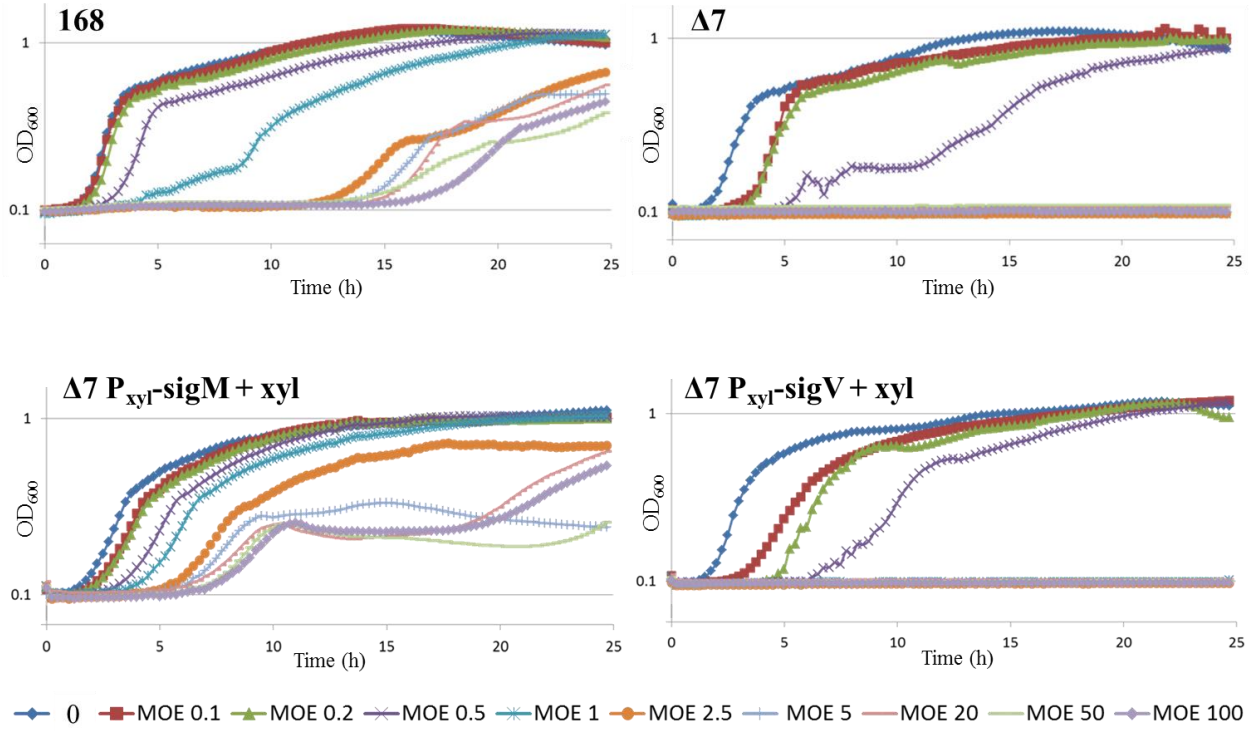


Figure 4.1. σ^M confers resistance to moenomycin in *B. subtilis*. Liquid growth assays were performed in LB medium using a Bioscreen C growth analyzer. The wild-type (168), Δ7ECF (Δ7) (top panel), and the Δ7ECF overexpression strains for *sigM* (left) and *sigV* (right) under the induced condition, were grown in the presence of increasing concentrations of moenomycin (MOE in µg/ml). Overexpressing σ^M in the Δ7ECF background completely restores resistance to MOE whereas overexpressing σ^V (σ^X or σ^W, not shown) does not.

4.3.2 σ^M –exclusively regulated genes and moenomycin resistance

In an approach to determine which of the genes on the σ^M regulon are involved in moenomycin resistance, we looked at those that are only regulated by σ^M. Due to the large amount of overlap in regulation of ECF σ factors, we subtracted from the known regulon of σ^M (10) those genes that are also present in the regulons of σ^W (17), σ^X (5), and σ^V (12), to produce a σ^M-exclusive gene list (Table 4.2).

We constructed knockout mutations of most of these genes and tested for moenomycin sensitivity using disk diffusion experiments (Figure 4.2). None of the mutations tested showed an increased sensitivity to moenomycin as that observed for the *sigM* mutant or the ΔECF strain. Nevertheless, we do not discard the possibility that these genes are involved in moenomycin resistance, especially those with very interesting characteristics.

For example, *ponA* encodes for a penicillin binding protein (pbp1A) involved in peptidoglycan synthesis (33), however, under the conditions tested, a *ponA* mutation showed no sensitivity phenotype. It is important to note that on these disk diffusion experiments, there is no zone of inhibition observed for the wild-type strain (the lawn grows all the way to the edges of the disk which is 7mm in diameter) therefore, small increases in resistance cannot be observed in this experiment.

DisA is one of three paralogous diadenylate cyclases required for the production of c-di-AMP, an essential signal molecule required for cell wall homeostasis (23), however a mutation on this gene showed no sensitivity to moenomycin phenotype on bioscreen experiments (data not shown). As with *ponA*, there may be more than one mutation required to obtain a phenotype.

The only σ^M –exclusively regulated gene we did not test was *murG*. *murG* is part of an essential cell wall synthesis operon, and the σ^M promoter is within the open reading frame of the

Table 4.2. Genes exclusively regulated by σ^M.

Gene	Annotation
ypbG	Putative phosphoesterase.
(*recU*)*ponA*	Promoter internal to *recU* (double strand break repair); *ponA*: pbp 1A
ydaH	Putative integral inner membrane protein
yfnI	(*ltaAS*) lipoteichoic acid synthase
(*sms*)*disAyacLM*	Promoter internal to sms (*radA*: DNA repair). *disA*: diadenylate cyclase. c-di-AMP
(*murG*)*murB divIBylxXW sbp*	Promoter internal to *murG* (essential). Peptidoglycan synthesis and cell division operon.

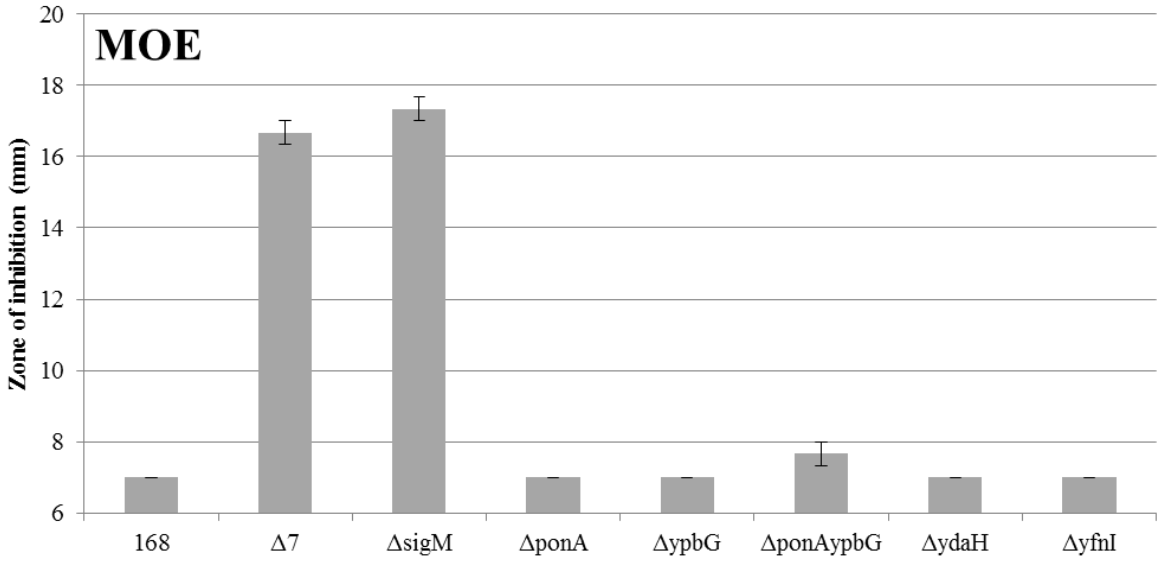


Figure 4.2. Genes exclusively regulated by σ^M and moenomycin resistance. Disk diffusion experiments performed with the wildtype (168), the Δ7ECF (Δ7), the *sigM* mutant (Δ*sigM*) and several σ^M-exclusively regulated genes mutations in the presence of 50 µg of moenomycin (MOE). None of the genes tested seemed to be directly or individually involved in moenomycin resistance.

gene. Therefore, the optimal strategy for functional characterization requires a promoter modification in which the σ^M-dependent promoter (P_M) is inactivated by a point mutation (as done for a P_W in (21)). There are many other internal σ^M-dependent promoters, and these will be studied as part of a different project with the overall goal of understanding how the σ^M regulon contributes to cell wall homeostasis and antibiotic resistance.

It is possible that several of these genes are involved in moenomycin resistance and that single or double mutations will not lead to a phenotype. A more thorough genetic analysis of multiple combined mutations would be ideal to test this.

4.3.3 σ^M – positively co-regulated genes and ypmB

In a second approach to find the σ^M-regulated genes responsible for moenomycin resistance, we looked at those positively co-regulated under different conditions of induction of σ^M. The transcriptome of *B. subtilis* has been studied exhaustively, and more recently, a thorough study of its transcriptomic response to a wide range of environmental and nutritional conditions that it might encounter in nature has been performed (30).

Most of the genes that appear to be co-regulated with σ^M had been already reported in the several σ^M regulon studies; however, we found a new region that appears to be σ^M-regulated as well. There are two transcripts in the *ypmAB* region of the chromosome that are shown to be up-regulated with σ^M induction. This region had not come up in our regulon studies; however, it was not completely unfamiliar to us.

YpmA is a small (56 aminoacids) hypothetical protein conserved in Bacilli, however not much is known about its structure or function. YpmB is a 161 aminoacid hypothetical protein also conserved in Bacilli. The protein structure of YpmB has been resolved and it shows a single transmembrane domain suggesting this protein is anchored to the cytoplasmic membrane of *B.*

subtilis (31). Additionally, sequence homology shows that YpmB has a PepSY domain similar to that of M4 family of metallopeptidases. For M4 peptidases, the PepSY domain has been shown to have a inhibitory role, which is released after cleavage (48). Whether YpmB serves a similar role in *B. subtilis* remains to be tested.

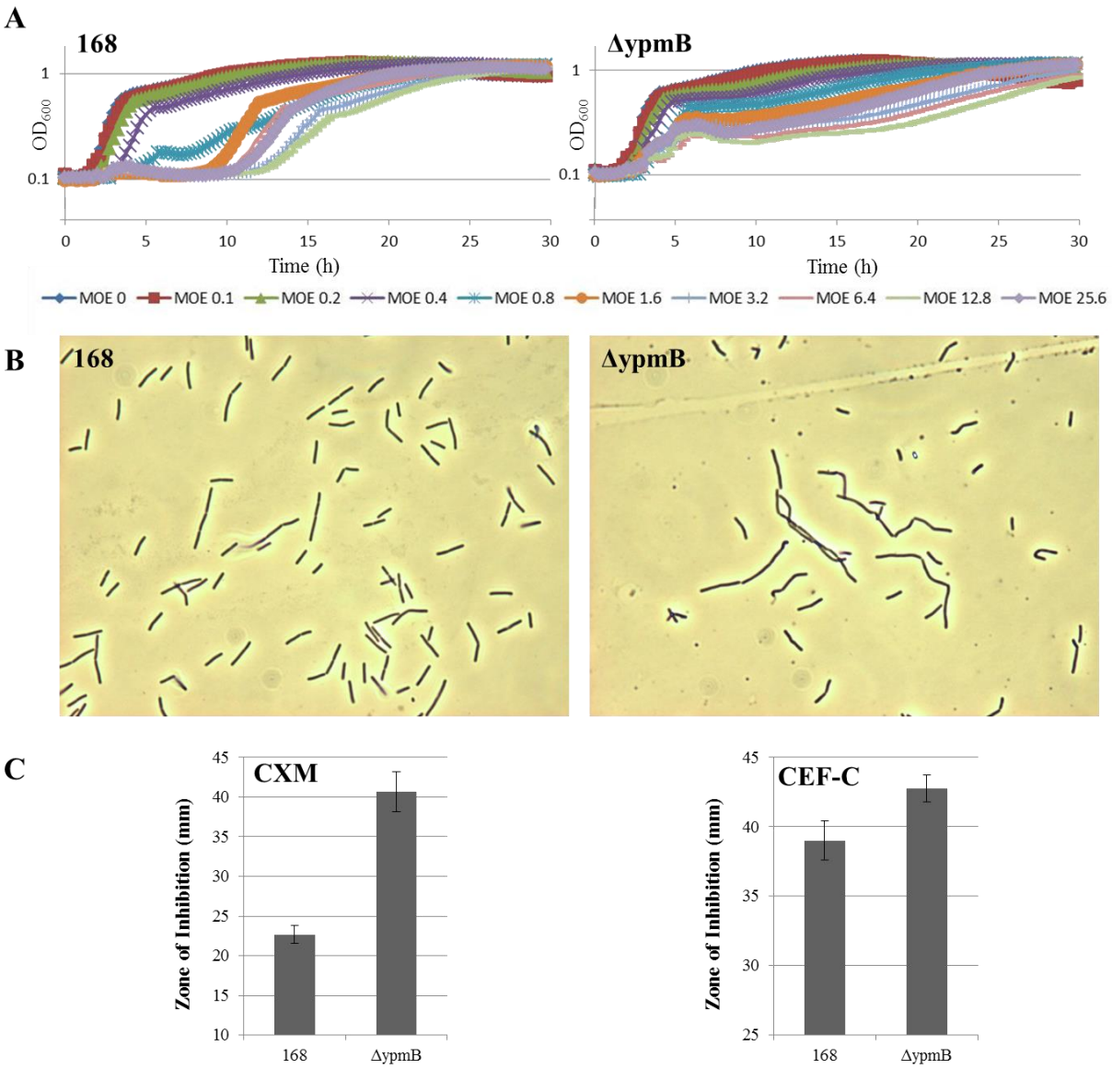
Even though the roles of *ypmA* and *ypmB* remain unknown, they have appeared in a few studies of relevance to this dissertation. *ypmB* was found to be one of the most frequent insertions in a Tn7 screen for insertions that increase resistance to moenomycin, with a second insertion in *dinG*, the gene directly upstream of *ypmAB* (15). Furthermore, YpmB has been shown to copurify with MreB, albeit together with 98 more proteins, suggesting a possible role in peptidoglycan biosynthesis (20). Together, this suggests that YpmB could have an important role in cell wall synthesis and could help us understand the role σ^M plays in moenomycin resistance.

4.3.2 A ypmB mutant is highly resistant to moenomycin and has pleiotropic phenotypes

We first constructed a *ypmB* knockout mutation to corroborate the moenomycin resistant phenotype. The *ypmB* mutant showed an increased resistant phenotype compared to the wild-type strain (Figure 4.3A). Interestingly, a *ypmA* mutant and a *ypmAB* double mutant are also resistant to moenomycin (data not shown), however when combined with a *sigM* mutation, the moenomycin sensitivity phenotype prevails (Figure S4.1). This reinforces that the key moenomycin resistance determinants are σ^M dependent.

Apart from its moenomycin phenotype we also noticed a slight lag phase in growth (~2h) characterized with morphologically altered cells with increased length and bending compared to

Figure 4.3. Characterization of the *ypmB* mutant. A) Liquid growth assays were performed in LB medium using a Bioscreen C growth analyzer. The wildtype (168) and the *ypmB* mutant (Δ*ypmB*) were grown in the presence of increasing concentrations of moenomycin (MOE in µg/ml). The *ypmB* mutant shows a highly increased resistant phenotype. Data shown is representative of experiments produced in duplicates and done at least three times. B) Phase contrast microscopy images of the wildtype (168) and the *ypmB* mutant (Δ*ypmB*) grown on LB at OD_{600} below 0.1. The *ypmB* mutant shows a higher percentage of filaments and longer cells as well as bendy cells. C) Disk diffusion experiments performed with the wildtype (168) and the *ypmB* mutant (Δ*ypmB*) in the presence of 50 µg of cefuroxime (CXM) or cephalosporin-C (CEF-C). The *ypmB* mutant shows increased resistance to both antibiotics.


A
168
ΔypmB
OD600
1
0.1
0
5
10
15
20
25
30
Time (h)
MOE 0
MOE 0.1
MOE 0.2
MOE 0.4
MOE 0.8
MOE 1.6
MOE 3.2
MOE 6.4
MOE 12.8
MOE 25.6
B
168
ΔypmB
C
CXM
CEF-C
Zone of Inhibition (mm)
45
40
35
30
25
20
15
10
168
ΔypmB

wild-type (Figure 4.3B), which resembles those of cells with altered peptidoglycan synthesis pathways (25, 29).

We tested the *ypmB* mutant for resistance to several cell wall-acting stresses like different antibiotics and detergents. We found the *ypmB* mutant to be more sensitive to the β-lactam antibiotics cefuroxime and cephalosporin-C (Figure 4.3C). The fact that the *ypmB* mutant is affected in such a range of phenotypes suggests that it could have an important role in cell wall synthesis.

4.3.3 Regulation of ypmB expression

We next sought to investigate how *ypmB* expression is regulated. The *ypmAB* region of the chromosome seems to be under tightly regulated control as it appears to have at least six different predicted promoters (30), two of which could be σ^M regulated according to consensus searches. The two proposed σ^M promoters point in opposing directions, one promoter being within *dinG* (labeled P_M1) and the one antisense to *ypmB* (labeled P_M2) (Figure 4.4 Top).

We fused these two promoters to the reporter gene *lacZ* and tested for β-galactosidase activity driven from them under inducing conditions of σ^M in both the wild-type and a *sigM* mutant strain. P_M1 seems to be active under normal growth conditions and its activity is increased under induction conditions for σ^M. Additionally, the activity of this promoter is completely abolished in the *sigM* mutant, suggesting that it is indeed a σ^M-dependent promoter (Figure 4.4 bottom). P_M2 showed no activity under any of the conditions tested (data not shown). Interestingly, the transcript that is up-regulated under σ^M-inducing conditions is that of *ypmA*, not *ypmB* (30) possibly due to activity of P_M1. Interestingly, when we tried to overexpress *ypmB* for complementation of our mutation it failed due to toxicity in *E. coli*, however we were able to

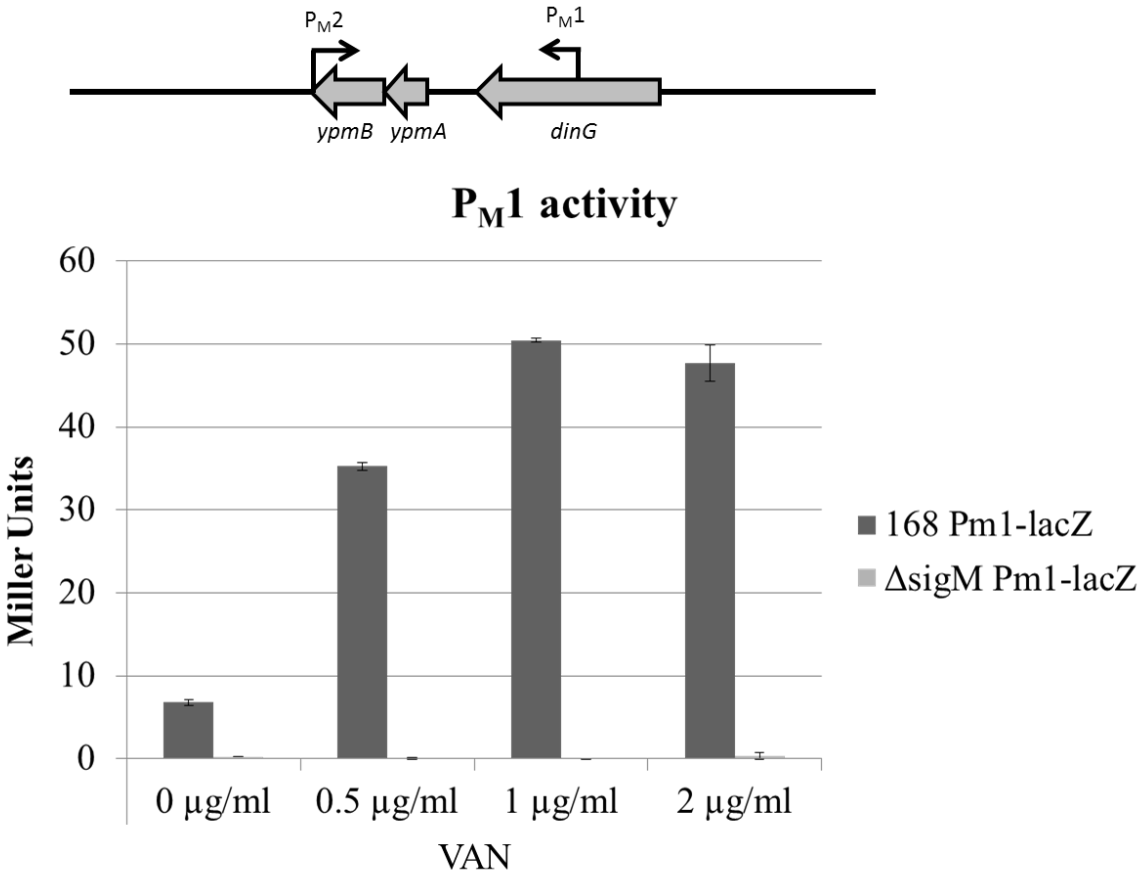


Figure 4.4. Regulation of expression of *ypmB*. Illustration of the *ypmAB* region and the two predicted σ^M-regulated promoters in it (Top). β-galactosidase activity of P_M1 in both the wildtype (168) and a *sigM* mutant (Δ*sigM*) background under induction of σ^M with increasing concentrations of vancomycin (VAN).

overexpress *ypmAB* together, suggesting a possible negative role of *ypmA* over *ypmB* (data not shown). Whether *ypmA* and *ypmB* are co-transcribed, or if YpmA somehow inhibits YpmB activity remains to be further tested.

4.3.4 The ypmB mutant transcriptome is characterized by the induction of a cell envelope stress response driven by σ^M

To further characterize the *ypmB* mutant and to gain insight into the different phenotypes it exhibits, we used DNA microarray hybridization to monitor transcriptional changes during normal growth (OD_{600} 0.4) compared to wild-type. Analysis of the resulting transcriptomic response of the *ypmB* mutant revealed the up-regulation of ~20 genes including many known cell wall synthesis components (Table 4.3).

We also observed induction (2 fold) of ECF σ factors σ^M and σ^X, which explains the resulting up-regulation of their known regulons. The up-regulation of σ^M in the *ypmB* mutant might also explain the moenomycin resistance phenotype, since we have shown that over-expression of σ^M can restore resistance to this antibiotic even in the absence of all other ECF σ factors.

In addition to the genes up-regulated, there is a large list of genes down-regulated in the *ypmB* mutant (Table S4.1). The list is largely composed of genes from the Fnr, Fur and Spo0A regulons, and the reasoning for this phenomenon still remains to be elucidated. However, within this list an interesting one came to our attention, *yoeB*. YoeB is a cell wall-associated protein that protects *B. subtilis* from autolysis (35), its down-regulation could be due the disruption of cell wall synthesis in the *ypmB* mutant.

Table 4.3. Genes up-regulated (foldchange Δ*ypmB*/WT > 3) in the *ypmB* mutant.

Genes	Foldchange	Regulator	Annotation	Reference
yraE	13	G	conserved hypothetical protein	(44)
pbpX	6	X(WV)	penicillin-binding endopeptidase X	(5, 12)
dctP	6		C4-dicarboxylate transport protein	
manR	4.5		regulation of the mannose operon	
bglH	4.4	A	aryl-phospho-beta-d-glucosidase	(2)
mreB	3.6	M	cell-shape determining protein	(10)
maf/radC	3.5/3	M	putative septum formation DNA-binding protein and putative DNA repair protein	(10)
rnpA	3.4		protein component of ribonuclease P (RNase P) (substrate specificity)	
tagE	3.3	A	wall teichoic acid glycosyltransferase	(1)
rluD	3.2		pseudouridylate synthase	
swrC	3.2		transporter involved in surfactin self-resistance	
gntK	3.2	A	gluconate kinase	(11)
motP	3.1		putative flagellar motor component	(39)
speA	3.1		arginine decarboxylase	
yrhG	3.1		putative formate/nitrite transporter	
yokF	3		SPbeta phage DNA nuclease, lipoprotein	
ykaA	3	Spo0A	putative Pit accessory protein	(28)
ypjD	3		nucleotide phosphohydrolase	

V refers to σ^V, M refers to σ^M, X refers to σ^X, W refers to σ^W, A refers to σ^A and G refers to σ^G. For those where the reference is not listed the function annotation is based on BsubCyc (http://bsubcyc.org/)

To corroborate *sigM* induction, we performed β-galactosidase studies with a *lacZ* fusion reporter gene fused to the auto-regulatory P_{sigM} promoter (P_M) in the *ypmB* mutant compared with the wild-type (Figure 4.5). We found that indeed P_M induction is higher throughout growth in the *ypmB* mutant, consistent with our microarray results.

Overall the transcriptomic data suggest that the *ypmB* mutant does in fact have alterations in its peptidoglycan synthesis pathway, and additionally has a mounted cell wall stress response characterized by the induction of σ^M and σ^X which may explain some of the antibiotic resistance phenotypes.

4.3.5 The ypmB mutant is unable to grow on defined media but readily generates suppressor mutants that restore growth

As mentioned earlier, the *ypmB* mutant exhibits a range of phenotypes possibly involved with cell envelope synthesis defects. In addition to these phenotypes, we found that the *ypmB* mutant is unable to growth on DSM as well as other defined media. Interestingly, after 48h of incubation, suppressor colonies appear on the plates (Figure 4.6).

As a tool to look into the role of YpmB in the cell, we collected and characterized ten of these suppressor mutants. We first corroborated that they have had their growth ability restored and also checked for the presence of the mutation marker. Once ten suppressors were selected, they were labeled PU1-10 and characterized based on the *ypmB* mutant phenotypes.

4.3.6 The ypmB suppressor mutations have pleiotropic phenotypes

We first characterized the suppressors based on their colony size and growth rate (Figure 4.7). Just like the *ypmB* mutant, most of the suppressors show a normal colony size, except for PU5

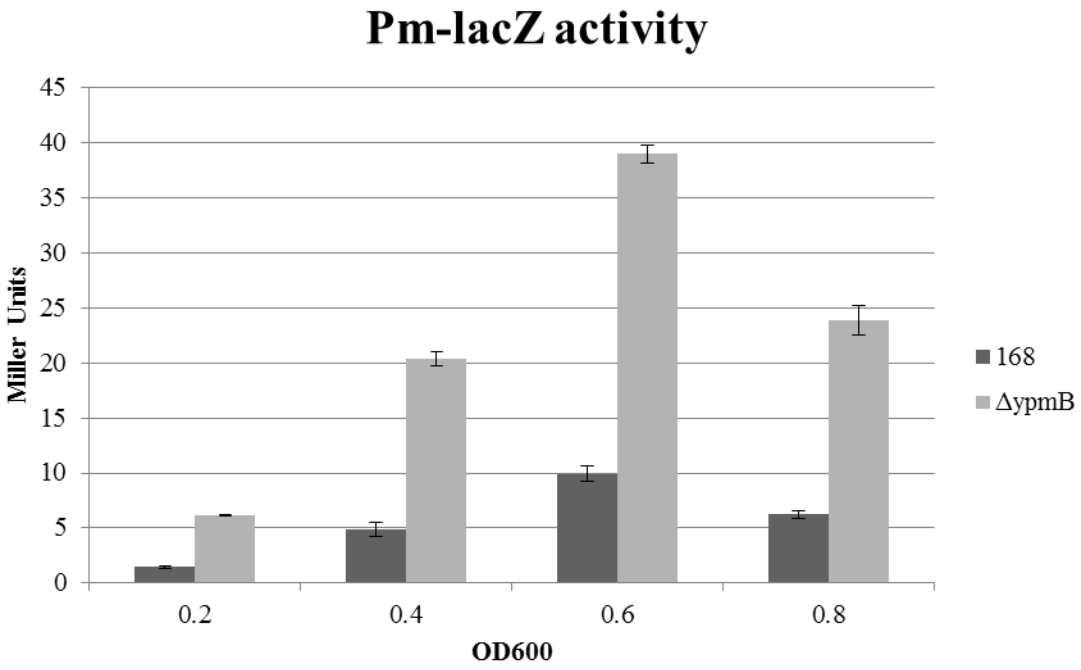


Figure 4.5. σ^M expression in the *ypmB* mutant. β-galactosidase activity of P_{sigM}-*lacZ* in the wild-type (168) and *ypmB* mutant (Δ*ypmB*) backgrounds. Experiment was performed in 3 biological replicas and repeated at least three times. Bars represent mean values with error bars indicating the standard deviation.

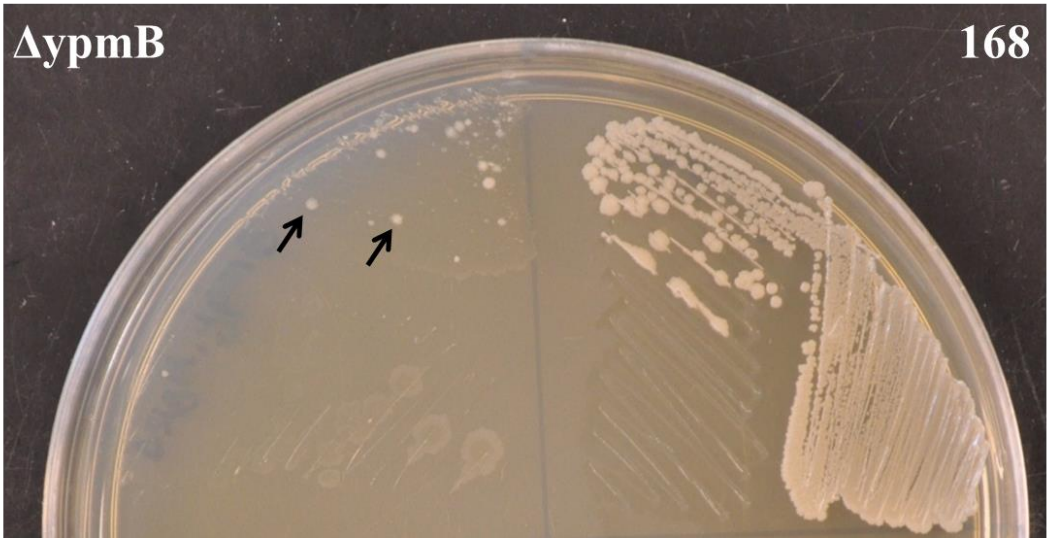
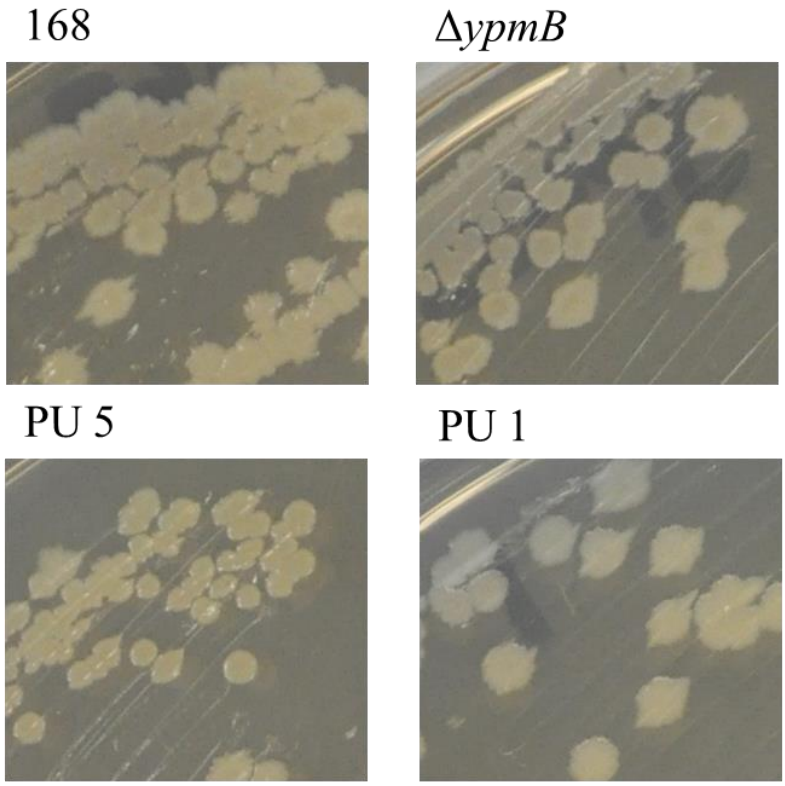


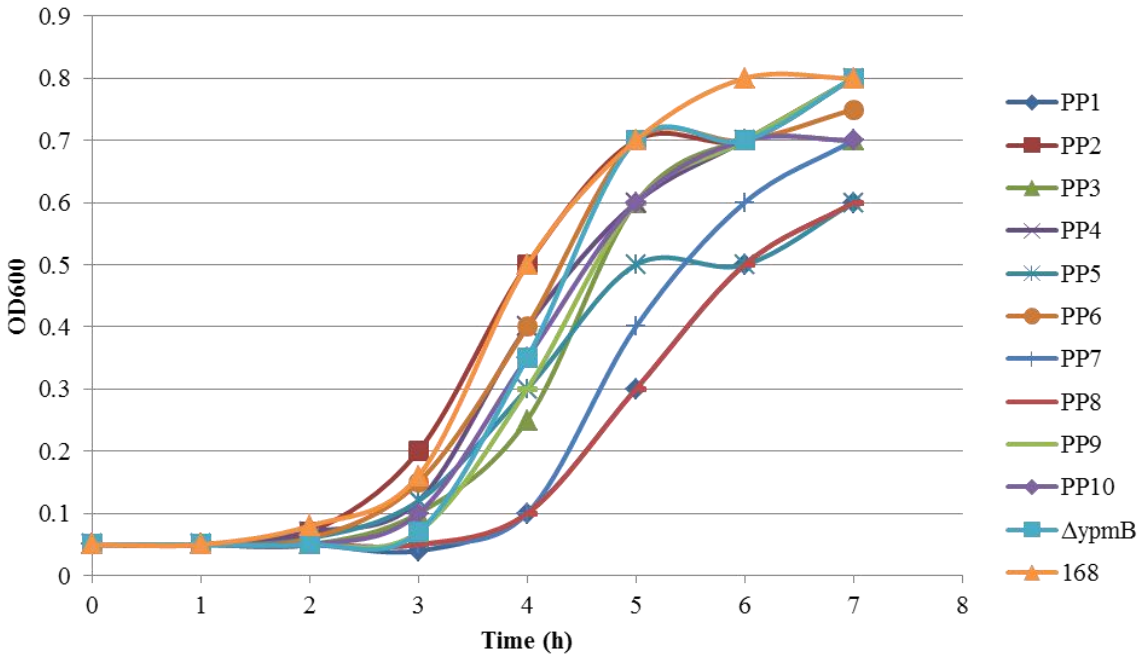
Figure 4.6. The *ypmB* mutant develops suppressor mutations that restore growth. Image of a DSM plate with a streak of the wild-type (168) and *ypmB* mutant (Δ*ypmB*) strains. The arrows depict suppressor colonies that arise after 48-72 h of incubation.

Figure 4.7. Characterization of the *ypmB* mutant suppressors. A) Photograph showing the colony size of wild-type (168), the *ypmB* mutant (Δ*ypmB*), and two suppressor mutants (PU5 and PU1) grown on LB plates overnight. Both the *ypmB* mutant and PU1 (as well as the rest of the suppressors (not shown)) show a wild-type colony size. However, PU5 shows a reduced colony size. Pictures are representative of experiments performed in triplicate on three different days. B) Liquid growth experiments performed with the wild-type (168), the *ypmB* mutant (Δ*ypmB*), and the ten suppressors (PU1-10) in LB and OD_{600} was monitored over the course of seven hours using a spectrophotometer 21.

A


168
ΔypmB
PU 5
PU 1

B


OD600
Time (h)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
1
2
3
4
5
6
7
8
PP1
PP2
PP3
PP4
PP5
PP6
PP7
PP8
PP9
PP10
ΔypmB
168

which shows a decreased colony size phenotype (4.7A). In a similar way, most of the suppressors exhibited the same 2h lag phase observed on the *ypmB* mutant, except PU2 which had no lag phase and grew like wild-type, and PU7 and PU8 which had a longer (~3h) lag phase (4.7B). From this data, we can assume that the different suppressors have not restored all of the *ypmB* mutant phenotypes to wild-type, but only its ability to grow in defined media.

We also tested if the suppressors had restored moenomycin sensitive to wild-type levels and were surprised to find that for the most part, it hadn't (Figure 4.8). In fact, most of the suppressors are highly resistant to moenomycin, even more than the *ypmB* mutant. The only suppressor that was sensitive to moenomycin was PU5, which is more sensitive than wild-type as well.

As far as sensitivity to β-lactams, the results were variable (Figure 4.9). For cefuroxime, most of the suppressors remained more sensitive than wild-type (although to different levels), except for PU4 and PU6 which are now at wild-type level of resistance. In a similar way, most of the suppressors are still sensitive to cephalosporin-C, except for PU8 which is now at wild-type level, and PU5 which is more resistant.

Finally, we looked at the cell morphology of the suppressors to see if the defects in growth were still observed. Surprisingly, all of the suppressors still showed defects in growth (Figure 4.10). Most of the suppressors now had a smaller size cell type (PU2, PU3, PU4, PU6, PU8 and PU10), except for PU1, PU7 and PU9 which still showed the longer, bendy cell type similar to that of the *ypmB* mutant, and PU5 which showed an aberrant "curly" morphology.

The results of the suppressors' characterization are summarized on Table 4.4. As far as we can tell all of the suppressors, except for PU4 and PU6, have different phenotypes and

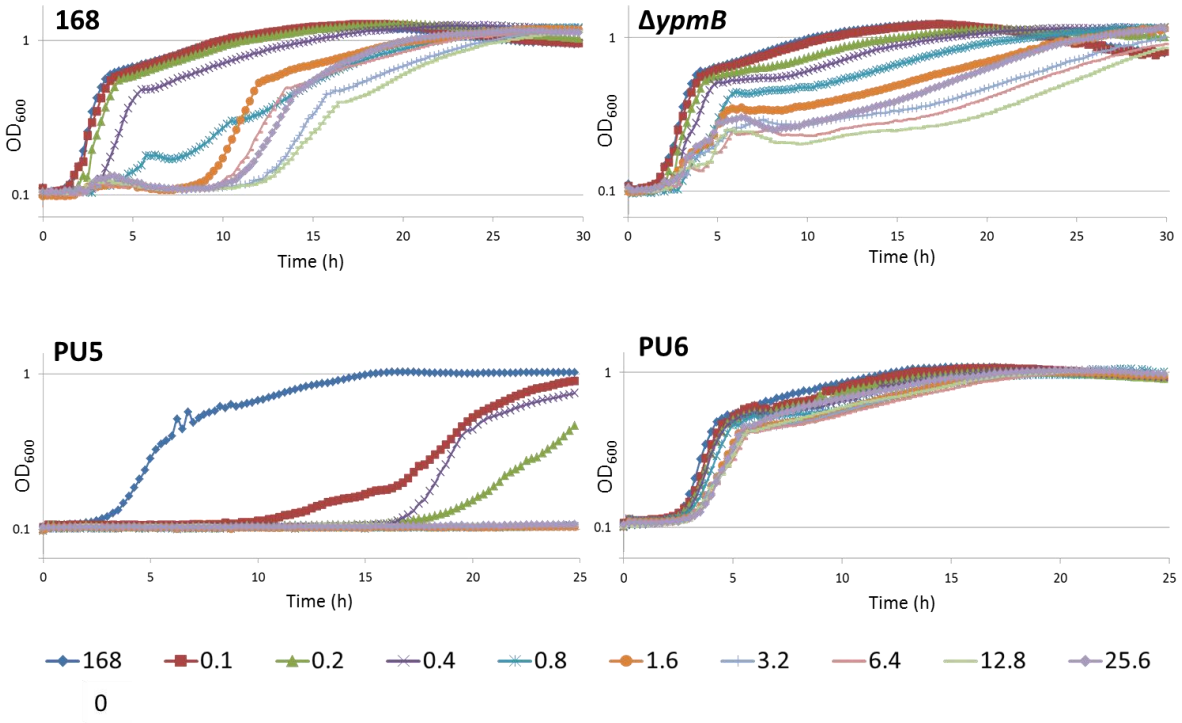
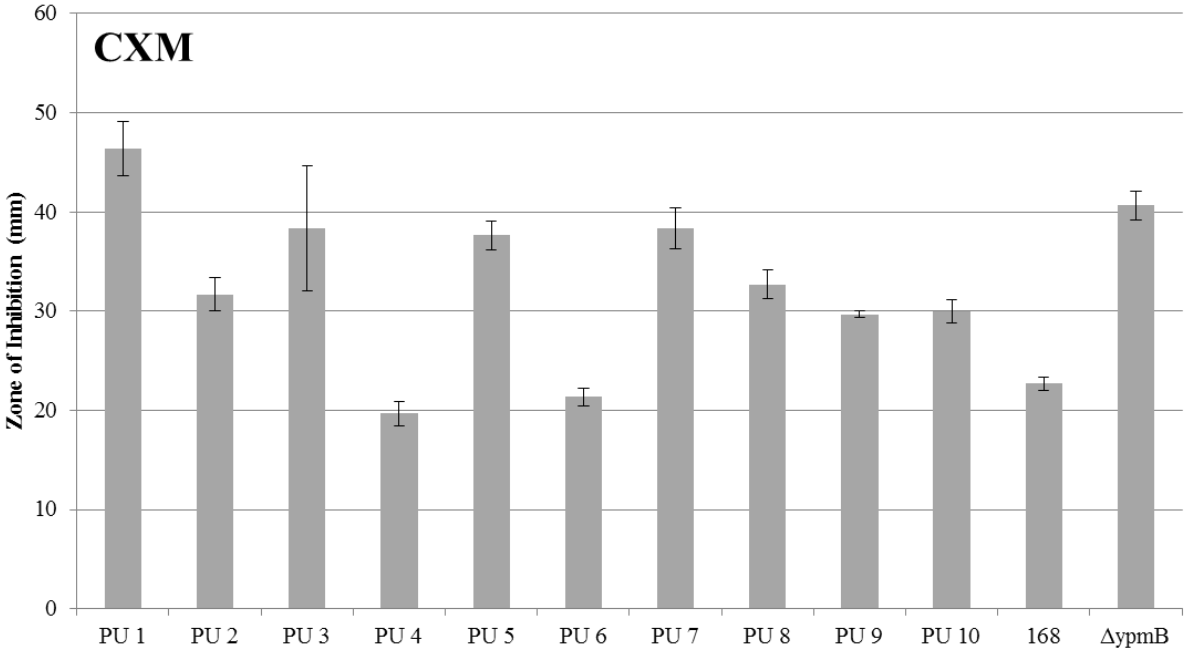
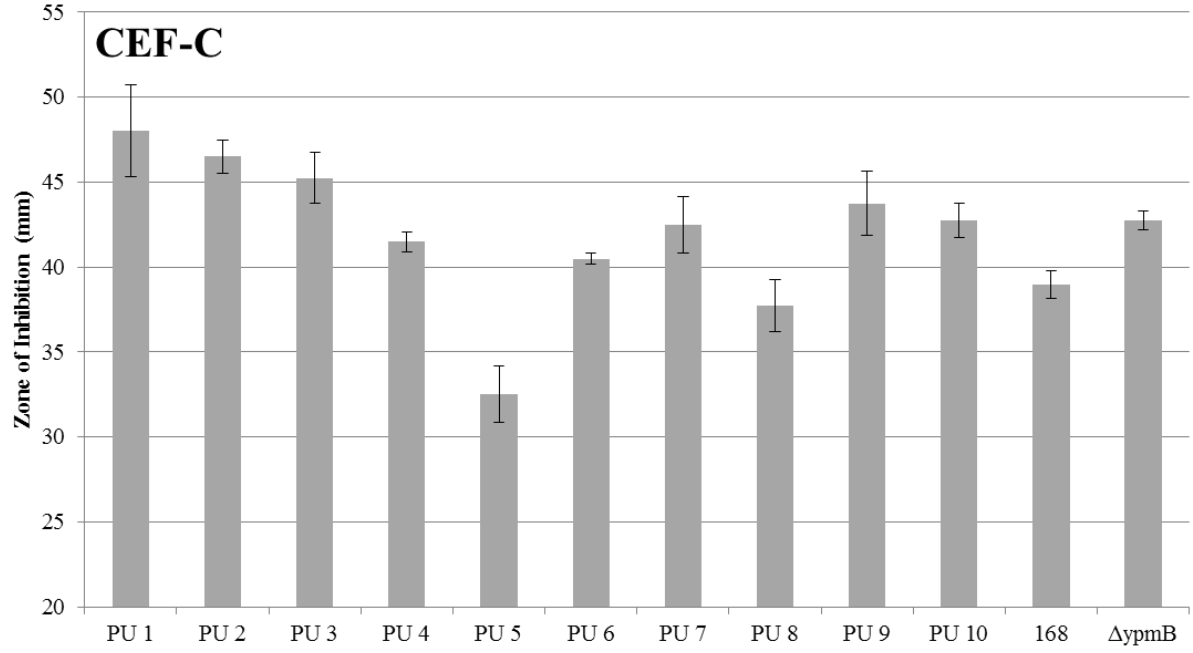


Figure 4.8. Moenomycin resistance of *ypmB* suppressor mutations. Liquid growth assays were performed in LB medium using a Bioscreen C growth analyzer. The wild-type (168), the *ypmB* mutant (Δ*ypmB*), and the ten suppressor mutants (only PU5 and PU6 shown as representatives) were grown in the presence of increasing concentrations of moenomycin (MOE in µg/ml). The curves shown are representative of experiments performed in duplicates and at least three times.

Figure 4.9. The *ypmB* mutant suppressors have variable resistance phenotypes to β-lactam antibiotics. Zone of inhibition experiments were used to quantify β-lactam sensitivity in *B. subtilis* wild-type (168), the *ypmB* mutant (Δ*ypmB*) and the ten suppressors (PU1-10). Strains were grown to an OD600 of 0.4 and an inoculum of this culture was used to make a lawn of cells on 0.75% LB agar. Disks containing 50 µg of cefuroxime (CXM) or cephalosporin C (CEF-C) were placed on top of the lawn and the inhibition of growth was measured after incubation at 37°C for 16 h. Each bar represents the average zone of inhibition of a least three assays performed with three biological replicas of each strain. The zone of inhibition is expressed as the total diameter (± standard error) of the clear zone.


CXM
Zone of Inhibition (mm)
60
50
40
30
20
10
0
PU 1
PU 2
PU 3
PU 4
PU 5
PU 6
PU 7
PU 8
PU 9
PU 10
168
ΔypmB


CEF-C
Zone of Inhibition (mm)
55
50
45
40
35
30
25
20
PU 1
PU 2
PU 3
PU 4
PU 5
PU 6
PU 7
PU 8
PU 9
PU 10
168
ΔypmB

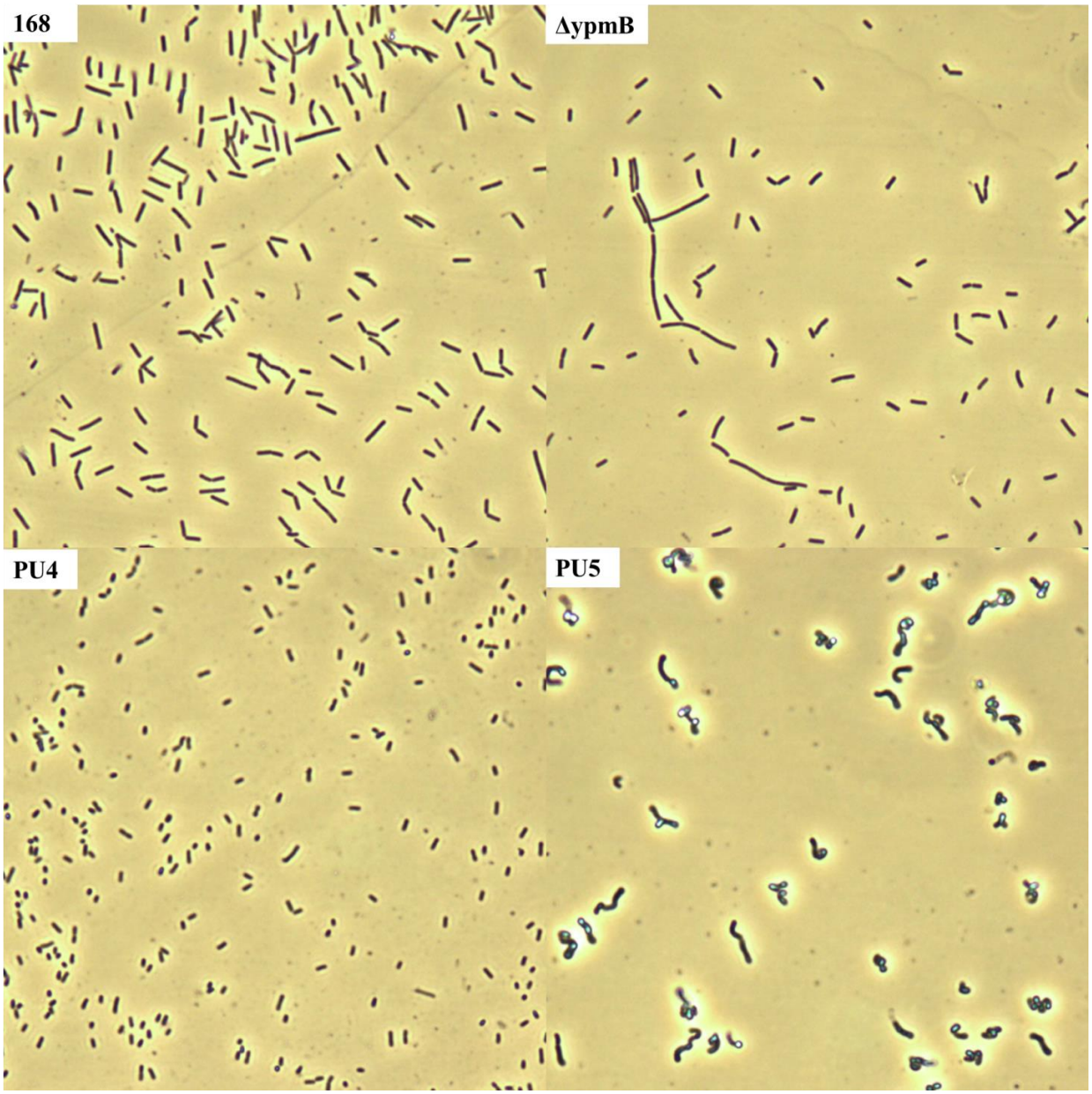


Figure 4.10. The *ypmB* suppressor mutants show defects in growth. Phase contrast microscopy images of the wild-type (168), the *ypmB* mutant (Δ*ypmB*) and two representative suppressors (PU4 and PU5) grown on LB at OD_{600} below 0.1.

Table 4.4. Characterization of the *ypmB* mutant suppressors.

Strain	Lag Phase	DSM growth	Colony size	MOE resistance	CXM resistance	CEF-C resistance	Cell morphology
PU1	Δ*ypmB*	+	wt	R	Extra S	Extra S	Δ*ypmB*
PU2	wt	+	wt	R	S	Extra S	small
PU3	Δ*ypmB*	+	wt	Extra R	S	Extra S	small
PU4	Δ*ypmB*	+	wt	Extra R	R	S	small
PU5	Δ*ypmB*	+	small	Extra R	S	Extra R	curly
PU6	Δ*ypmB*	+	wt	S	R	S	small
PU7	worse	+	wt	Extra R	S	S	Δ*ypmB*
PU8	worse	+	wt	R	S	R	small
PU9	Δ*ypmB*	+	wt	Extra R	S	S	Δ*ypmB*
PU10	Δ*ypmB*	+	wt	R	S	S	small
ΔypmB	Δ*ypmB*	-	wt	R	S	S	Δ*ypmB*

R: more resistant than 168. S: more sensitive than 168. MOE: moenomycin. CXM: cefuroxime. CEF-C: cephalosporin-C. Δ*ypmB*: phenotype similar to that of the *ypmB* mutant.

therefore must have different sets of mutations that arose to compensate for the growth defect of the *ypmB* mutant (i.e. they are not siblings).

Interestingly, most of the suppressor mutations have a reduced cell size. It has been well documented that during rapid growth in rich media, *B. subtilis* cells are approximately twice the length of cells grown in nutrient poor conditions (36), and that increasing cell size during rapid growth may be a means of ensuring that division is coordinated with segregation of the fully replicated chromosome (45). It seems plausible to think that the *ypmB* mutant somehow has the coordination between division and elongation affected, giving rise to longer cells, which fail to grow in limited nutrient conditions. The reversion to a smaller cell phenotype could show compensation for this phenomenon.

Although analysis is still preliminary, we have re-sequenced the genomes of four of these suppressors, and the data produced seems very encouraging (Table S4.2). However, more analysis and more experiments are needed before stronger conclusions can be drawn. Out of the four suppressors re-sequenced, we were able to identify multiple single nucleotide polymorphisms (SNPs) in all but one.

The genome sequence of PU2 revealed a G → T SNP in *gtaB* that if translated would change the asparagine in position 133 to a tyrosine. GtaB is an UTP-glucose-1-phosphate uridylyltransferase, involved in glucolipid biosynthesis and which functions as a metabolic sensor to coordinate cell size with growth rate in *B. subtilis* (42). It has been previously shown that mutations in *gtaB* result in reduced cell size, and that it can suppress blocks in cell division (as those observed with overexpression of MinCD) (45) .

As for the other suppressors they all show very interesting SNPs when compared to the Δ*ypmB* parental strain. For example, PU10 has, amongst other ones, a silent mutation at the start

of walK, part of the two-component system controls peptidoglycan metabolism in exponentially growing cells (4); and one that changes a proline to leucine in position 240 of RpoC. Mutations in RpoC can often lead to variable phenotypes such as antibiotic resistance or optimal growth in minimal media (8, 22).

One of the suppressors, PU5, has a SNP in *ytkD* which has been shown to increase the spontaneous mutation frequency of growing cells (7); this might explain why there is over twenty SNPs on that strain, including a C → T SNP in *ftsH* which would change threonine 327 to isoleucine in FtsH, a metalloprotease that accumulates in the midcell septum of dividing cells (46). Mutations in *ftsH* are known to cause defects in growth, characterized by the up-regulation of the σ^W regulon and more specifically *pbpE* (PBP4) (49).

A few other suppressor strains are currently being re-sequenced and once the mutations are confirmed and reconstructed we will be able to draw a conclusion from each suppressor. Nevertheless, it seems that mutations in cell envelope-related pathways are the common theme found in these strains which would indicate an important role for YpmB.

4.4 Conclusions and future directions

Moenomycin is a mimic of the transition state for peptidoglycan transglycosylation and binds to PBPs inhibiting their activity. Resistance depends critically on σ^M: A *sigM-null* mutant is as sensitive to moenomycin as the Δ7ECF mutant, and overexpression of σ^M only completely restores, even increases, wild-type levels of resistance.

The σ^M-dependent genes responsible for resistance to moenomycin remain unknown, and further genetic analysis might be needed to single them out. However, we found a previously uncharacterized gene, *ypmB*, which could be indirectly regulated by σ^M through *ypmA*. We have

initiated a genetic analysis of the *ypmAB* region. It is still unclear whether these two genes are transcribed as a unit, but it is apparent that this region is under complex regulation involving at least one σ^M-dependent promoter.

We have found that the *ypmB* mutant is highly moenomycin resistant, morphologically altered, sensitive to β-lactams, and unable to grow on certain media. This growth defect could be restored by spontaneous suppressor mutations, several of which also have aberrant cell morphology, suggestive of cell wall synthesis defects, which has been supported in some of them by whole genome re-sequencing.

Although preliminary, our data suggest that YpmB plays an important role in cell wall synthesis, and expanding these studies to look at YpmB localization using proteomics and fluorescence microscopy, will provide novel insights into YpmB and its postulated role in modulating the composition and function of cell wall synthesis complexes.

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4.5. Supplementary information

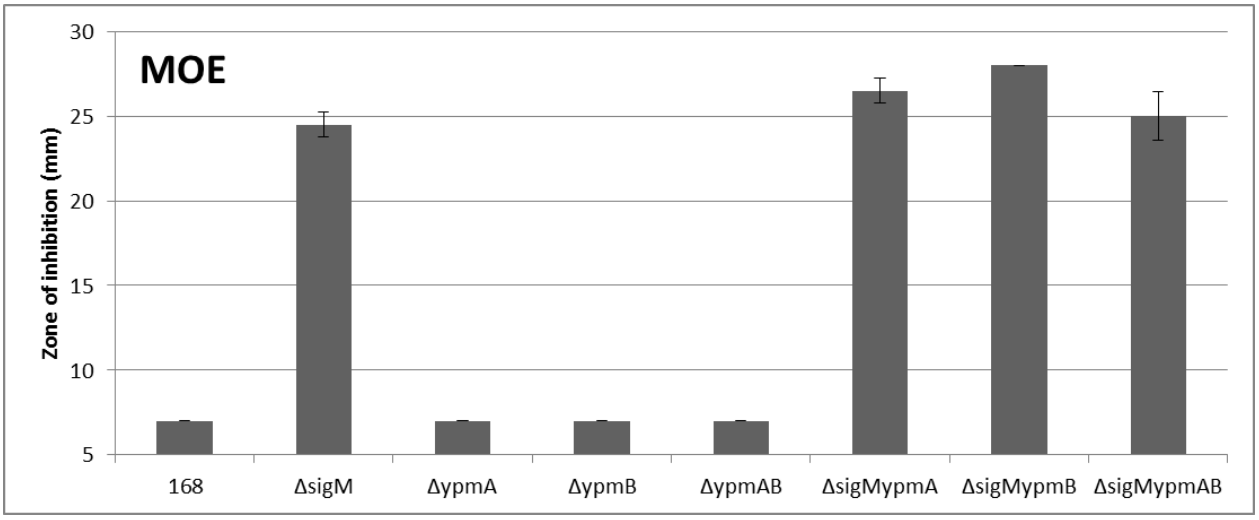

Figure S4.1 Disk diffusion experiments performed with the wild-type (168) and *sigM*, *ypmA*, *ypmB* and *ypmAB* mutations as well as the double mutations. 50 µg of moenomycin (MOE) was used. Experiments were performed at least three times an in duplicate.

Table S4.1. Genes downregulated (foldchange Δ*ypmB*/WT < 0.2) in the *ypmB* mutant.

Gene	Foldchange	Regulator	Gene	Foldchange	Regulator
narH	0.007	fnR	*dhbE*	0.15	fur
narJ	0.01	fnR	*sboA*	0.15	
narK	0.012	fnR	*ybaC*	0.15	
cydD	0.012		*nasB*	0.16	fnR
narI	0.018	fnR	*albC*	0.16	Spo0A-AbrB
aspB	0.029		*licB*	0.17	
fnr	0.032	fnR	*yvqE*	0.17	
comER	0.059		*ywjC*	0.17	
narG	0.073	fnR	*dhbC*	0.17	fur
lctP	0.075		*albF*	0.17	Spo0A-AbrB
arfM	0.076	fnR	*nasD*	0.18	fnR
ytiB	0.077		*ecsA*	0.18	
yoeB	0.080		*albB*	0.18	Spo0A-AbrB
ccpB	0.089		*ybcL*	0.18	
levD	0.10		*yrhH*	0.18	
nasA	0.11	fnR	*yqgZ*	0.18	
yvyD	0.11		*ydjC*	0.19	
yvkC	0.12		*cotJC*	0.19	
leuA	0.13		*pstS*	0.19	
dhbB	0.14	fur	*leuD*	0.19	
albA	0.14	Spo0A-AbrB	*ldh*	0.19	
cydC	0.16		*ykgA*	0.19	
ykfC	0.15	Spo0A-AbrB			
dhbF	0.15	fur			
fruR	0.15	fruRBA			

Table S4.2 Single nucleotide polymorphisms found in *ypmB* mutant suppressors

Strain	Reference position	Gene	Mutation	Aminoacid change
PU2	3666025	*gtaB*	G → T	Asp133Tyr
PU5	15564	*yaaC*	C → G	Gln77His
	77963	*ftsH*	C → T	Thr327Ile
	114846	*cysS*	A → G	Glu466Gly
	127903	*rpoC*	A → G	His781Arg
	221457	*ybdJ*	T → A	Ile67Asn
	277868	*ycbK*	C → G	Ala176Gly
	456589	*ycsE*	C → T	
	462512	*lipC*	C → T	Arg28Cys
	572052	*ydeL*	G → A	Asp222Asn
	627010	*gmuA*	C → T	
	1009364	*yhcY*	G → C	Ala233Pro
	1026236	*mcsB*	T → C	
	1308602	*exuT*	T → C	Phe386Ser
	1608788	*thiQ*	T → C	
	2470067	*dsdA*	G → A	Ala287Val
	3135269	*ytkD*	G → C	Ala64Gly
	3631239	*yvyD*	C → T	Gly112Ser
	3746948	*flhO*	C → T	
	3981152	intergenic *comS-srfAC*	C → T	
	3988145	*yxkD*	C → T	Ala207Thr
PU7	117891	*nusG*	T → C	Met1
	153429	*truA*	C → G	Gln165Glu
	216806	*skfC*	A → G	His468Arg
	446132	intergenic *gdh-ycnI*	A → G	
	492178	*mntH*	C → T	Ala83Thr
	793922	*yetO*	C → T	Thr414Ile
	3233682	*yuxJ*	T → C	Leu348Pro
	3393171	*gerAB*	C → T	Pro325Ser
	4006089	*yxjA*	T → C	Leu113Ser
PU10	126280	*rpoC*	C → T	Pro240Leu
	151001	*ybxA*	C → T	His187Tyr
	1365812	*rplD*	G → A	
	4097044	*tcyC*	A → G	
	4130991	*yydD*	C → T	Gly450Arg
	4151895	*walK*	A → C	
	4173610	*dtpT*	A → G	

CHAPTER 5

CONCLUSIONS AND FINAL PERSPECTIVES

The cell envelope of bacteria is of pivotal importance for growth and survival, and hence it is often the target of antimicrobial compounds. Studying the mechanisms that ensure cell envelope maintenance under stressful conditions has great significance for the establishment of new pathways to target with antimicrobial therapies — a growing problem in antibiotic development — enhancement of industrial practices, and could even offer insight into developmental processes such as sporulation and biofilm formation.

B. subtilis is the best characterized Gram positive bacterium (9). It is a soil and rhizosphere-associated microbe, and also a gut commensal in animals. The ease with which it grows, and the multiple genetic, physiological, and biochemical techniques available for use with this organism, ensures that *B. subtilis* continues to be an excellent tool for the study of cell envelope stress responses (CESRs). Furthermore, analysis of *B. subtilis* and its associated CESRs has provided information relevant to many important pathogens including *Staphylococcus*, *Mycobacterium*, *Clostridium*, *Listeria*, the enterococci and streptococci.

One of the main components involved in CESRs are extracytoplasmic function (ECF) σ factors (5). The genome of *B. subtilis* encodes for seven ECF σ factors, σ^M, σ^W, σ^X, σ^Y, σ^V, σ^Z and σ^{YlaC} (4). In this dissertation, I have performed an in depth analysis of one of them, σ^V, which had been previously uncharacterized. I have described the regulon of σ^V, the role that it plays in lysozyme resistance, and provided evidence for a novel promoter element important for σ^V recognition. Additionally, I have studied the role that σ^M plays in moenomycin resistance, and

discovered a previously uncharacterized gene, *ypmB*, that seems to play an important role in cell envelope synthesis. Taken together, this dissertation was aimed at taking further steps into the understanding of the role that ECF σ factors play in regulating the stress response triggered by cell envelope acting antimicrobials.

In chapter 2 a mutant strain lacking all seven ECF σ factors (1) was used to ectopically induce the expression of σ^V to study the transcriptomic response ensued by the activation of this ECF σ factor. The regulon of σ^V revealed abundant overlap with the regulons of σ^M, σ^X and σ^W. Two of the operons regulated by σ^V, *dltABCDE* and *oatA*, proved to be indispensable in conferring σ^V-dependent lysozyme resistance in *B. subtilis*.

There are several groups currently working on σ^V and its regulation (3, 10). So far it has been found that the activation of σ^V involves the activity of RasP, as in activation of σ^W (11), however the site 1 protease involved in cleavage of RsiV, has not been determined. It would be interesting to test whether ClpP, ClpX, and ClpE are also involved in σ^V activation, since they have been shown to modulate the RasP-dependent σ^W stress response (11).

Little is known about the nature of the signal of activation. Is it a product of degradation what induces the response, or the actual physical disruption of the peptidoglycan. It would be interesting to assess whether other peptidoglycan degrading enzymes are able to induce σ^V. We have performed some preliminary studies using the N-acetylmuramidase Mutanolysin, but we did not observe any quantifiable induction. We don't discard however that lytic transglycosylases, amidases or endopeptidases could have an effect. Furthermore, the continuation of our intra- and inter- species studies could reveal a possible role of σ^V in resistance to other peptidoglycan degradation enzymes found in the soil which would be more relevant to the niche where *B. subtilis* grows.

The ECF σ factors of *B. subtilis* play a major role in the regulation of important processes such as the cell envelope stress response and antibiotic resistance, however, the functional overlap these proteins display complicates the study of their individual functions (6). From the studies derived in chapter 2, it was observed that a stretch of Ts in the spacer region of the promoters regulated by σ^{MXWV} was highly conserved, and virtually absent from promoters only regulated by σ^{MXW}. In chapter 3 we show *in vivo* data that supports that the -30/-26 stretch of Ts constitutes a new promoter element that is important for σ^{V} recognition and specificity, however, how it affects recognition by other σ factors, and if it requires other *cis*- or *trans*- acting factors (as many overlapping σs do) remains unclear.

One of the most striking results was obtained when mutating a single base, the -28 T, from a T to an A base. We presume that the effect shown is not due to base specific contacts, however at the moment, we have no evidence for this. One way we could test this hypothesis would be to mutate the T to a G or a C and test to see if the effect is observed when the stretch of Ts is disrupted regardless of the base used.

Furthermore, we would like to know if the role of the stretch of Ts is provided by the overall topology of the promoter and its influence in the trajectory or flexibility of the DNA. This study could be further improved by experiments testing the overall bending of the promoters and the different mutant variants studied. The continuation of these experiments should provide a better understanding of the role that the stretch of Ts plays in σ factor specificity in *B. subtilis* and enhance our understanding of their overlap in regulation.

Finally, in chapter 4 we aimed to make progress into elucidating the mechanism by which σ^{M} confers moenomycin resistance in *B. subtilis*. Over time our lab has taken several approaches to determine the key determinants of moenomycin resistance in *B. subtilis*. In a previous study, it

was found that even though moenomycin appears to have one cellular target; its resistance can be achieved by many mechanisms (2). Using Tn7SX transposon mutagenesis, 95 insertions in 25 different genes were found to increase resistance to moenomycin, most of them linked to cell wall synthesis but no specific mechanisms could be elucidated. In chapter 4 we took a different approach: given the amount of overlap in regulation by the ECF σ factors of *B. subtilis*, combined with the fact that over producing σ^M in the Δ7ECF background is sufficient to restore resistance to Moenomycin, we sought to investigate the role of σ^M-exclusively dependent genes as moenomycin resistance determinants. Unfortunately, none of the single and double mutations tested had an effect in moenomycin resistance, suggesting two or more of these genes could be involved in resistance.

Taking an alternate approach, we searched for genes positively co-regulated with σ^M under a number of different growth conditions (7). Most of the genes found had already been reported as part of the σ^M regulon, however, we found a previously uncharacterized gene, *ypmB*, which could be indirectly regulated by σ^M through *ypmA*. We have initiated a genetic analysis of the *ypmAB* region. It is still unclear whether these two genes are transcribed as a unit, but it is apparent that this region is under complex regulation involving at least one σ^M-dependent promoter. There is much more to be explored about *ypmAB* regulation, we could study their transcription using RT-PCR or Northen-blots, and map out their transcriptional start using 5' RACE.

We have found that *ypmB* mutants are (in addition to moenomycin resistant) morphologically altered, sensitive to β-lactams, and unable to grow on certain media. This provided a selection for suppressors, several of which also have aberrant cell morphology, suggestive of cell wall synthesis defects. These suppressor strains were targeted for whole-

genome re-sequencing and will continue to be analyzed. The reconstruction of the mutations will provide insight into how the *ypmB* mutation was suppressed.

In parallel, we would like to investigate the effects of YpmB deletion and/or overproduction on the composition and localization of cell wall biosynthetic component as monitored using proteomics and fluorescence microscopy. Not much is known about YpmB, however this protein has been crystalized and its structure shows a single transmembrane domain, suggesting this is a membrane protein (8). We anticipate that continuing biochemical analyses will provide novel insights into YpmB and its postulated role in modulating the composition and function of cell wall synthesis complexes.

Overall, the work presented in this dissertation revealed an important role for ECF σ factors in antimicrobial resistance. Since the ECF σ factors of *B. subtilis* play a crucial role in regulating the setup of a defense mechanism in response to such stresses, the study of such mechanisms is of pivotal importance. Future work continuing these projects would help us understand the complex cell envelope stress response mediated by ECF σ factors, and the key components of the cell envelope biogenesis machinery.

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