BACILLUS SUBTILIS CELL ENVELOPE STRESS RESPONSES INDUCED BY ANTIBIOTICS AND GENETIC PERTURBATIONS OF MEMBRANE COMPOSITION

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Bacteria thrive in many different habitats, and therefore will encounter a multitude of environmental stresses. Survival requires the development of sophisticated response mechanisms that can identify potential threats and initiate regulatory cascades orchestrating the expression of defensive components.

The cell envelope is the first layer of the cell to come in contact with any environmental agent. In Gram-positive bacteria this envelope is comprised of a cytoplasmic membrane surrounded by a thick multilayered cell wall. The cytoplasmic membrane forms an essential permeability barrier and is made of different types of complex lipids which vary not only in the length and modifications of the acylated fatty acid groups but also in the composition of their headgroups. The cell wall is a complex matrix consisting predominantly of equal amounts of peptidoglycan and teichoic acids with the addition of surface attached proteins. Cell wall synthesis and maturation requires both incorporation of new peptidoglycan by penicillin-binding proteins, as well as cleavage of the existing peptidoglycan by autolysins.

The work presented here describes the use of external stimuli or genetic manipulations to elucidate cell envelope stress responses. First, I have identified a possible novel post-translational modifier of autolysin activity, which is induced by cell wall-targeting antibiotics. This modifier is proposed to inhibit autolytic enzyme activity under conditions of impaired cell wall synthesis, thereby reducing the rate of antibiotic-dependent cell death. Second, I studied a transcriptional regulator

controlling the expression of efflux pumps proposed to be involved in resistance to cell-wall targeting antibiotics. Finally, by genetic perturbation I created a series of *B. subtilis* strains with altered cytoplasmic membrane composition, and characterized them for growth, antibiotic resistance, morphology, and alterations in global gene expression patterns. *B. subtilis* retains viability and even rapid growth when the membrane is comprised predominantly, if not exclusively, of one type of complex lipid, suggesting that the cell can tolerate even large changes in membrane composition.

BIOGRPHICAL SKETCH

Letal Salzberg was born in Rochester, NY. At the age of three her parents decided to move to Israel, where Letal lived till she returned to the USA for graduate school. After graduating from high school in 1995 she served for two years in the intelligence corps of the Israeli Defense Force. After her army service she attended the Ben-Gurion University of the Negev in Beer-Sheva, Israel and received a B.Sc in Life Sciences in 2002. During her undergraduate studies she worked in the lab of Prof. Arieh Zaritsky on the bacteriophage T4 and its host *Escherichia coli*. It was during this time she first developed a love of and an appreciation for the weird and wonderful world of Microbiology. Letal began her Ph.D. studies in the Department of Microbiology at Cornell University in the fall of 2002. She joined the lab of Dr. John D. Helmann in the spring of 2003 where she investigated many different aspects of cell envelope stress responses in *Bacillus subtilis*.

"מכל מלמדי השכלתי..."

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Reaching the end of this wild journey I realize that although there were times when I felt completely alone, this was never actually the case. Whatever I may have accomplished would not have been possible without the love and assistance of many.

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I value all of the moments - cherishing the good and learning from the bad

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CHAPTER ONE

The Cell Envelope of the Gram-positive *Bacillus subtilis*

The cell envelope of *Bacillus subtilis* and other Gram-positive organisms is comprised of a cytoplasmic membrane surrounded by a thick multilayered cell wall. Unlike Gram-negative bacteria, which possess an additional outer membrane, the Gram-positive cell wall serves as a surface organelle, the first layer of the cell to come in contact with any environmental agent. The following chapter outlines the structure and biosynthesis of the *Bacillus subtilis* cell envelope as well as outlining how the cell detects stress signals.

1. The Cell Wall

The cell wall of Gram-positive bacteria forms a rigid structure that defines the cell-shape and provides protection against mechanical and osmotic pressures (20,51). The cell wall is a complex matrix consisting predominantly of equal amounts of peptidoglycan (PG) and teichoic acids (TA) (4), with the addition of surface attached proteins.

1.1 Peptidoglycan synthesis and assembly

PG is comprised of chains of the alternating disaccharides *N*-acetylglucosamine (NAG) and *N*-acetylmuramic (NAM) acid. These chains are crossed-linked to neighboring chains by short stem peptides attached to the NAM (63), thereby providing increased tensile strength.

The synthesis of PG is shown in Figure 1.1. Briefly, the PG precursors UDP-NAG and UDP-NAM, and the subsequent linking of the pentapeptide chain (5P) containing a terminal D-Ala-D-Ala to UDP-NAM take place in the cytoplasm (51). Then the UDP-NAM-5P is linked to an undecaprenyl- pyrophosphate carrier

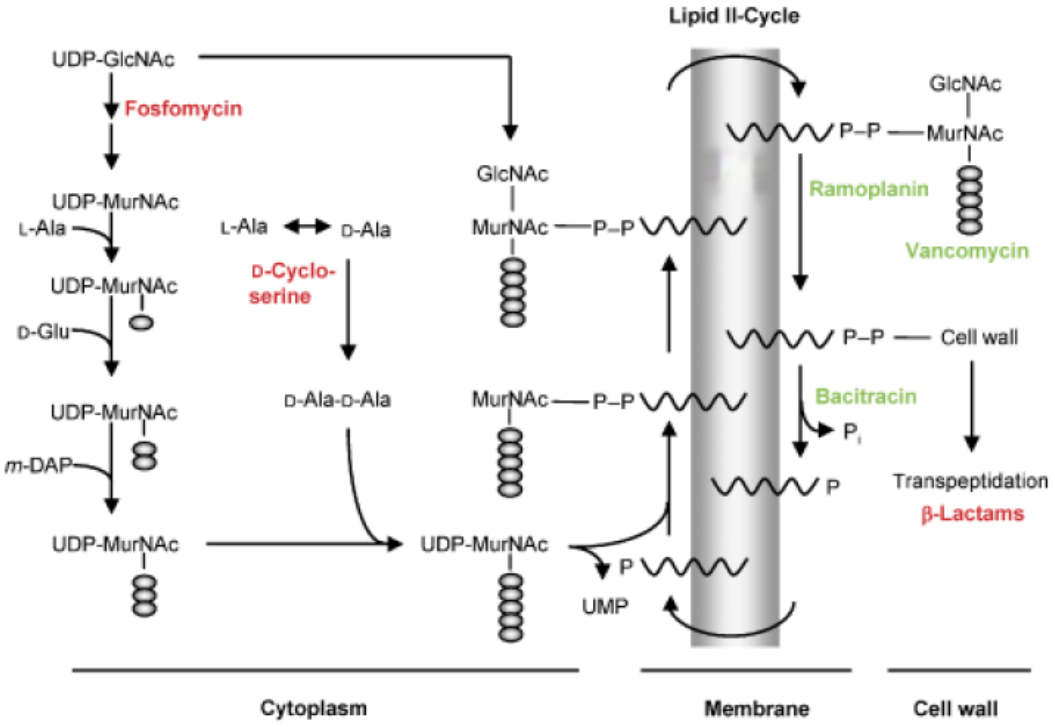

Figure 1.1. Cell wall biosynthesis and its inhibition by antibiotics. Important steps in cell wall biosynthesis are schematically depicted, and their cellular location is indicated below. GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid. Amino acids are symbolized by grey circles. Cell wall antibiotics mentioned in this chapter are indicated next to the steps they inhibit. Antibiotics in green sequester the substrate of the given step; those in red inhibit the corresponding enzymatic function. Adapted from Jordan *et al*. (32)

molecule (C_{55}-PP) forming Lipid I followed by the addition of UDP-NAG to NAM resulting in Lipid II (Fig. 1.1) (51). Lipid II is then translocated across the membrane to the site of peptidoglycan polymerization where it is incorporated into the growing glycan chain (24). This last step is catalyzed by penicillin binding proteins (PBPs), which can be divided into two main categories: high molecular mass (HMM) PBPs, and low molecular mass (LMM) PBPs (63). Polymerization of the glycan chains (transglycosylation; TG) and insertion into the pre-existing cell wall is carried out by HMM-PBPs (63). HMM-PBPs contain a C-terminal domain where the transpeptidation (TP) activity responsible for peptide cross-linking between two adjacent glycan chains is located (63). These PBPs can be further divided into either class A or class B based on the structure of their N-terminal domain (63, 24). In the case of class A HMM-PBPs this domain is responsible for the elongation of the glycan chains (TG). The LMM-PBPs can be divided mainly into carboxypeptidases or endopeptidases, that target the peptide chain (7) thus affecting the degree of cross-linking in the cell wall (51).

The classical "inside-to-outside" model for peptidoglycan assembly proposes that new wall material is inserted at the inner face of the wall, adjacent to where the PBPs are located (64). As new layers of PG are added, the wall moves outward and the outer layers become more stressed, leading to greater autolytic activity (64). Thus newly closed layers of cell wall are formed prior to degradation of older outer layers (64). This paradigm has been challenged by recent findings using fluorescently labeled antibiotics that target different steps in peptidoglycan synthesis. Studies imaging peptidoglycan biosynthesis with fluorescent ramoplanin and vancomycin revealed helical patterns along the cylindrical wall of cells (71). In addition, nascent peptidoglycan incorporation was found to be dependent on the presence of helical filaments of Mbl, the bacterial actin homolog (18). These findings have given rise to a

model for the helical assembly of the cell wall, predicting a non-random and spatially restricted mechanism of nascent peptidoglycan incorporation into the cell wall (4).

1.2 Peptidoglycan degradation

Autolysins are bacteriolytic enzymes that degrade the peptidoglycan of the bacteria that produce them (68). These enzymes have been proposed to play a role in many cellular processes including cell growth, cell-wall turnover, peptidoglycan maturation, cell division, motility, chemotaxis, competence, protein secretion, sporulation and pathogenicity (7,39, 68).

Left unchecked, autolysins will degrade the cell wall leading to its rupture and eventually to cell death. Therefore, fine-tuning of autolysin activity through efficient and strict regulation is crucial for bacterial survival. Autolysin activity and expression are controlled on a number of levels. Hypothetical mechanisms for post-translational regulation of autolysin activity include: proton motive force (36), secreted proteases (70), or the binding of positively charged autolysins to anionic polymers in the cell wall (28). Transcriptional control is often orchestrated by complex interdependent regulatory machinery, including a set of alternative sigma factors, thus ensuring that the autolysins are expressed specifically during the growth stage at which their activity is required (68).

Amino acid similarity searches of the *Bacillus subtilis* genome identified 35 genes coding for candidate peptidoglycan hydrolases (68). These autolysins are classified according to the peptidoglycan bond they target: muramidases, glucosaminidases, N-acetylmuramoyl-L-alanine amidases (amidase) and endopeptidases (68; Fig 1.2).

During vegetative growth, *B. subtilis* produces two major autolysins, a 50 kDa N-acetylmuramoyl-L-alanine amidase encoded by *lytC* and a 90 kDa endo-β-N-acetylglucosaminidase encoded by *lytD*, which together account for ~95% of the cells

autolytic activity (68). These autolysins play roles in several cellular functions including: cell wall turnover during vegetative growth by dissolving the outermost murein layer (37), separation of cells after septation by splitting the newly constructed septum (37), cell lysis induced by the addition of cell-wall-synthesis-inhibiting antibiotics (7), as well as facilitating motility by separating linked cells, and enabling release of mature endospores by lysis of the mother cell at the end of sporulation (68).

These two major autolysins along with the γ-D-glutamate-*meso*-diaminopimelate muropeptidase, LytF (42) are under the transcriptional control of the alternative sigma factor σ^D. σ^D is active in late exponential phase and is involved in the regulation of several cellular functions including flagellar synthesis, motility, chemotaxis and expression of autolysins (43). Additionally, the extracytoplasmic-function (ECF) sigma factor σ^X (12) and the YvrGH two-component system (TCS) (66) are also potentially involved in the transcriptional regulation of autolysins.

1.3 Teichoic acid structure and function

Teichoic acids (TA) are highly charged anionic polymers (20). In *B. subtilis* this polymer is comprised of repeating units (45-60) of glycerol phosphate, and its biosynthetic machinery is encoded by the *tag* genes (*tagABDEFGHO*) (5). TAs can be divided into wall teichoic acids (WTA) and lipoteichoic acids (LTA). WTAs are covalently bound to NAM residues in the cell wall via a linkage unit comprised of two glycerol phosphate residues linked to *N*-acetylmannosaminyl-*N*-acetylglucosaminyl-phosphate (24). LTAs are linked to the cytoplasmic membrane via a glycolipid anchor (24), synthesized by the diglucosyldiacylglycerol synthase, UgtP (52, 38).

The sugar residues of TAs can be further modified by esterification with D-alanine. Modification with D-Ala reduces the net charge of the cell envelope by incorporation of free amino groups (NH_3^+) (58). The *dltABCDE* operon responsible

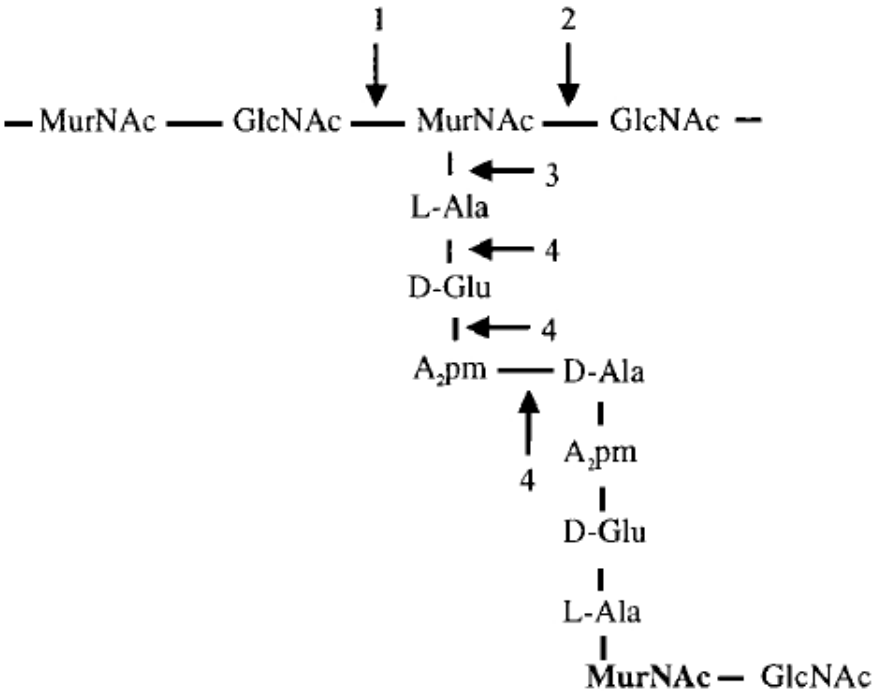

Figure 1.2. Schematic example of bonds hydrolyzed by the different classes of autolysins: (1) Glucosaminidase, (2) Muramidase, (3) Amidase, (4) Endopeptidase. Adapted from Smith *et al*. (68)

for D-alanine esterification of both WTA and LTA in *B. subtilis*, has been shown to be transcriptionally controlled by extracytoplasmic function sigma factor, σ^X (12).

D-Ala modified TAs have been proposed to be involved in various functions related to their anionic nature: modulation of autolysin activity, maintaining cation homeostasis, assimilation of metal cations for cellular function, and defining the electrochemical properties of the cell wall (52).

Although TAs are considered essential for cell viability, when phosphate levels are limited, the *tag* operon is repressed and the *tua* operon, which encodes the enzymes involved in the teichuronic acid, is induced (10). Teichuronic acids (TUA) contain the anionic glucuronic acid but are phosphate free; therefore they can satisfy the need for an anionic polymer while freeing up phosphate (5).

In addition, recent findings (20) have shown that deletion of the gene encoding for the first biosynthetic step (*tagO*) results in a viable mutant, albeit demonstrating slow growth and altered colony morphology. This suggests that while teichoic acids play a crucial role in maintaining the shape of *B.subtilis* cells, they are not essential for viability.

1.4 Cell-wall targeting antibiotics

Due to the great importance of the cell wall in providing protection against mechanical and osmotic pressures, it serves as a target for many antibiotics, which can affect different steps in its biosynthetic pathway. Below are detailed the modes of action of some of the better studied cell-wall targeting antibiotics.

Fosfomycin is a simple three-carbon epoxy propyl phosphonate produced from phosphoenolpyruvate (PEP) by *Streptomyces wemorensis* (67, 74). Fosfomycin acts as a PEP analog, and irreversibly inhibits the first step of peptidoglycan synthesis, catalyzed by MurA, by covalently binding to its active site (67, 74).

D-cycloserine inhibits the interconversion of the commonly found L-Ala to D-Ala, by acting as a suicide inhibitor of the alanine racemase (23), as well as inhibiting the D-Ala- D-Ala ligase which incorporates this component into the pentapeptide chain (74).
Ramoplanin is a lipoglycopeptide antibiotic which inhibits the activity of bacterial transglycosylases by binding to Lipid II found at the external surface of the membrane (29).
Bacitracin, a nonribosomal decapeptide antibiotic, binds to and prevents the recycling of the C_{55}-PP lipid carrier thus inhibiting cell wall synthesis (49).
Cephalosporin is a member of the β-lactam family of antibiotics, which block transpeptidation of the glycan chains. β-lactams serve as suicide substrates for PBPs, which mistake them for a yet to be cross-linked PG chain (74).
Vancomycin, a glycopeptide antibiotic binds specifically to the D-Ala- D-Ala terminus of the pentapeptide chain, thus inhibiting both transpeptidation and transglycosylation activities by blocking access of the enzymes to their substrate (34).

Common resistance mechanisms for these antibiotics include efflux of the antibiotic via pumps (48,56), expression of enzymes that inactivate the antibiotic (11, 26), or modification of the target molecule. This latter mechanism is exemplified by vancomycin resistant *Enterococci* (VRE) that contain enzymes that can replace the D-Ala- D-Ala terminus with a D-Ala- D-lactate terminus which will not be recognized by vancomycin (34).

2. The cytoplasmic membrane

The cytoplasmic membrane forms an essential permeability barrier and is a defining feature of life. In most bacteria, the membrane is a lipid bilayer composed of fatty esters linked to *sn*-glycerol-3-phosphate (G3P) (59). These complex lipids vary not only in the length and modifications of the acylated fatty acid groups but also in

the composition of their headgroups, which differ significantly in charge and propensity to form non-bilayer structures within the membrane. The membrane of the soil bacterium *Bacillus subtilis* is a complex structure comprised mainly of the anionic phospholipid phosphatidylglycerol (PhG) and the zwitterionic phosphatidylethanolamine (PhE). Other components include a relatively large amount (~30%) of neutral glycolipids (GL), a variable amount of positively charged lysylphosphatidylglycerol (LPG), and a small amount of anionic cardiolipin (CL) (Fig 1.3).

The complex composition of the membrane is presumed to be important since the physical properties of membrane lipids influence many cell processes including division (55), DNA replication (30), and protein transport (16). For example, the anionic lipids PhG and CL mediate the cycling of the DNA replication protein DnaA from the ADP-DnaA to ATP-DnaA (active) state, and inhibit the binding of DnaA to *oriC* via sequestration (8). *E. coli* cells with reduced amounts of anionic phospholipids accumulate outer membrane proteins in the cytoplasm and some proteins insert incorrectly into the membrane (72). Furthermore, CL and PhE facilitate the formation of non-bilayer structures (16) important in cell division and sporulation (47). Interestingly, the functional requirement for specific lipid characteristics does not restrict membrane composition, and bacteria exhibit great diversity not only in the content of their lipid head groups, but also in their relative abundance within the membrane (Table 1.1). This diversity represents an adaptation to environmental conditions. For example, in oligotrophic marine environments phospholipids can be replaced by sulfolipids (73; Table 1.1).

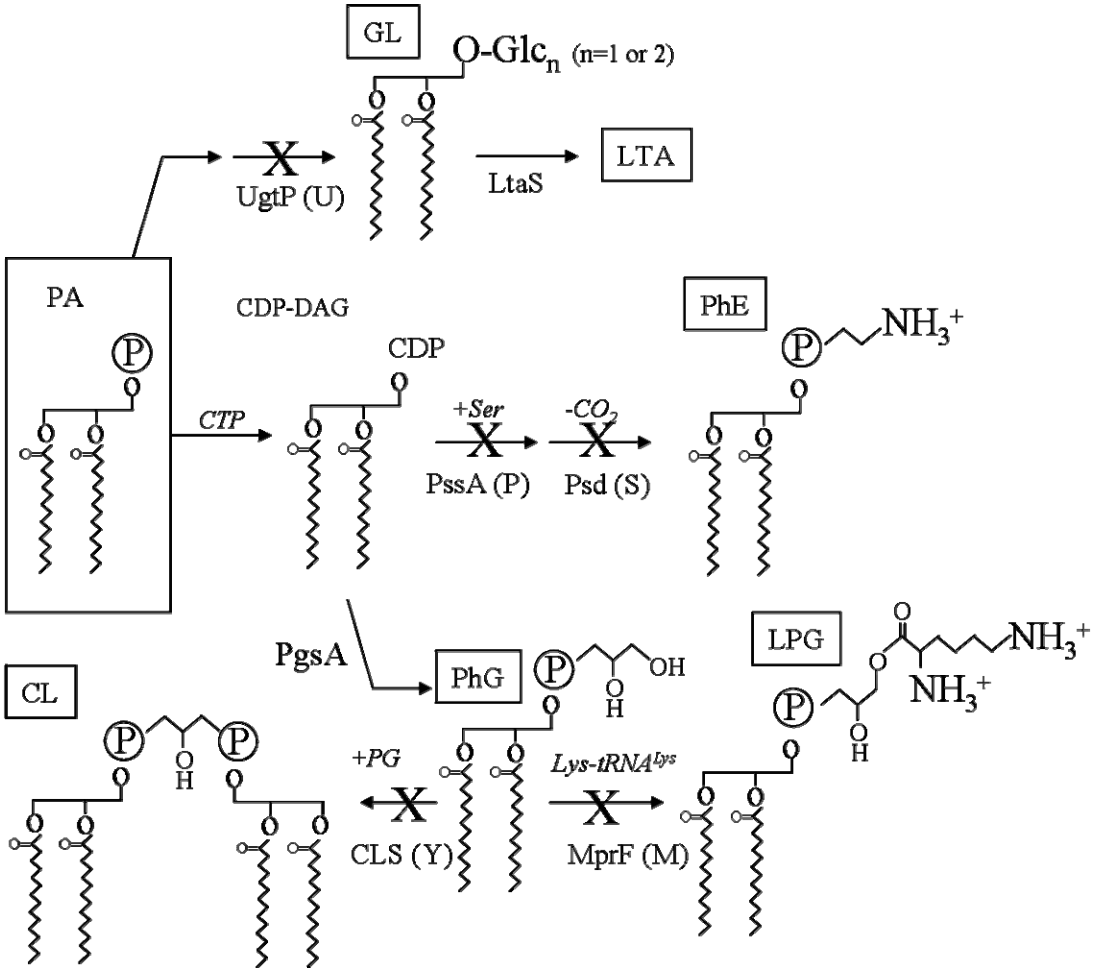

Figure 1.3. Pathways for membrane lipid synthesis in *B. subtilis*. Membrane lipid synthesis begins with the common precursor phosphatidic acid (PA) and leads to the generation of glycolipids (GL), phosphatidylglycerol (PhG), phosphatidylethanolamine (PhE), cardiolipin (CL), and lysylphoshatidylglycerol (LPG). Key enzymes and substrates are indicated and steps blocked by mutation are denoted with an "X" (see chapter four). Note that in the absence of UgtP, LTA is still synthesized but the glycerol-phosphate copolymer is linked to the membrane by diacylglycerol (DAG) rather than by diglucosyl-DAG.

Recent evidence suggests that these various lipid species may assort into spatially distinct lipid micro-domains (69), which in turn may affect the localization of membrane proteins (47). In *B. subtilis* CL-rich and PhE-rich domains are localized to the septal regions and the poles (35, 54), corresponding with the FtsZ-dependent subcellular localization of the lipid biosynthesis enzymes PssA, YwnE, and PgsA (phosphatidylglycerophosphate synthase; 54).

In *B. subtilis*, membrane lipids are synthesized from the common precursor phosphatidic acid (PA; Fig 1.3). In the case of PhE, PA is converted to CDP-diacylglycerol (CDP-DAG) which is condensed with serine and then rapidly decarboxylated to generate PhE. Condensation of CDP-DAG with glycerol-3-phosphate followed by removal of the phosphate leads to PhG, the only essential complex lipid in *B. subtilis*. PhG can be further modified to form two minor complex lipids CL and LPG. CL is formed by the condensation of two PhG molecules by cardiolipin synthase (CLS). *B. subtilis* contains two CLS enzymes: the major form (YwnE; also called ClsA (35)) is expressed during vegetative growth, while the minor (YwjE) is involved in sporulation (35). LPG is formed when MprF transfers a lysyl group from lysyl-$tRNA^{Lys}$ to PhG. Finally, GL are created by dephosphorylation of PA to diacylglycerol which is then modified by the transfer of one or two glucose molecules from UDP-glucose by UgtP (31). Fatty acid synthesis and desaturation are controlled by the FapR (65), and DesRK (17) regulatory systems and both chain length and desaturation may be regulated by various stress conditions (41, 50, 40, 61). In contrast, little is known about how membrane headgroup composition is regulated, although the extracytoplasmic-function (ECF) σ factor σ^X has been shown to contribute to expression of PhE biosynthesis genes (12).

Table 1.1. Complex lipid composition in bacteria

	PG	PE	CL	LPG	GL	PIM	PC	PDME	PMME	OL	SPM	SQDG	PS
B. subtilis (35)	39.4	24.4	1.4	15.6	10.6								
E. coli (16)	20	75	5										
S. aureus (53)	90		1	3.4	5.6								
M. tuberculosis (62)		13.7	19.8			68.9							
Zymomonas mobilis (15)	20	61	2				13						
Corynebacterium glutamicum (57)	50		19			25							
Hyphomicrobium vulgare NP-160 (1)	10	9	6				35	33	1	6			
Spiroplasma citri (19)	38		15				14				33		
Prochlorococcus (MED4) (73)	2				32							66	
Roseobacter denitrificans (73)	44						39						
Psuedomonas T-13 (3)	21.4	54	12.6										12

PIM - phosphatidylinositol mannosides
PDME - phosphatidyl-N-N-dimethylethanolamine
PMME - phosphatidyl-N-monomethylethanolamine
PC - phosphatidylcholine
OL - ornithinolipid
SPM - egg sphingomyelin
SQDG - sulfoquinovosyldiacylglycerol
MGDG - monogalactosyldiacylglycerol
DGDG - digalatosyldiacylglycerol
PS - phosphatidylserine

3. Cell envelope stress response in *Bacillus subtilis*

Bacteria thrive in many different habitats, and therefore will encounter a multitude of environmental stresses. Survival requires the development of sophisticated response mechanisms that can identify potential threats and initiate regulatory cascades orchestrating the expression of defensive components. Components of the cell envelope are often involved in these response mechanisms, since the cell-envelope serves as a first line of defense against environmental challenges.

In *B. subtilis*, control of gene expression patterns in response to external stress signals is coordinated by a suite of alternative sigma factors and two-component regulatory systems both containing a transmembrane sensory component (Fig 1.4).

3.1 Extracytoplasmic-function (ECF) σ factors

ECF σ factors often control functions associated with cell surface, transport or secretion. A typical ECF σ factor is cotranscribed with a downstream regulatory gene, or anti-σ factor. This anti-σ factor is usually located in the cytoplasmic membrane and negatively regulates the activity of the σ factor by binding it directly (27). When an extracytoplasmic stress signal is sensed, the ECF σ factor is released, binds to core RNA polymerase (RNAP), and directs transcription initiation from specific promoter sites (27). ECF σ factors often regulate their own transcription (autoregulation) and that of their anti-σ factor, therefore determination of the upstream promoter sequence can be used to help identify other genes with similar regulatory elements.

B. subtilis possesses seven ECF σ factors, σ^X, σ^W, σ^M, σ^Y, σ^V, σ^Z and σ^{ylaC} identified based on sequence similarity (27). The function of these σ factors has been studied through a combination of genetic, biochemical and bioinformatics tools.

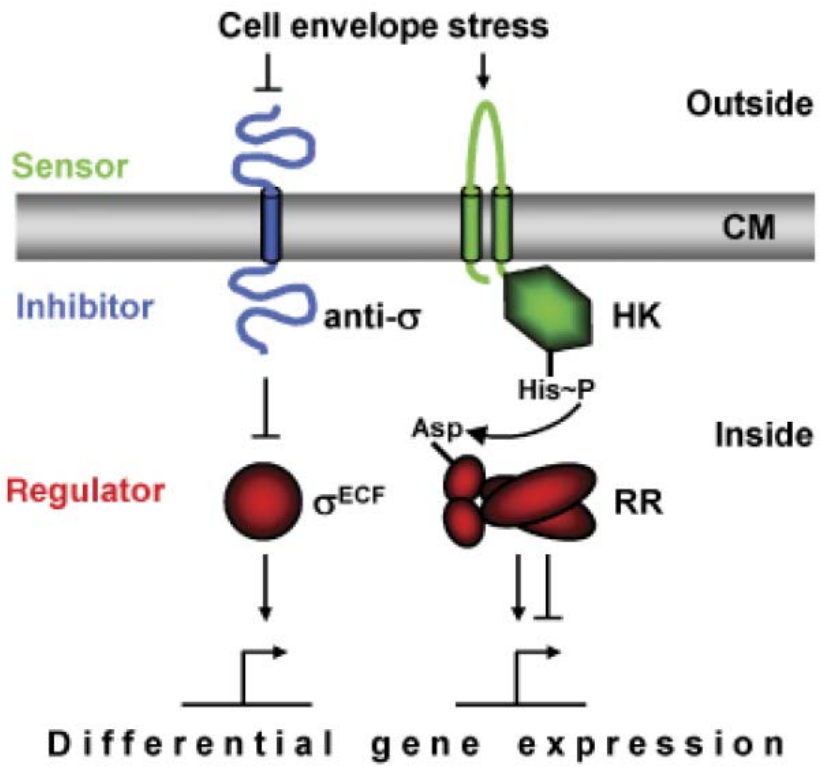

Figure 1.4. Regulatory principles orchestrating cell envelope stress response in Gram-positive bacteria. On the left, ECF σ factors, and on the right, two-component systems (HK, histidine kinase; RR, response regulator). Sensor proteins are shown in green, inhibitor in blue, transcriptional regulators in red. Arrows indicate activation, T-shaped lines repression. CM, cytoplasmic membrane. Adapted from Jordan *et al*. (32)

To date, the best understood of these σ factors are σ^X, σ^W and σ^M which control genes involved in cell envelope synthesis and / or modification (27).

σ^X, the first ECF σ factor to be sequenced in *B. subtilis* (27), and its cognate anti-σ-factor are encoded for by a bi-cistronic operon (*sigXrsiX*). The σ^X regulon was defined using promoter consensus search and *in vitro* runoff transcription-macroarray analysis (ROMA) (12). σ^X was found regulate transcription of the *dltABCDE* operon, responsible for D-alanine esterification of TAs, and to partially control the transcription of the *pssAybfMpsd* operon responsible for the biosynthesis of PhE (12). In addition, *sigX* mutants demonstrate increased sensitivity to cationic antimicrobial peptides (CAMPs), and an increased rate of autolysis (12). Based on these findings it was concluded that σ^X regulates resistance to CAMPs via modification of cell surface properties (12).

The σ^W regulon, comprised of 30 operons (~60 genes), was identified using promoter consensus search, ROMA and transcriptional profiling approaches (13). The σ^W regulon is induced by alkali shock (75), vancomycin (14), and the mammalian cationic peptide LL37 (60), but does not seem to confer resistance to these stresses (14, 60). However, σ^W was found to control the transcription of a major fosfomycin resistance determinant (11). In addition, using a panel of *B. subtilis* mutants disrupted for each of the 30 operons it was discovered that genes in the σ^W regulon provide intrinsic resistance to antimicrobial compounds produced by *Bacilli* species, thus enabling *B. subtilis* to maintain its niche in the face of other antibiotic-producing bacteria (9).

A comprehensive study of the σ^M regulon was conducted using promoter consensus search, ROMA and transcriptional profiling approaches and σ^M was found to affect the transcription of genes involved in cell wall biosynthesis, shape determination and cell division, DNA damage responses and detoxification enzymes

(21). A subset of these genes are indirectly regulated by σ^M via induction of Spx (21), which can act both as both a positive and negative transcriptional regulator (76).

An interesting topic arising from these studies is the overlap in transcriptional regulation between σ^M and other ECF σ factors (21). This overlap further complicates the precise definition of regulons, as well as the elucidation of in vivo regulatory networks.

3.2 Two-Component regulatory Systems (TCS)

TCS are comprised of a membrane bound sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). An external stress signal is sensed by the HK, causing the kinase to autophosphorylate a histidine residue. The phosphoryl group is then transferred to an aspartic acid residue on the RR, thus activating it (22).

Transcriptional profiling in response to treatment with CAMPs (60) and cell wall targeting antibiotics (45) have revealed differential expression patterns for a number of TCS. Treatment with bacitracin resulted in the induction of three TCS: LiaRS, BceRS and YvcPQ (45). The HK of these systems are classified as intra-membrane sensing HK, and are proposed to sense stimuli either directly inside or at the surface of the cytoplasmic membrane (44).

BceRS represents a large group of TCS associated with an ATP-binding cassette (ABC) transporter. The genes encoding for the ABC transporter are usually organized in a separate operon, the expression of which is completely dependent on the associated TCS (44). BceRS regulates the expression of the BceAB ABC transporter, which is the major bacitracin resistance determinant in *B. subtilis* (2).

The LiaRS TCS is actually a representative of a three-component system. The LiaRS TCS is linked to a third protein LiaF, which inhibits LiaR-dependent transcription (33). In addition to bacitracin, LiaRS is induced by other antibiotics

which interfere with the cycling of lipid II such as ramoplanin or vancomycin and the CAMP LL37 (46).

Although not directly induced by antibiotics, the YycFG essential TCS has been implicated in cell wall homeostasis (Fig 1.5). YycFG was found to activate the expression of autolysins, and repress the expression of an autolysin activity modulator, and that of a peptidoglycan deacetylase (the activity of which affects susceptibility to lysozyme; 6) In addition, the YycG HK was found to localize to the division septum, and its effective kinase activity requires the presence of the division septum (25). This data supports a model according to which the YycFG TCS coordinates cell growth and division with external events such as cell wall restructuring (25).

The bacterial cell envelope is an essential component determining cell shape, and providing protection against mechanical and osmotic pressure. The cell envelope also serves as a first line of defense against external stress, and therefore the ability to sense and respond to the presence of challenges to the envelope is crucial for the cell's survival.

The overall goal of the work presented in this dissertation is to elucidate cell envelope stress responses in the model Gram-positive bacteria *B. subtilis*, in particular those induced by treatment with antibiotics. The strategy for studying these responses was to stress cells either by the use of external stimuli or by genetic manipulation.

Chapter two characterizes a novel post-translational modifier of autolysin activity, YoeB, that is under the transcriptional regulation of the YycFG essential TCS. This modifier is induced by cell wall-targeting antibiotics and is proposed to inhibit autolytic enzyme activity under conditions of impaired cell wall synthesis, thereby reducing the rate of antibiotic-dependent cell death (Fig 1.5).

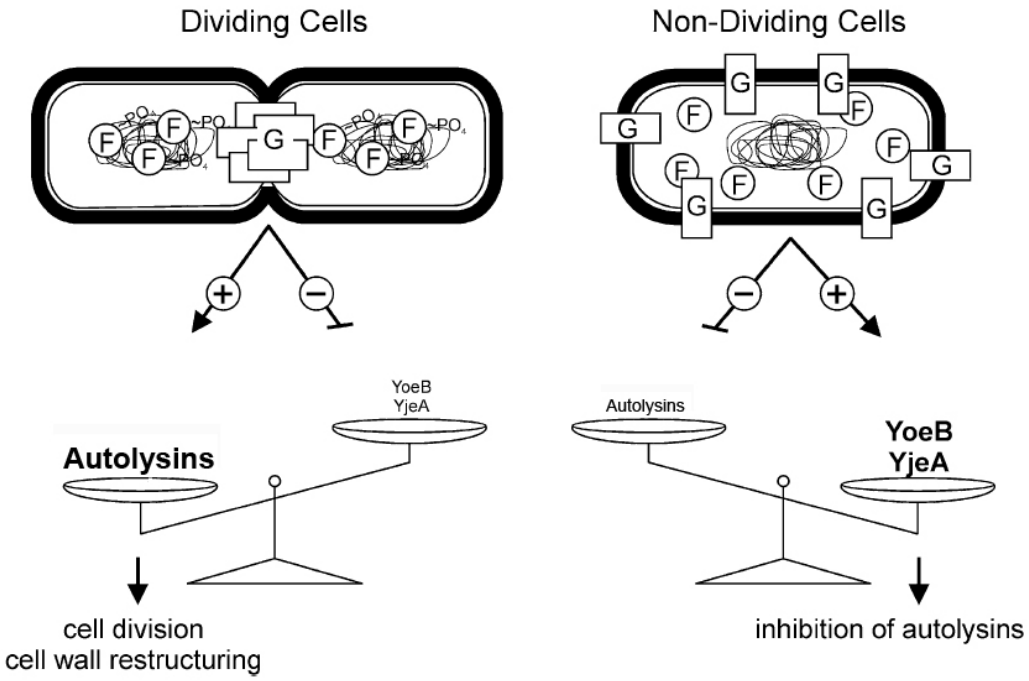

Figure 1.5. Model for the role of YycFG in coordinating cell division with cell wall homeostasis. YycG is activated upon localization to the septum in dividing cells. This activation increases phosphorylation of YycF and subsequently leads to increased expression of autolysins and other genes involved in cell division, and repression of genes involved in inhibiting cell wall remodeling (*yoeB* and *yjeA*). In the absence of septa in non-dividing cells, YycG fails to localize, adopts a state of low activity, and hence YycF remains unphosphorylated. Under these conditions, cell wall remodeling is not required and the expression balance is tipped towards the genes involved in autolysin inhibitory process. Adapted from Fukushima *et al*. (25).

Chapter three describes a transcriptional regulator induced in response to glycopeptide antibiotics. The regulon of this regulator includes efflux pumps proposed to be involved in resistance to cell-wall targeting antibiotics.

Finally, chapter four characterizes a series of *B. subtilis* strains with genetically altered membrane composition. Mutant strains were characterized for growth, antibiotic resistance, morphology, and alterations in global gene expression patterns. The results suggest that the cell can tolerate even large changes in membrane composition.

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CHAPTER TWO

An antibiotic-inducible cell wall-associated protein that protects *Bacillus subtilis* from autolysis*

1. Summary

In *Bacillus subtilis*, antibiotics that impair cell wall synthesis induce a characteristic stress response including the σ^W and σ^M regulons and the previously uncharacterized *yoeB* gene. Here, I demonstrate that YoeB is a cell wall-associated protein with weak sequence similarity to a non-catalytic domain of Class B penicillin-binding proteins. A null mutant of *yoeB* exhibits an increased rate of autolysis in response to cell wall targeting antibiotics or nutrient depletion. This phenotype does not appear to be correlated with gross alterations in peptidoglycan structure or levels of autolysins. Promoter dissection experiments define a minimal region necessary for antibiotic-mediated induction of *yoeB*, and this region is highly conserved preceding *yoeB* homologs in close relatives of *B. subtilis*. These results support a model in which induction of YoeB in response to cell envelope stress decreases the activity of autolysins and thereby reduces the rate of antibiotic-dependent cell death.

* Letal I. Salzberg and John D. Helmann (2007). J Bacteriol 189(13): 4671-4680

2. Introduction

The bacterial cell wall defines cell morphology and provides it with the structural rigidity required to withstand osmotic pressure. In Gram positive bacteria the scaffold of the cell wall is comprised of a thick layer of peptidoglycan (PG), a polymer of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid glycan chains crosslinked by peptide side chains (37). PG synthesis requires the transfer of the nucleotide sugar-linked precursors across the membrane where they are incorporated into the growing cell wall by penicillin-binding proteins (PBPs) (37). PBPs polymerize the glycan strands by transglycosylation, and catalyze cross-linking of the glycan chains by transpeptidation (37).

Insertion of new material into the cell wall also requires cleavage of the existing PG. It has been proposed that the PBPs form complexes with the enzymes responsible for cleavage, the peptidoglycan hydrolases (5). These enzymes, also known as autolysins, have been proposed to play a role in many cellular processes including cell growth and division, cell-wall turnover, peptidoglycan maturation, motility, chemotaxis, competence, protein secretion, sporulation, and pathogenicity (2, 23, 40). Obstruction of cell-wall synthesis has been proposed to lead to an imbalance between synthesis (PBPs) and wall turnover (autolysins) and thereby lead to cell rupture (19). Indeed, autolysin deficient mutants of *B. subtilis* have increased resistance to D-cycloserine and β-lactam antibiotics (36). Left unchecked, autolysins will degrade the cell wall leading to its rupture and eventually to cell death. Therefore, fine-tuning of autolysin activity through efficient and strict regulation is crucial for bacterial survival.

In *Bacillus subtilis*, autolysins are transcriptionally regulated by several alternative σ factors and two component systems (TCS). Transcription of the major

vegetative autolysins LytC and LytD is under the control of alternative sigma factor σ^D (20, 25), which is also involved in the regulation of flagellar synthesis, motility, and chemotaxis (26). Transcription of *lytC* is also under the negative control of the YvrGHb TCS (39). The essential YycFG TCS has been implicated in the regulation of two genes encoding potential autolysins, *yocH* and *ykvT* (15).

The post-translational control of autolysins is more complicated. Dissipation of the proton-motive force (PMF) across the *B. subtilis* membrane leads to rapid lysis of the cells (17). It has also been reported that the cell wall of *B. subtilis* is protonated during respiratory growth causing acidification of the local environment of the autolysins (6). The low pH in the cell wall may prevent the activity of the autolysins, and only when the enzymes are exposed to a higher pH would they become active (18). Indeed, it has been suggested that the bacteriolytic effect of some β-lactams is caused by a depolarization of the membrane potential and the consequent activation of autolysins (33).

We have investigated the transcriptional responses of *B. subtilis* to treatment with cell wall active antibiotics (7, 27). Vancomycin led to the rapid induction (3 min.) of ~100 genes, including the regulons controlled by the extracytoplasmic function (ECF) σ factors, σ^W and σ^M (7). However, one of the most strongly induced genes was *yoeB*, which is not under control of either of these ECF σ factors. A subsequent comprehensive study of the transcriptional responses of *B. subtilis* to 37 antibacterial compounds revealed induction of *yoeB* by cell wall- and membrane-targeting antibiotics such as cycloserine, oxacillin, vancomycin, fosfomycin, ristocetin, and triclosan (16).

I here demonstrate that YoeB is a cell wall-associated protein that moderates the activity of autolysins. A *yoeB* null mutant displayed an increased rate of autolysis in response to various cell envelope stress conditions, but not when autolysis was

induced by dissipation of the proton motive force. Thus, YoeB functions as a component of a cell envelope stress response that inhibits autolytic enzyme activity under conditions of impaired cell wall synthesis.

3. Materials and Methods

Bacterial strains and growth conditions. All *B. subtilis* strains used were derivatives of CU1065 (W168 *trpC2 attSPβ*) (Table 2.1). *Escherichia coli* strain DH5α was used for standard cloning procedures. Bacteria were grown in Luria-Bertani (LB) medium, Difco sporulation medium (DSM) or modified Spizizen minimal medium (SMM) without citrate supplemented with 0.5 % glycerol (w/v), 0.02 % casamino acids (w/v) and 50 μg/ml of tryptophan for auxotrophic requirements (17) at 37°C with vigorous shaking. Antibiotics were added to the growth medium when appropriate: 100 μg/ml ampicillin for *E. coli*, and 1 μg/ml erythromycin plus 25 μg/ml lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 μg/ml chloramphenicol and 8 μg/ml neomycin for *B. subtilis*. OD_{600} readings were taken on a Spectronic 21 (Milton Roy) spectrophotometer.

Sensitivity to cell wall targeting antibiotics and sodium azide was measured by growing strains in LB medium at 37°C with vigorous shaking. Antibiotics were added at the indicated concentration during early exponential growth (OD_{600} ~ 0.25). Lysis was monitored by measuring the decrease of OD_{600} at 30 min intervals. For D-cycloserine-treated cells, direct colony counts were performed at 0, 45, and 90 min. after antibiotic addition and compared to the corresponding OD_{600} measurements. Susceptibility to lysozyme was measured by resuspending 1 ml of early logarithmic (OD_{600} ~ 0.3) cell culture in 1 ml of 10 mM Tris-HCl (pH 8.0). Lysozyme was added at a final concentration of 2 μg/ml. The resuspended cells were incubated at 37°C without shaking and lysis was monitored by the decrease in OD_{450}.

Construction of the *yoeB* null mutant. A chromosomal deletion was created using long-flanking homology PCR (LFH-PCR) as described (27) with the following changes: flanking fragments were amplified using *Pfu* DNA Polymerase (Stratagene) and the flanking fragments and antibiotic resistance gene were joined using Expand Long Template PCR System (Roche). Primers #1589 & #2002 were used to amplify the upstream fragment, and primers #2087 and #2088 were used to amplify the downstream fragment. Detailed protocols are available upon request.

Overproduction of YoeB in *B. subtilis* with a xylose-inducible system. The *yoeB* open reading frame was PCR amplified using primers #2004 and #2005. The resulting PCR fragment was digested with EcoRI and HindIII and cloned into pXT, a derivative of pDG1731 that places the cloned gene under the control of a xylose-inducible promoter (P_{xylA}) (9), creating plasmid pLS20. The sequence of the insert was verified by DNA sequencing (Cornell DNA sequencing facility). *B. subtilis* CU1065 and HB5345 (*yoeB::MLS*) were transformed to Spc^r with ScaI-linearized pLS20 which integrates into the *thrC* locus. The resulting strains were designated HB5364 (CU1065, with P_{xylA}-*yoeB* at the *thrC* locus) and HB5365 (HB5345, with P_{xylA}-*yoeB* at the *thrC* locus).

Construction and analysis of P_{yoeB} transcriptional fusions. DNA fragments of the *yoeB* regulatory region were PCR amplified using primers: #1713 #1714 (-514), #2133 #1714 (-68), #2132 #1714 (-42), #1995 #1714 (-22), #1713 #2404 (+18). The fragments were digested with HindIII and BamHI, and cloned into vector pDG1661, containing a promoterless *lacZ* gene (12), resulting in plasmids pLS08, pLS13, pLS14, pLS15 and pLS16, respectively. The sequences of the inserts were verified by DNA sequencing (Cornell DNA sequencing facility). *B. subtilis* CU1065 was transformed to Cm^r with the ScaI-linearized plasmids which integrated into the *amyE* locus, creating strains HB5377, HB5372, HB5371, HB5370 and HB5366, respectively.

For quantitative measurements of β-galactosidase activity, strain HB5377 was grown in either LB or DSM medium at 37°C with vigorous shaking, and samples were collected at different growth stages as determined by OD_{600}. To test promoter induction, strain HB5377 was grown in LB until an OD_{600} ~ 0.1, and then the culture was split into aliquots, which were challenged with either vancomycin (2.5 μg/ml), D-cycloserine (100 μg/ml), oxacillin (2.5 μg/ml), fosfomycin (50 μg/ml), triclosan (1 μg/ml), monensin (0.125 μg/ml), nigericin (0.008 μg/ml), polymyxin (64 μg/ml), tetracycline (0.5 μg/ml), diamide (1 mM final), paraquat (100 mM final), or arsenate (10 μM final). To test the activity of the different length promoters strains HB5377, HB5372, HB5371, HB5370 and HB5366 were grown in LB until an OD_{600} ~ 0.1, the cultures were split into aliquots and challenged with vancomycin (2 μg/ml). The cultures were returned to 37°C, and samples were collected after 30 min. β-galactosidase activity was measured according to the method of Miller (28) except that cells were lysed by the addition of lysozyme to a final concentration of 20 μg/ml followed by a 30 min incubation at 37°C.

RNA isolation and slot blot hybridization. Strains CU1065, HB5377, HB5372, and HB5366 were inoculated into LB and grown at 37°C with vigorous shaking until an OD_{600} ~0.3, at which time the culture was split into 10 ml aliquots. To test induction of *yoeB* with various stress conditions (Fig. 2.5A), cells were challenged for 10 min at 37°C with either vancomycin (2 μg/ml final), diamide (1 mM final), paraquat (100 mM final), arsenate (10 μM final), triclosan (1 μg/ml), nigericin (0.008 μg/ml), monensin (0.125 μg/ml), or tetracycline (0.5 μg/ml). For activity measurements of strains containing different length promoter fusions (Fig. 2.6C) the cultures were split into two 10 ml aliquots, one of which was challenged with diamide (1 mM final) for 10 min at 37°C. RNA isolation and quantification was performed as described previously (30). RNA samples were loaded onto Zeta-Probe Blotting Membrane (Bio-

Rad). Signals from samples containing 2 and 0.2 μg are presented in Fig 2.5A; the signal from a blot with 0.8-μg loaded per lane was used to generate Fig. 2.6C. DNA probes were constructed by PCR. The *yoeB* probe was amplified using primers #2006 and #2007 with *Pfu* DNA Polymerase (Stratagene) and the *lacZ* probe was amplified using primers #2640 and #2641 with Thermo-Start Master Mix (ABgene), as per the manufacturers' specifications. The probes were purified with a QIAquick PCR Purification Kit (Qiagen), labeled with [α-^{32}P]ATP using DECAprime II Random-primed DNA labeling kit (Ambion), and unincorporated [α-^{32}P]ATP was removed by using NucAway Spin columns (Ambion). Slot blot hybridizations were performed overnight at 42^{o}C using the NorthernMax Hybridization Kit (Ambion) according to the manufacturer's instructions. Low stringency washes were performed at room temperature and high stringency washes at 42^{o}C. The membranes were imaged on a Storm 840 PhosphorImage scanner (Molecular Dynamics) after overnight exposure of a PhosphorImage screen. The resulting signals were quantified using ImageQuant data analysis software.

Construction and analysis of FLAG-tagged YoeB. Primers #1713 and #2430 were used to PCR amplify (*Pfu* DNA Polymerase) a 1093 bp fragment, containing the entire promoter region and ORF except for the terminal stop codon. The fragment was digested using HindIII and EagI and cloned into plasmid pJL070 (21) creating plasmid pLS22 containing *yoeB*-FLAG under control of its native promoter. pJL070 is a derivative of pDG1730 (12) which contains *perR*-FLAG cloned into the HindIII and EcoRI sites. pJL070 was digested with HindIII and EagI, which leaves the FLAG tag undisturbed. The digested plasmid was then gel purified prior to ligation. The sequence of *yoeB*-FLAG was verified by DNA sequencing (Cornell DNA sequencing facility). Strain CU1065 was transformed to Spc^{r} with ScaI-linearized pLS22, which integrates into the *amyE* locus, creating strain HB5384. To determine YoeB

localization, strain HB5384 was inoculated into 2 x 50 ml LB and grown at 37°C with vigorous shaking until OD_{600}~ 1.0, at which point 2 x 50 ml were centrifuged for 10 min at 4,500 x g after which one of the 50 ml pellets was resuspended in 1 ml Buffer A (100 mM Tris-HCl pH8.0, 1 mM $MgCl_2$, 0.1 mM PMSF) and then sonicated (Crude). The second 50 ml pellet was resuspended in 1 ml of iron-starvation minimal medium (4) supplemented with 0.2 mM PO_4, 40 mM $MnCl_2$ and 1 mM $FeSO_4$ (MMa) and centrifuged for 2 min at 5,900 x g. The pellet was then resuspended in 1 ml of MMb (1 x Bacillus salts (4), 40 mM MOPS (pH 7.4) and 2% glucose (w/v)) and centrifuged for 2 min at 5,900 x g. The pellet was then resuspended in 1 ml of MMa with the addition of 20% sucrose and 1 mg/ml lysozyme and incubated with agitation at 37°C for 40 min. Formation of protoplasts was verified by microscopy, which were then centrifuged for 4 min at 4,600 x g at 4°C. The supernatant was removed for use as cell wall fraction. The protoplasts were resuspended in 1 ml Buffer A, briefly sonicated and then ultracentrifuged at 45,000 rpm for 30 min (TLA 100.3 fixed angle rotor for Beckman TL-100 ultracentrifuge) to separate the membrane and cytoplasmic fractions. The membrane fraction was resuspended in 1 ml Buffer A. Cell crude extract, cell wall, membrane, and cytoplasmic preparations were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and boiled for 10 min prior to being resolved by SDS-PAGE (12% gel). After electrophoresis, proteins were blotted to a PVDF membrane (Bio-Rad), and YoeB-FLAG was detected by Western-blot analysis using polyclonal anti-FLAG antibody from rabbit and anti-rabbit antibody conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO.).

Preparation of purified cell wall substrates. A 50 ml CU1065 culture was grown in LB to late exponential stage (OD_{600} = 0.6). Cells were spun down at 4,500 x g for 10 min at 4°C, and the pellet was resuspended in 1 ml cold H_2O. The suspension was

added slowly to 15 ml of boiling 4% SDS and boiled for 20 min. Cell wall material was washed three times in 1 ml ddH_2O, and finally resuspended in 1 ml sterile ddH_2O.

Zymogram renaturing gel electrophoresis. Zymograms were performed as previously described (11). Fractionated samples were run on a 12% SDS-polyacrylamide gel containing 4% of the peptidoglycan extract. Following eletrophoresis at 20mA (constant current) the gel was washed in 250 ml dH_2O at room temperature for 30 min with agitation. The gel was then transferred to 250 ml of renaturation solution (0.1% Triton-X-100, 10 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.5)) and soaked for 30 min at room temperature with agitation. The gel was transferred to 250 ml renaturation solution and soaked for 16 h at 37^{o}C with agitation. The gel was then stained for 3 h in 100 ml of 0.1% Methylene blue, 0.01% KOH (at 37^{o}C with agitation), and then destained in dH_2O. Autolysin activity appeared as zones of clearing on a blue background.

Succinate dehydrogenase activity assay. A 50 µl aliquot of each subcellular fraction was added to 950 µl of a reagent mixture containing 25 mM 2-hydroxyethylpiperazine (HEPES), 25 mM sodium succinate, 100 µg/ml Brij 58, 30 µg/ml 3(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and 300 µg/ml phenazine methosulfate (PMS). Succinate dehydrogenase activity was measured at 570 nm and units of nmol MTT reduced/min/mg protein were calculated using an extinction coefficient of 17,000 $M^{-1}cm^{-1}$ for reduced MTT (43).

Table 2.1. Strains, plasmids and oligonucleotides used in this study

	Description	**Created / reference**
E.coli		
DH5α	ϕ80*lacZ*Δm15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^-, m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169	Lab strain
B. subtilis		
CU1065	W168 *trpC2 attSPβ*	Lab strain
HB5345	CU1065 *yoeB::MLS*	LFH-PCR → CU1065
HB5364	CU1065 *thrC::*P_{xylA}*-yoeB*	pLS20 → CU1065
HB5365	CU1065 *yoeB::MLS thrC::*P_{xylA}*-yoeB*	pLS20 → HB5345
HB5366	CU1065 *amyE::*P_{yoeB}*-lacZ* (+18)	pLS16 → CU1065
HB5370	CU1065 *amyE::*P_{yoeB}*-lacZ* (-22)	pLS15 → CU1065
HB5371	CU1065 *amyE::*P_{yoeB}*-lacZ* (-42)	pLS14 → CU1065
HB5372	CU1065 *amyE::*P_{yoeB}*-lacZ* (-68)	pLS13 → CU1065
HB5377	CU1065 *amyE::*P_{yoeB}*-lacZ* (-514)	pLS08 → CU1065
HB5384	CU1065 *amyE::yoeB-FLAG*	pLS22 → CU1065
Plasmids		
pXT	Cloning vector for overexpression	(9)
pDG1661	Vector for integration of *lacZ* fusions at *amyE* locus	(12)
pLS08	P_{yoeB}*-lacZ* (-514) in pDG1661	This work
pLS13	P_{yoeB}*-lacZ* (-68) in pDG1661	This work
pLS14	P_{yoeB}*-lacZ* (-42) in pDG1661	This work
pLS15	P_{yoeB}*-lacZ* (-22) in pDG1661	This work
pLS16	P_{yoeB}*-lacZ* (+18) in pDG1661	This work
pLS20	*yoeB* cloned into pXT	This work
pLS22	*yoeB-FLAG* in pDG1730	This work

Table 2.1. (Continued)

Oligos	
#1589	GCAGCAGGAGAACCAGCAGTTCC
#1713	CCGTAAGCTTGTCCTATCAG
#1714	GCAGGATCCGCTCATGGTGTTCC
#1995	CCCAAGCTTCCGTAATTATTGTAACATTTGTAAC
#2002	GAGGGTTGCCAGAGTTAAAGGATCTCATTATCGCTCTCTCCTTC
#2004	CCCAAGCTTTTGTAACATAAGAGAAAGAGAT
#2005	CGGAATTCTTCACAAAAAGACAACAGGC
#2006	GTTGGCATTATCCGCTAGAG
#2007	CTTTCCGTTTTGCAGAAGGG
#2087	CGATTATGTCTTTTGCGCAGTCGGCACTGTCCCTACATTAGACGG
#2088	GCATATTGTAGAGCCGATTCGC
#2132	CCCAAGCTTGCAAAAGTATTGTAATCTATCCG
#2133	CCCAAGCTTGAGTAAAAGAATAAAAAATAATTTATGC
#2404	GCGGATCCATCTCTTTCTCTTATGTTACAAATG
#2430	CTGCACGGCCGTATAACTGCGTCAAATTGATTG
#2640	ACAGTACGCGTAGTGCAACC
#2641	CGCCACTTCAACATCAACGG

Restriction sites are underlined

4. Results

The *yoeB* mutant demonstrates an increased rate of lysis in response to cell wall antibiotics. A *yoeB* null mutant was constructed by allelic replacement with an MLS resistance gene using LFH-PCR (42). This mutant did not demonstrate any growth defects in the media tested (LB, LB+ 2% glucose, DSM), nor did it exhibit any changes in cellular morphology as observed by phase contrast microscopy (data not shown).

I next tested the sensitivity of the *yoeB* mutant to different cell wall antibiotics. Upon exposure of logarithmically growing cells to a variety of PG synthesis inhibitors (vancomycin, bacitracin, cephalosporin, D-cycloserine and fosfomycin) the *yoeB* mutant exhibited an increased rate of lysis relative to wild-type (Fig. 2.1A-E). This increased rate of lysis is still observed when antibiotics were added at later logarithmic stages (OD_{600} = 0.4, 0.6), but not once cells entered transition stage (OD_{600} = ~0.8) (data not shown). Despite this increased rate of lysis, there was no apparent difference in sensitivity (<2-fold) as judged by minimal-inhibitory-concentration (MIC) assays or by measuring zones of growth inhibition on solid media (data not shown). Indeed, both wild-type and mutant demonstrated a comparable extent of lysis after prolonged exposure (Fig. 2.1A-E).

To determine if YoeB protects cells against antibiotic-mediated killing, in addition to its obvious effects on rates of lysis, viable counts (CFU) were determined for D-cycloserine treated cultures of the wild-type and *yoeB* mutant strains. In both cases, a significant decrease in CFU was noted 45 min. after antibiotic treatment (~ 3-4-fold decrease for the wild-type and ~10-fold for the *yoeB* mutant) despite the fact that the OD_{600} had not yet begun to decrease. After 90 min of treatment, there was a ~5-fold drop in OD_{600} for the *yoeB* mutant and the CFU had decreased to ~2% of the starting value. In contrast, the wild-type cells displayed little net drop in OD_{600} at this

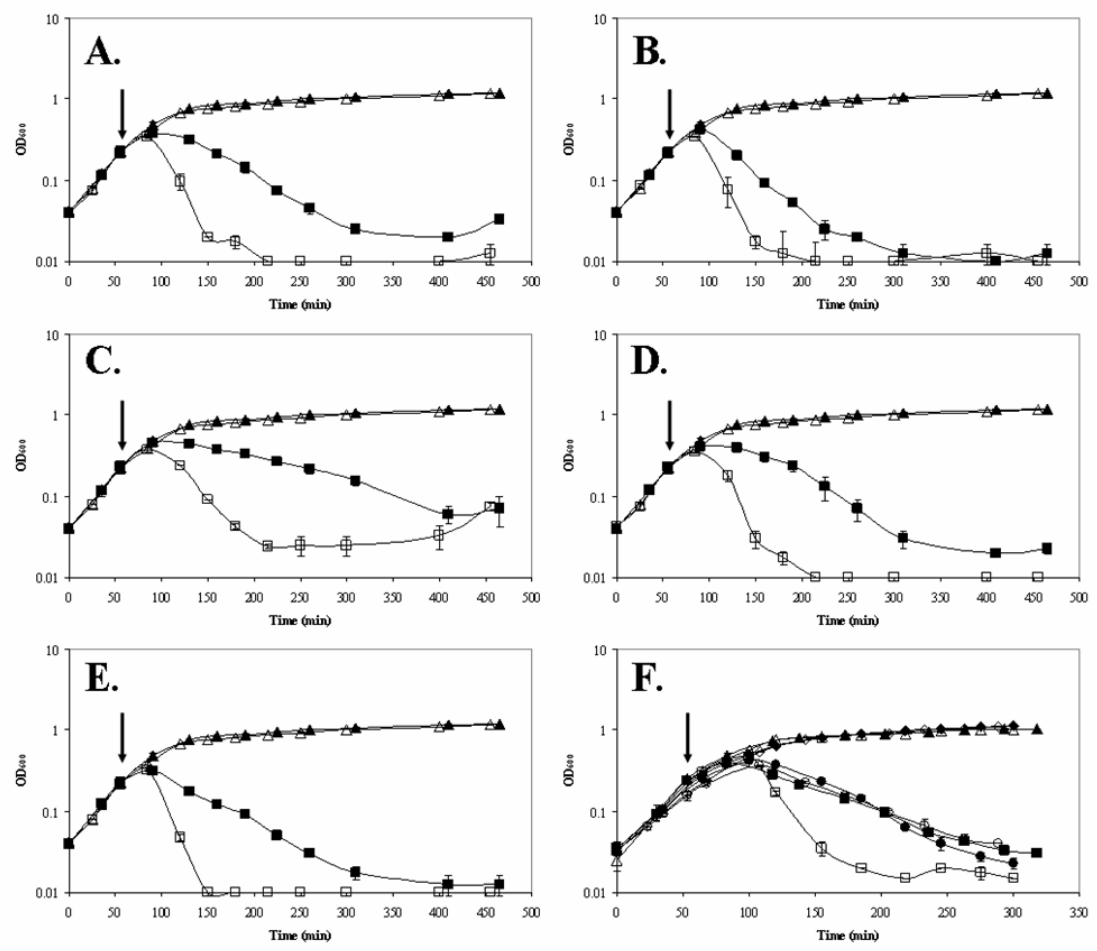

Figure 2.1. The *yoeB* mutant demonstrates an increased rate of lysis in response to cell wall antibiotics. *B. subtilis* wild-type (filled symbols) and an isogenic *yoeB* mutant (open symbols) grown in LB were either untreated (triangles) or treated at OD_{600} ~ 0.25 (squares) with: A, vancomycin (1 μg/ml); B, cephalosporin C (20 μg/ml); C, fosfomycin (50 μg/ml); D, bacitracin (250 μg/ml); E, D-cycloserine (100 μg/ml). (F) The increased rate of lysis is dependent on the absence of *yoeB*. Complementation experiments with WT and *yoeB* mutant strains containing P_{xyl}-*yoeB*. Filled symbols, WT cultures; open symbols, *yoeB* mutant strain. Cells were grown in LB (triangles), LB + 1% xylose (diamonds), LB and then treated with D-cycloserine (squares) or LB + 1% xylose and then treated with D-cycloserine (100 μg/ml) (circles). The arrows indicate the time at which antibiotics were added. Results are representative of three independent experiments.

time (see. Fig. 2.1E) and retained 13-17% of the starting CFU. These results suggest that although death may precede lysis, the presence of YoeB significantly decreases the rate of both events. Thus, YoeB likely protects cells in environments where cell wall active compounds are present at low (sub-lethal) levels or exposure is transient.

Expression of *yoeB* under the control of a xylose-inducible promoter complemented the phenotype of a *yoeB* null mutant and restored the slower rate of lysis characteristic of wild-type cells (Fig. 2.1F). To determine whether overexpression of YoeB could confer resistance to cell wall antibiotics, I tested lysis rates in wild-type cells containing a second, inducible copy of *yoeB* under P_{xyl} control. Overexpression did not result in decreased lysis when cells were treated with cell wall-targeting antibiotics, suggesting that wild-type levels of YoeB are sufficient for the maximal protective effect.

The *yoeB* mutant demonstrates an increased susceptibility to lysis upon nutrient depletion. To determine if there is an innate difference in autolysis in the *yoeB* mutant strain I examined the effects of autolysis as a consequence of nutrient depletion. When grown in modified Spizizen's minimal medium (17) supplemented with 5% glycerol, WT and *yoeB* mutant demonstrated comparable growth rates. However, once exponential growth ceased the *yoeB* mutant began to lyse (Fig. 2.2A) suggesting that it is susceptible to autolysis caused by de-energization of the cell upon nutrient depletion. Similar results were observed when cells grown in LB were resuspended in buffer (data not shown). This is consistent with the hypothesis that the gradual de-energization of the *yoeB* mutant cells leads to an imbalance in the synthesis and degradation of PG. In principle, the increased autolysis of the *yoeB* mutant could be due to alterations in either the substrate (PG) or effectors (autolysins) responsible for cell lysis.

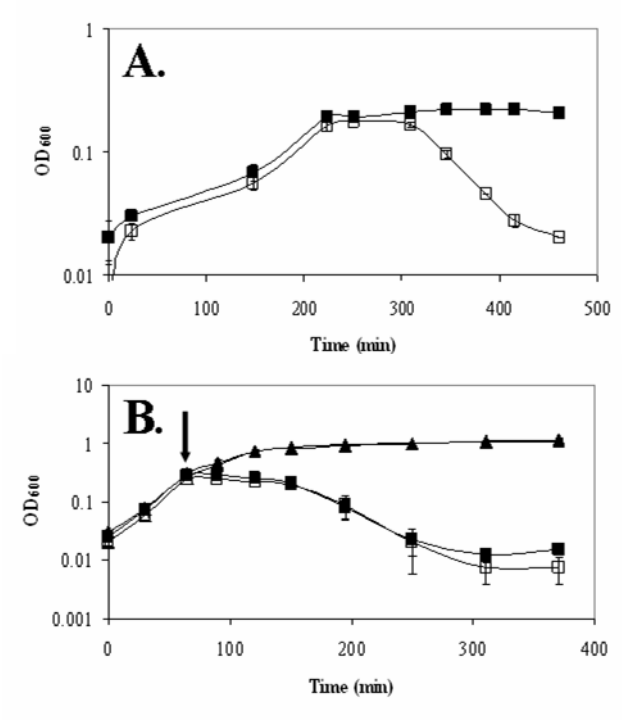

Figure 2.2. The *yoeB* mutant demonstrates an increased susceptibility to lysis in de-energized cells, but not in response to rapid dissipation of the proton motive force**.** (A**)** Cells were grown in a modified Spizizen's minimal medium supplemented with 5 % glycerol. WT (filled squares), *yoeB* mutant (open squares). Results are representative of three independent experiments. (B) *B. subtilis* WT (filled symbols) and *yoeB* mutant (open symbols) grown in LB were either untreated (triangles) or were treated with 0.05M sodium azide (added at OD_{600} ~ 0.25, indicated by arrow) (squares).

YoeB is a cell wall-associated protein. YoeB encodes an ~19 kD protein containing a predicted signal sequence. To monitor localization of YoeB, I expressed a FLAG epitope-tagged allele of *yoeB* under regulation of its native promoter. The FLAG-tagged YoeB protein was at least partially functional as judged by complementation of the autolysis phenotype of the *yoeB* null mutant. YoeB-FLAG was found in highest abundance in the cell wall fraction, with lesser amounts detected in the membrane fraction, as judged by immunoblot analysis (Fig 2.3A). The portion detected in the membrane fraction may correspond to cross-contamination with cell wall-associated proteins as judged by the fractionation of a known wall-associated protein (autolysin) by zymogram analysis (Fig. 2.3B). As expected, a known membrane-associated enzyme, succinate dehydrogenase, was found primarily (~85%) in the membrane fraction. Together, these results suggest that YoeB is a wall-associated protein. Semi-quantitative analysis of the YoeB-FLAG signal intensity, by comparison with a dilution series of a purified FLAG-tagged protein (data not shown), suggests that YoeB is present in ~50-100 molecules per cell at OD_{600} ~1.

Increased susceptibility to autolysis is not correlated with altered peptidoglycan. I hypothesized that YoeB might affect PG structure and thereby reduce the susceptibility of the cell to autolysis. Precedent for such effects derives from studies of PG deacetylases, cell wall-associated proteins that modulate the susceptibility of the cell to lysozyme treatment (3, 35, 41). Indeed, intact cells of the *yoeB* mutant are more sensitive to lysozyme-triggered cell lysis than wild-type (Fig. 2.4B). However, interpretation of this result is complex, since in these buffer conditions the *yoeB* mutant undergoes significant autolysis even in the absence of lysozyme (Fig. 2.4A).

To more directly test the possible effects of YoeB on PG structure, cell walls were purified from WT and *yoeB* mutant cells and tested for their susceptibility to degradation by lysozyme and mutanolysin. Both the rate and extent of hydrolysis

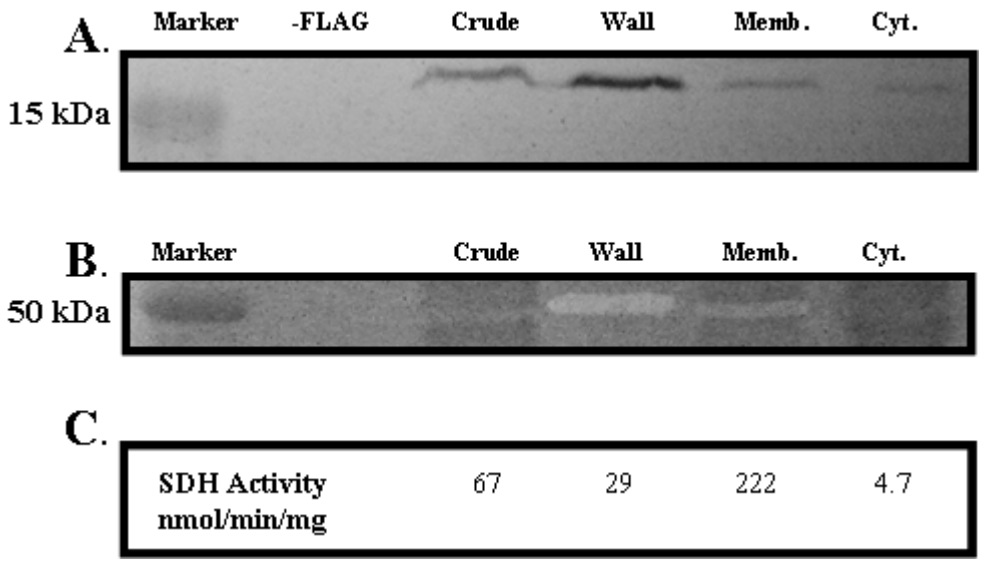

Figure 2.3**.** Localization of YoeB. (A) Western blot analysis using anti-FLAG antiserum. Subcellular fractions were obtained from cells containing an ectopically integrated FLAG-tagged copy of *yoeB* under regulation of its native promoter. (B) Zymogram assay for subcellular protein fractions. (C) Succinate dehydrogenase activity of subcellular fractions. Results are representative of at least three independent experiments.

were indistinguishable (data not shown). Similarly, when I used native *B. subtilis* autolysins (extracted from cells with LiCl) to digest PG from WT and *yoeB* mutant cells, there were no apparent differences. Finally, HPLC analysis of muropeptides prepared from vegetative WT and *yoeB* mutant cells demonstrated overall similar structural parameters: there were no significant changes in either the fraction of crosslinking, or the pattern or amount of sugar modifications (D. Popham, personal communication). These results suggest that the *yoeB* mutant phenotype cannot be obviously linked to alterations to the PG.

Increased susceptibility to autolysis is not correlated with differences in the level of autolysins. I hypothesized that YoeB might affect the levels of autolytic enzymes associated with the cell wall. To test this hypothesis we treated cells with sodium azide, a metabolic poison that rapidly dissipates the proton motive force leading to strong activation of autolytic enzymes. Under these conditions, the rates of lysis of WT and *yoeB* mutant cells were indistinguishable (Fig. 2.2B). This suggests that the autolytic potential of the cells is very similar. Further, zymogram analysis of the major autolytic activities present in the cell wall fractions from WT and *yoeB* mutant cells did not reveal any apparent differences in either the number or intensity of bands (data not shown). This technique, however, only detects the four or five most abundant autolysins that efficiently renature after SDS-PAGE. Finally, I note that the ability of the extracted autolysins from WT and *yoeB* mutant cells to degrade PG (from either WT or *yoeB* cells) was indistinguishable.

I reasoned that the *yoeB* mutant strain might be altered in the abundance of a minor autolysin or a cell wall protein not detected by zymography. However, comparison of the extracellular proteome of WT and *yoeB* mutant cells by 2D-PAGE also failed to reveal any obvious differences (H. Antelmann, personal

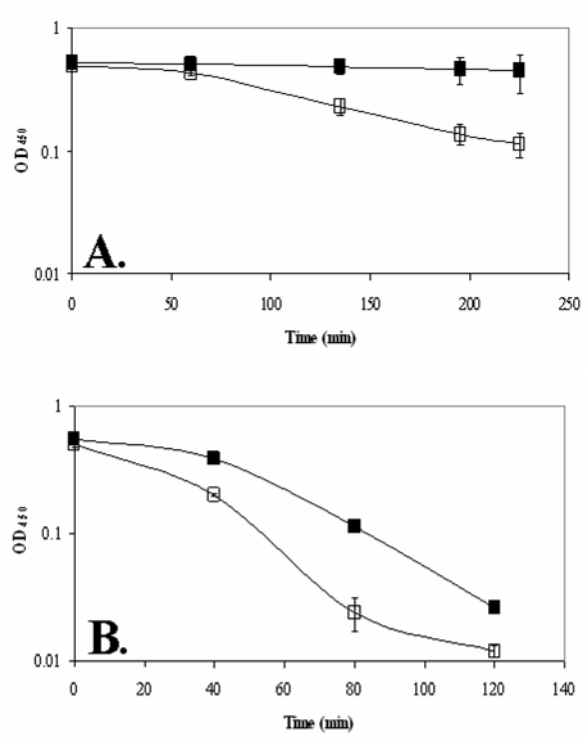

Figure 2.4. The *yoeB* mutant demonstrates an increased susceptibility for autolysis independent of treatment with lysozyme. Early log-phase (OD_{600} ~0.3) cultures of both wild-type and the *yoeB* mutants were resuspended in 10mM Tris-HCl (pH 8.0), without addition of lysozyme (A); or, with addition of 2μg/ml lysozyme (B). Lysis was monitored by a drop in OD_{450}. Wild-type (filled squares) and *yoeB* mutant (open squares).

communication). In summary, these results suggest that the complement of autolysins is not detectably altered in the *yoeB* mutant strain.

Together, these results suggest that neither the chemical composition or cross-linking of the PG nor the composition or potential activity of autolysins are grossly affected by YoeB. Therefore, I am led to a model in which YoeB functions at the post-translational level as an inhibitor of one or more autolysins. This inhibition is apparently ineffective under conditions of sodium azide treatment (leading to strong activation of autolytic activity) or when autolysins are fractioned by SDS-PAGE and analyzed by zymography, but is clearly of biological relevance.

***yoeB* is induced by cell-envelope targeting antibiotics.** Previous transcriptome analyses have demonstrated that *yoeB* is strongly induced by vancomycin (7) and several other cell wall- and membrane-targeting antibiotics such as cycloserine, oxacillin, fosfomycin, ristocetin, and triclosan (16) (summarized in Table 2.2). Using a combination of β-galactosidase and slot blot analyses, I have confirmed induction of *yoeB* under many of these stress conditions. Treatment with the cell wall biosynthesis inhibitors vancomycin, D-cycloserine, oxacillin, and fosfomycin resulted in a ~3-4 fold induction of the P_{yoeB}-*lacZ* reporter fusion (Fig. 2.5B) and vancomycin induction was also observed using slot-blot analysis (Fig. 2.5A). The highest level of induction was obtained by treatment with triclosan (~7 fold; Fig. 2.5B), while treatment with the membrane-active compounds nigericin and monensin resulted in lower levels of induction (~2 fold; Fig. 2.5B), consistent with the induction levels noted in a previous transcriptome study (16) (Table 2.2). Although generally considered to be a specific inhibitor of fatty acid biosynthesis, triclosan also acts non-specifically as a membrane-active compound (10, 13). Using slot blot analyses (Fig. 2.5A), I was also able to confirm induction of *yoeB* by diamide (2 fold) and the protein biosynthesis inhibitor tetracycline (~2 fold), although I could not detect induction using β-galactosidase

Table 2.2. Reported induction of *yoeB* under various stress conditions

Treatment	Fold change*	Reference
Cell wall biosynthesis		
Vancomycin		
(2 μg/ml)	19 (10 min)	(7)
(0.5 μg/ml)	7.7 (40 min)	(16)
Cycloserine (16 μg/ml)	15 (40 min)	(16)
Oxacillin (0.25 μg/ml)	7 (40 min)	(16)
Ristocetin (0.5 μg/ml)	7.8 (40 min)	(16)
Fosfomycin (256 μg/ml)	10 (80 min)	(16)
Membrane active compunds		
Monensin (0.125 μg/ml)	59 (40 min)	(16)
Polymyxin B (64 μg/ml)	4.9 (40 min)	(16)
Nigericin (0.008 μg/ml)	3.8 (40 min)	(16)
LL-37	9.1 (20 min)	(34)
Fatty acid biosynthesis		
Triclosan (1 μg/ml)	31 (10 min)	(16)
Cerulenin (4 μg/ml)	8.6 (10 min)	(16)
Protein biosnythesis		
Tetracycline (0.5 μg/ml)	5.1 (40 min)	(16)
Oxidants		
Diamide (1 mM)	65 (50 min)	(22)
Paraquat (100 μM)	13 (10 min)	(31)
Arsenate (10 μM)	~65 (3 min)	(29)

* In all cases the fold change noted is the highest for the conditions tested.

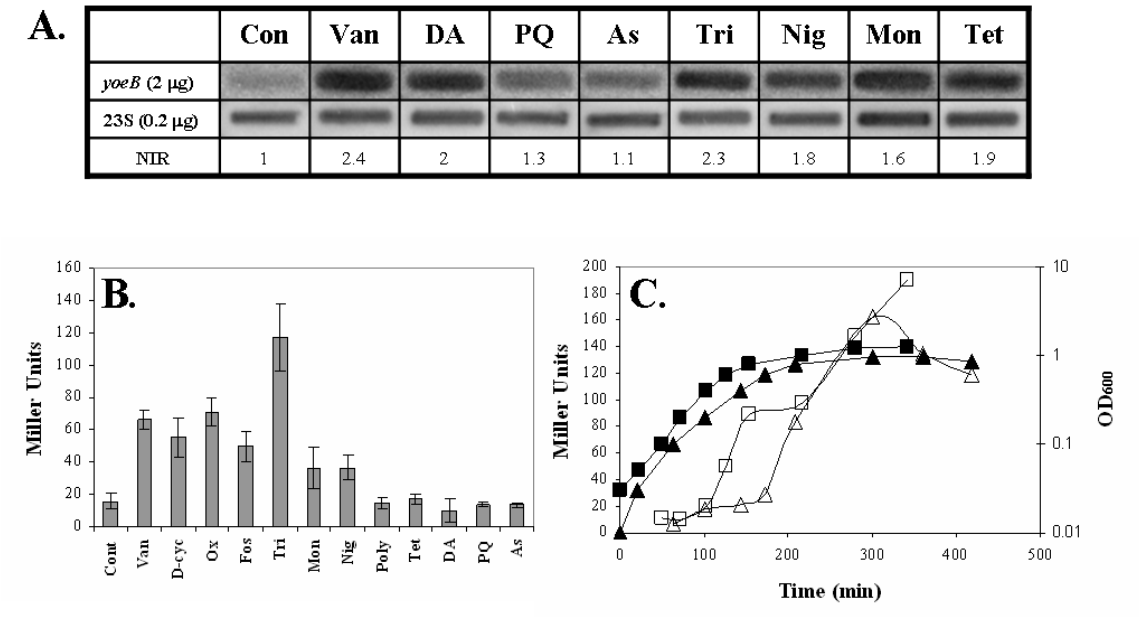

Figure 2.5. *yoeB* is induced by cell-envelope targeting antibiotics. Induction of P_{yoeB} compared to untreated control cells (Con) by Van (vancomycin 2.5 μg/ml), D-cyc (D-cycloserine 100 μg/ml), Ox (oxacillin 2.5 μg/ml), Fos (fosfomycin 50 μg/ml), Tri (triclosan 1 μg/ml), Mon (monensin 0.125 μg/ml), Nig (nigericin 0.008 μg/ml), Poly (polymyxin 64 μg/ml), Tet (tetracycline 0.5 μg/ml), DA (diamide 1 mM), PQ (paraquat 100 mM), and As (arsenate 10 μM) was measured using (A) RNA slot blot analyses and (B) β-galactosidase assays. RNA slot blot results are representative of three independent experiments. Normalized induction ratios (NIR) were derived using the signal measured for the 23S probe. β-galactosidase results are the average (± SD) of at least four independent cultures. (C) Growth phase regulation of P_{yoeB}-*lacZ* (measured using a -514 promoter fusion; see Fig. 2.6A) was monitored in either LB (squares) or DSM (triangles). Growth was determined by absorbance at 600 nm (filled symbols, axis on right) and β-galactosidase activity was measured as Miller units (open symbols, axis on left). Results shown are representative of three independent experiments.

assays (Fig. 2.5B). In some cases, the induction observed in DNA microarray studies was not reproducible under our conditions. For example, I was unable to confirm the observed *yoeB* induction by paraquat (31) or arsenate (29) (Fig. 2.5A and 2.5B). The lack of induction by paraquat or arsenate (Fig. 2.5B) was confirmed at the mRNA level (Fig. 2.5A).

Many of the enzymes involved in cell wall biosynthesis and turnover are regulated during growth transitions. To examine the dependence of *yoeB* expression on growth phase, cells were grown in either LB or DSM media and the activity of P_{yoeB}-*lacZ* was examined as a function of growth (Fig. 2.5C). Expression was low during vegetative growth and increased upon entry into transition/stationary phase. This suggests that YoeB may normally function during transition phase, perhaps to stabilize the cell wall against autolysis in response to energy depletion.

Dissection of the *yoeB* promoter region. To define regulatory elements involved in *yoeB* expression, I mapped the +1 site of transcription using 5' RACE-PCR and constructed a nested series of transcriptional fusions (Fig. 2.6A). β-galactosidase activity was measured in the presence and absence of 2 μg/ml vancomycin (Fig. 2.6B). A region of 68 bp upstream of the +1 of transcription was sufficient for maximal induction levels. Although activity of the –42 fusion was diminished (14), induction was still evident. As expected, the –22 fusion did not show any activity. To confirm that the same minimal promoter region could mediate induction by 10 mM diamide, slot blot analyses were performed (Fig. 2.6C). Comparable induction levels were observed for fusions -514, -68 and +18, confirming that the minimal promoter and regulatory elements are within the –42 to +18 region. Since most positive-acting transcription factors bind upstream of the -35 element, this result is most consistent with the hypothesis that *yoeB* is under negative control (see Discussion). Indeed, inspection of the promoter and regulatory region reveals the presence of a candidate

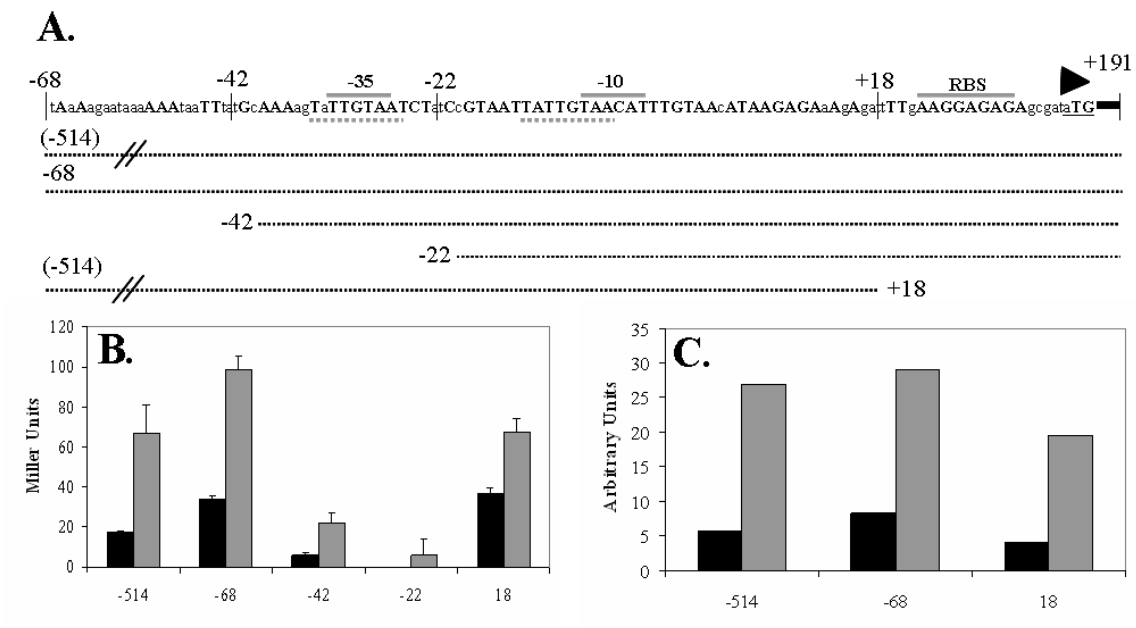

Figure 2.6. Dissection of the *yoeB* promoter region. (A**)** Schematic of *yoeB* promoter region. Capital letters designate bases that are identical in *B. subtilis* 168, *B. licheniformis* 14580, and *B. amyloliquefaciens* FZB45. Conserved -35, -10 promoter elements and the RBS are designated by grey overlines and the start of translation by a triangle, dashed underlines designate the conserved 8 bp direct repeat. Dotted lines represent promoter fusions. (B) β-galactosidase assay for induction of various promoter fusions by vancomycin (2 μg/ml) (Grey) compared to uninduced controls (Black). Results are representative of two independent experiments, and are the average of two independent samples. (C) Graphic representation of RNA slot blot analysis for expression of P_{yoeB}-*lacZ*. RNA samples extracted from strains containing the different promoter fusions either uninduced or induced by 10 mM diamide were applied to the blot. The blot was hybridized with a probe for *lacZ*, and the resulting signals were quantified with the ImageQuant data analysis software. Results are representative of two independent experiments.

regulator binding site (the 8 bp direct repeat: TATTGTAA-N_{12}-TATTGTAA) that is also conserved in the promoter regions of the *yoeB* homologs in *B. licheniformis* and *B. amyloliquefaciens* (Fig. 2.6A).

5. Discussion

Bacteria must develop mechanisms for dealing with a variety of stresses. The genes induced by a particular stress define a stimulon, and often include genes controlled by several different regulatory proteins or pathways (regulons). Transcriptome analysis of the vancomycin stimulon revealed that *yoeB* is one of the most highly induced genes (~19 fold). As summarized here (Table 2.2), *yoeB* is also induced by several other inhibitors of cell-wall biosynthesis (including D-cycloserine, fosfomycin, and oxacillin), lipid-targeting antibiotics such as triclosan and membrane active compounds (16), and disulfide stress (22). I have confirmed these results here, and additionally provide evidence that *yoeB* is induced upon entry into stationary phase (Fig. 2.5).

YoeB is a 181 aa protein with a predicted amino-terminal signal sequence. Here, I demonstrate that YoeB co-fractionates with the cell wall (Fig. 2.3). Interestingly, YoeB was recently detected as a cell wall protein induced by 1% acidic phytate (YoeB had not been detected in any prior proteome analyses), a condition that results in apparently increased levels of several autolysins (1). The only known homologs of YoeB are a hypothetical protein 01880 from *B. amyloliquefaciens* (77% identity and 81% similarity) and YoeB from *B. licheniformis* (65% identity and 78% similarity) (Fig. 2.7).

It is intriguing to note that YoeB has a low level of identity (25% identity, 47% similarity) with an ~80 aa region of MecA1 from *Staphylococcus sciuri* (Fig. 2.7). The corresponding *mecA1* gene is thought to be the ancestor of the *mecA* methicillin

Figure 2.7. Alignment of YoeB from *B. subtilis* (Bsu), *B. amyloliquefaciens* (Bam) and *B. licheniformis* (Bli). The predicted signal sequence cleavage site is after Ala23 as designated by the black triangle. Boxed sequences are an alignment of partial sequences from *S. sciurii* (Ssc) PBP2a (aa 73-131 of 665), *S. sciurii* MecA1 (aa 61-131 of 666) and *B. subtilis* (Bsu) PbpC (aa 64-140 of 668). Black shading (non-boxed sequences): conserved in all three YoeB proteins. Black shading (boxed sequences): conserved in all six proteins. Dark grey shading (non-boxed sequences): conserved in two out of three YoeB proteins. Light grey shading (boxed sequences): conserved in at least four of the six proteins. Alignments performed using ClustalW and shaded using Genedoc program (http://www.psc.edu/biomed/genedoc).

resistance gene in *Staphylococcus aureus* encoding PBP2a (44). PBP2a has a low affinity for β-lactam antibiotics, and therefore allows continued cell wall synthesis in the presence of otherwise lethal levels of β-lactams (8). Despite the similarity to MecA proteins, YoeB is unlikely to function as a PBP: the region of homology between YoeB and PBP2a corresponds to an N-terminal extension found in high molecular mass PBPs of subclass B1 hypothesized to play a non-catalytic, structural role (24). In *B. subtilis*, PBP3 (PbpC) is also a member of this class and also has weak similarity to YoeB in this region.

The most dramatic phenotype of a *yoeB* null mutant is an increased rate of lysis in response to treatment with cell wall antibiotics that target several different steps of the PG biosynthetic pathway. Direct measurements of viable cell counts demonstrates that this increased rate of lysis is correlated with an increased rate of cell death in D-cycloserine treated cultures. An increased rate of autolysis could be explained by an imbalance in autolysin composition or activity and/or an alteration in the cell wall structure. I currently favor a model in which YoeB modulates the activity of autolysins. Evidence for this model includes the localization of YoeB to the cell wall and the altered rates of cellular autolysis observed in both antibiotic-treated and nutrient depleted cells. It is formally possible that YoeB could bind to PG, and thereby impede autolysin action. However, the low abundance of YoeB (~50-100 molecules /cell) makes this model less attractive.

B. subtilis encodes as many as 35 different proteins known or postulated to have PG hydrolase activity (40). It is currently unclear how YoeB affects autolysin activity, and the large number of autolysins makes identification of a specific target challenging. An increased rate of autolysis was still observed even when the effect of *yoeB* was tested in a *sigD* mutant background in which the major vegetative autolysins (LytC and LytD) are present at greatly reduced levels (data not shown). However, cell

lysis in response to the metabolic poison sodium azide was unaffected in a *yoeB* mutant (Fig. 2.2B), suggesting that under conditions which strongly activate autolytic activity there was no gross difference in either autolysin levels or activity. The levels of autolysins (and other cell wall binding proteins) extracted from WT and *yoeB* mutant cells were indistinguishable by SDS-PAGE, 2D-gel electrophoresis, zymographic analysis, and autolysin activity assays (data not shown and H. Antelmann, personal communication). Nor were there any obvious differences in PG structure (D. Popham, personal communication). These results are most consistent with a model in which YoeB moderates the activity of one or more autolysins, presumably by protein-protein interaction.

Promoter dissection experiments demonstrate that the minimal regulatory region for *yoeB* is included within the region from –42 to +18. This region contains recognition elements for the σ^A holoenzyme together with a 8 bp direct repeat element conserved in the two orthologous genes from *B. licheniformis* and *B. amyloliquefaciens* (Fig. 2.6A). Notably, the genes are not found in the same genetic context in these organisms. In *B. subtilis* and *B. amyloliquefaciens, yoeB* is monocistronic and divergently transcribed from *yoeA*, which encodes a protein similar to a Na^+ driven multidrug efflux pump in *B. cereus* (58% identity). In *B. licheniformis*, *yoeB* is divergent from BLi01309, a putative cell wall-binding protein containing a LysM domain found in enzymes involved in bacterial cell wall degradation. BLi01309 is homologous to YocH in *B. subtilis*, a putative autolysin under the control of the YycFG TCS (15). The proximity to *yocH* in *B. licheniformis* led me to hypothesize that YoeB might regulate YocH activity specifically. However, a *yoeB yocH* double mutant in *B. subtilis* is indistinguishable from the *yoeB* single mutant in the rate of lysis in response to antibiotic treatment (data not shown).

My initial efforts to identify the negative regulator(s) of *yoeB* were unsuccessful. Selection for mini-Tn*10* transposon insertions that led to constitutive expression of a *yoeB-cat-lacZ* transcriptional fusion did not identify any candidates, nor did biochemical experiments using the *yoeB* regulatory region to affinity purify DNA-binding proteins. However, recent work from K. Devine's laboratory (personal communication) has identified *yoeB* as a direct target for negative regulation by YycF, the response regulator of the essential YycFG TCS. This is entirely consistent with my promoter dissection studies and there is clear similarity between the proposed YycF consensus site (15) and the conserved repeat motifs noted above in the *yoeB* regulatory region. The essential nature of the *yycFG* TCS may also account for my inability to identify this system using Tn*10* mutagenesis.

The YycFG TCS functions to maintain cell wall homeostasis by positively regulating the expression of cell division (*ftsZA*) and wall synthesis functions (autolysins) (15). Similarly, the orthologous system in *Streptococcus pneumoniae* activates expression of the essential murine hydrolase PcsB (32). I suggest that activation of the *B. subtilis* YycFG system during growth both enables autolysin expression and cell wall expansion while repressing the expression of YoeB, shown here to inhibit autolytic activity. Conversely, upon nutrient depletion or exposure to antibiotics that inhibit cell wall synthesis, a decrease in active YycF will lead to a reduced synthesis of autolysins and a concomitant increase in expression of YoeB.

In summary, my studies demonstrate that YoeB is a cell wall-associated protein that decreases the rate of lysis in response to cell-wall targeting antibiotics and upon nutrient depletion. The lack of apparent changes in overall autolytic activity or PG structure leads me to prefer a model in which YoeB directly interacts with one or more autolysins to down-regulate their activity. In light of the recent findings linking *yoeB* regulation to YycFG, one or more of the autolysins controlled by this TCS are

likely candidates, however it does not seem that YoeB is regulating a single autolysin since double mutants of *yoeB* and various autolysins under the control of the YycFG TCS did not decrease the rate of lysis to a level comparable to that seen with WT cells (data not shown). However, recent data has suggested that YoeB binds to three autolysins: LytF, LytE and CwlS, and completely inhibits their activity (38). The loss of this control mechanism in *yoeB* mutants leads to a decrease in the structural integrity of the cell wall under conditions of impaired cell wall synthesis or in response to growth transitions.

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CHAPTER THREE

A GntR family transcriptional regulator in *Bacillus subtilis* modulates resistance to cell wall-targeting antibiotics

1. Introduction

Antibiotic efflux pumps constitute a widespread and basic resistance determinant in bacteria, making up on average more than 10% of the transporters in an organism (17). These pumps are of great interest due to their broad substrate range, as well as the possibility that induction of pump expression by a certain substrate will result in resistance to other substrates (cross-resistance) (26). One of the major classes of these transporters is the ATP-binding cassette (ABC) transporter family, shown to effectively efflux structurally dissimilar antibiotic compounds (17, 24). A typical ABC transporter is usually encoded by genes in the same operon, and is comprised of two hydrophobic membrane-spanning domains (MSD), and two hydrophilic nucleotide-binding domains (NBD) localized on the cytoplasmic side of the membrane, which provide the energy for the transport process (19).

Although constitutively expressed multidrug ABC transporters have been reported (22), they are usually subject to various mechanisms of transcriptional regulation. An adjacent transcriptional regulator can act by binding directly to the effector molecule, leading to expression of the transporter as exemplified by the *Lactococcus lactis* LmrR transcriptional repressor that regulates the expression of the LmrCD multidrug ABC transporter (1). LmrR is thought to bind to toxic compounds that have permeated the cell membrane leading to a conformational change and subsequent derepression of *lmrCD* (1). However, global regulators responding to different sets of signals can also affect transcription of these systems. In *Escherichia coli*, inactivation of the MarR regulator leads to production of the MarA

transcriptional activator, which can then activate the transcription of the *acrAB-tolC* transport complex (9).

One approach to characterizing the regulatory networks involved in stress responses is to study transcriptional expression profiles in response to varying compounds. Previously we have investigated the transcriptional responses of *B. subtilis* to treatment with different cell wall active antibiotics (6, 14). Vancomycin led to the rapid induction (3 min.) of ~100 genes, including the regulons controlled by the extracytoplasmic function (ECF) σ factors, σ^W and σ^M (6). Among the genes induced by vancomycin, but not known to be under the control of either of these ECF σ factors, were genes in the *ytrABCDEF* operon. The *ytrABCDEF* operon was proposed to encode for a putative ABC transport system involved in acetoin utilization during stationary phase and sporulation, since deletion of the operon did not affect production of acetoin, but did reduce the efficiency of acetoin reuse (27). Expression of this operon was shown to be under the control of a GntR-type repressor encoded by the first gene of the operon, *ytrA*. GntR-type regulators have previously been reported to regulate antibiotic production and morphogenesis in *Streptomyces coelicolor* (11), as well as the expression of efflux pumps affecting resistance of *Staphylococcus aureus* to quinolones and unexpectedly to β-lactams, albeit those with more lipophilic side-chains that could partition to the membrane (23).

I here demonstrate that YtrA regulates the expression of both the *ytrABCDEF* operon and an additional locus, *ywoB*, and define the regulatory elements of these promoters. In addition I show that both P_{ytrA} and P_{ywoB} are induced by cell-wall targeting antibiotics, and that *ywoBCDEF* is involved in resistance to bacitracin. Thus I propose that YtrA functions as a component of a cell envelope response mechanism involved in resistance to cell-envelope targeting compounds.

2. Materials and Methods

Bacterial strains and growth conditions. All *B. subtilis* strains used were derivatives of CU1065 (W168 *trpC2 attSPβ*). *Escherichia coli* strain DH5α was used for standard cloning procedures. Bacteria were grown in Luria-Bertani (LB) medium at 37°C with vigorous shaking. Antibiotics were added to the growth medium when appropriate: 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for *E. coli*, and 1μg/ml erythromycin plus 25 μg/ml of lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 μg/ml chloramphenicol, 100 μg/ml spectinomycin and 10 μg/ml kanamycin for *B. subtilis*. OD_{600} readings were taken on a Spectronic 21 (Milton Roy) spectrophotometer.

Sensitivity to cell wall targeting antibiotics and was measured by growing the different strains in LB medium at 37°C with vigorous shaking. Antibiotics were added at the indicated concentration during early exponential growth (OD_{600} ~ 0.25). Lysis was monitored by measuring the decrease of OD_{600} at 30 min intervals.

Construction of null mutants. Chromosomal deletions were created in the CU1065 background using long-flanking homology PCR (LFH-PCR) as described (14) with the following changes: flanking fragments were amplified using *iProof* DNA Polymerase (Bio-Rad) and the flanking fragments and antibiotic resistance marker were joined using Expand Long Template PCR System (Roche). A detailed protocol is available at:

http://www.micro.cornell.edu/faculty/helmann/supplemental%20index.htm.

Construction of the *ytrA* null mutant was created using the pMAD shuttle vector (2). Briefly, a DNA fragment containing an in-frame deletion of the *ytrA* sequence was PCR amplified using primers #3676, #3677, #3678 and #3679. this fragment was then digested with EcoRI and BamHI, and cloned into vector pMAD, which contains a constitutively expressed *bgaB* gene, as well as being replication-thermosensitive (2),

resulting in plasmid pLS39. The sequence of the inserts was verified by DNA sequencing (Cornell DNA sequencing facility). *B. subtilis* WT strain CU1065 was transformed with pLS39, plated on media containing MLS and 80 μg/ml 5-Bromo-4-choloro-3-indoxyl-beta-D-galactopyranoside (X-gal) and incubated at 30°C. Blue colonies were inoculated into LB-MLS liquid broth and grown at 40°C for 6-8 hours, and then plated (in dilution) on LB-MLS-X-gal plates and incubated at 40°C. Resulting blue colonies were inoculated into LB liquid broth and grown for 6 hours at 30°C, next for 3 hours at 40°C, and then plated (in dilution) on LB-X-gal plates and incubated at 40°C. Resulting white colonies were restreaked to LB-X-gal and LB-MLS-X-gal plates (to verify loss of MLS^r). The *ytrA* deletion was verified by PCR amplification of the region followed by DNA sequencing (Cornell DNA sequencing facility). Primer sequences used for mutant construction are detailed in Table 3.1.

Construction and analysis of transcriptional fusions. DNA fragments of the *ytrA* and *ywoB* regulatory regions were PCR amplified using primers: #3700 #3701 (P_{ytrA}) and #3734 #3735 (P_{ywoB}). The fragments were digested with EcoRI and BamHI, and cloned into vector pDG1661, containing a promoterless *lacZ* gene (10), resulting in plasmids pLS33 and pLS34, respectively. The sequence of the inserts was verified by DNA sequencing (Cornell DNA sequencing facility). *B. subtilis* strains were transformed to Cm^r with the ScaI-linearized plasmids which integrated into the *amyE* locus. For quantitative measurements of β-galactosidase activity, cells were grown in either LB medium at 37°C with vigorous shaking, and samples were collected at different OD_{600} points.

Table 3.1. Strains, plasmids and oligonucleotides used in this study

	Description	**Created / reference**
E.coli		
DH5α	ϕ80*lacZ*Δm15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^-, m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169	Lab strain
BL21(DE3)pLysS	F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm*(DE3) / pLysS	Novagen
HE5322	BL21(DE3)pLysS containing pLS36	pLS36 → BL21(DE3)pLysS
B. subtilis		
CU1065	W168 *trpC2 attSPβ*	Lab strain
HB5447	CU1065 *ytrABCDEF::kan*	LFH-PCR → CU1065
HB5448	CU1065 *ywoBCDEF::MLS*	LFH-PCR → CU1065
HB5449	CU1065 *ywoHG::spc*	LFH-PCR → CU1065
HB5450	CU1065 *ytrABCDEF::kan ywoBCDEF::MLS*	LFH-PCR → HB5447
HB5451	CU1065 *ytrABCDEF::kan ywoHG::spc*	LFH-PCR → HB5447
HB5452	CU1065 *ywoBCDEF::MLS ywoHG::spc*	LFH-PCR → HB5448
HB5453	CU1065 *amyE::*P_{ytrA}-*lacZ*	pLS33 → CU1065
HB5454	CU1065 *ytrABCDEF::kan amyE::*P_{ytrA}-*lacZ*	pLS33 → HB5447
HB5455	CU1065 *ywoBCDEF::MLS amyE::*P_{ytrA}-*lacZ*	pLS33 → HB5448
HB5456	CU1065 CU1065 *ywoHG::spc amyE::*P_{ytrA}-*lacZ*	pLS33 → HB5449
HB5457	CU1065 *amyE::*P_{ywoB}-*lacZ*	pLS34 → CU1065
HB5458	CU1065 *ytrABCDEF::kan amyE::*P_{ywoB}-*lacZ*	pLS34 → HB5447
HB5459	CU1065 *ywoBCDEF::MLS amyE::*P_{ywoB}-*lacZ*	pLS34 → HB5448
HB5460	CU1065 CU1065 *ywoHG::spc amyE::*P_{ywoB}-*lacZ*	pLS34 → HB5449
HB5485	CU1065 with in-frame markerless deletion of *ytrA*	pLS39 → CU1065
HB5486	CU1065 with in-frame markerless deletion of *ytrA* *ywoBCDEF::MLS*	LFH-PCR → HB5485
HB5486	CU1065 with in-frame markerless deletion of *ytrA* *amyE::*P_{ytrA}-*lacZ*	pLS33 → HB5485
HB5486	CU1065 with in-frame markerless deletion of *ytrA* *amyE::*P_{ywoB}-*lacZ*	pLS34 → HB5485
HB6726	CU1065 *ytrA::kan*	LFH-PCR → CU1065
Plasmids		
pDG1661	Vector for integration of *lacZ* fusions at *amyE* locus	(10)
pET-11a	Overexpression vector	Novagen
pMAD	Vector for marker free allelic replacement	(2)
pLS33	P_{ytrA}-*lacZ* in pDG1661	This work
pLS34	P_{ywoB}-*lacZ* in pDG1661	This work
pLS36	pET-11a containing a *Nde*I- *BamH*I fragment encoding for *ytrA*	This work
pLS39	pMAD containing a 1.4-kb *BamH*I-*Eco*RI fragment for in-frame deletion of *ytrA*	This work

Table 3.1. (Continued)

Primers		
#	**Name**	**Sequence***
3676	ytrA up fwd (BamHI)	cgcggatccatctccagcaatgtatgcag
3677	ytrA up rev	catttttttgcctccttcgtaaatgggtgttgagcttcttggatcgatttg
3678	ytrA do fwd	caaatcgatccaagaagctcaacacccatttacgaaggaggcaaaaaaaatg
3679	ytrA do rev (EcoRI)	ccggaattcttccacacttttggcttcagc
3700	PytrA fwd (EcoRI)	ccggaattcgattgactttgtgagtcaaagt
3701	PytrA rev (BamHI)	cgcggatccccctactttctatacgatctga
3702	ytrA up fwd (LFH)	aattcatgtgcctccttgcg
3703	ytrA up rev (kan)	CCTATCACCTCAAATGGTTCGCTGgtgttgagcttcttggatcg
3704	ytrA do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGaaaccaacgtgctgacagcg
3705	ytrA do rev (LFH)	aacttgtcctcttccttcgc
3734	PywoB fwd (EcoRI)	ccggaattctcatgtaagatttcctgacatg
3735	PywoB rev (BamHI)	cgcggatccccctcagtgtattatttgatgt
3736	ywoB up fwd (LFH)	tgtaccgtctttccgtttgcc
3737	ywoB up rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCgggaatcgacttgcgtaccg
3738	ywoB do fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCcgttagatcctgattcacctgc
3739	ywoB do rev (LFH)	ttgatgagagtggtaagctttcc
3740	ywoH up fwd (LFH)	ccgaggtcaataccaatatccc
3741	ywoH up rev (spc)	CGTTACGTTATTAGCGAGCCAGTCgatgaatgaggtgtctgttgacgta
3742	ywoG do fwd (spc)	CAATAAACCCTTGCCCTCGCTACGgctgaaggaaaagtgtctattgcag
3743	ywoG do rev (LFH)	cacagttgcaggcaatgtgg
4020	ytrA fwd (NdeI)	gtacgcatatgattcaaatcgatccaagaagctc
4021	ytrA rev (BamHI)	gcggatccatcatttttttgcctcccttcac
4153	ytrA GPS1	ttcgcaataatgatcgttgc
4154	ytrA GPS2	gtgttgagcttcttggatcg
4155	ywoB GPS1	aacagctgccaggatttcac
4156	ywoB GPS2	ccaattaccactttcgccag

* Capped letters indicate sequences complementary to antibiotic resistance genes. Underlined sequences indicate restriction sites.

To test promoter induction, cells were grown in LB until an OD_{600} ~ 0.4, and then the culture was split into aliquots, which were challenged with either vancomycin (2 μg/ml final), bacitracin (20 μg/ml final) and ramoplanin (10 μg/ml final). The cultures were returned to 37°C, and samples were collected after 30 min. β-galactosidase activity was measured according to the method of Miller (15) except that cells were lysed by the addition of lysozyme to a final concentration of 20 μg/ml followed by a 30 min incubation at 37°C.

RNA isolation and 5'-RACE. Strain HB5485 (CU1065 containing in-frame deletion of *ytrA*) was grown in LB (37°C with vigorous shaking) to mid-exponential stage (OD_{600} ~ 0.4), and RNA isolation and was performed using the RNeasy mini kit (Qiagen). RNA was subsequently DNase treated with TURBO DNA-free™ (Ambion) and precipitated overnight. The RNA was dissolved in RNAse free water and quantified using a NanoDrop spectrophotometer (Nanodrop Tech. Inc., Wilmington, DE). 2 μg of total RNA was used with the 5' random amplification of cDNA ends (RACE) kit (Invitrogen) according to the manufacturer's specifications (primers #4153, #4154, #4155, #4156). Resulting DNA fragments were sequenced at the Cornell DNA sequencing facility**.**

Hierarchical clustering analysis. Hierarchical clustering for whole genome datasets was performed using Cluster 3.0 (7). Transcriptome datasets were derived from unpublished results from our lab or a previously published study (13). The datasets represent the following treatment conditions and times: 16 μg/ml D-cycloserine (10 min), 0.25 μg/ml oxacillin (10 min), 1.0 μg/ml moenomycin (20 min), 100 μg/ml bacitracin, (5 min) 2 μg/ml vancomycin (10 min), 0.5 μg/ml ristocetin (10 min), 5 μg/ml ramoplanin (10 min), 0.03 μg/ml gramicidin A (10 min), 0.125 μg/ml monensin (10 min), 64 μg/ml polymyxin B (10 min), 64 μg/ml Triton –X- 114 (10 min), 64 μg/ml puromycin (10 min), 0.5 μg/ml tetracycline (10 min), and 4 μg/ml

chloramphenicol (10 min). The hierarchical clustering was visualized using TreeView (8).

Overproduction and purification of YtrA. YtrA was purified using *E.coli* strain HE5322; BL21(DE3)pLysS (Novagen) containing pLS36, a pET11a derivative, which places *ytrA* under the control of a T7*lac* promoter. A 1-liter culture was grown from a fresh colony of HE5322 in LB broth containing 0.5% glucose, 100 μg/ml ampicillin and 34 μg/ml chloramphenicol to enhance plasmid stability. At an OD_{600} of 0.4, isopropyl-β-D-thiogalactopyranoside (IPTG; Gold BioTechnology) was added to 1mM, and growth continued for 2 hours, at which point cells were harvested. Cell pellets were resuspended in 10 ml resuspension buffer (40 mM Tris-HCl [pH 8.0], 1 mM EDTA, 5% [vol/vol] glycerol and 1 mM dithiothreitol [DTT]) to which 1 tablet of Complete mini, EDTA-free protease inhibitor cocktail (Roche) was added. Cell suspension was disrupted by freeze-thawing followed by pulsed sonication, and clarified by 15 min centrifugation. The resulting supernatant was applied to a heparin column. Bound proteins were eluted with a NaCl gradient (0.2 to 1M) in elution buffer (20mM Tris-HCl [pH 8.0], 1mM EDTA, 5% [vol/vol] glycerol and 1mM DTT). Samples were loaded onto a 15% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) gel to identify the fractions containing YtrA. Pooled samples containing YtrA were then loaded onto a Bio-Rad Bio-Scale^{TM} Mini Macro-Prep High S Cartridge, and proteins were eluted with a 0-1.0 M NaCl gradient in elution buffer (20mM Tris-HCl [pH 7.0], 1mM EDTA, 5% [vol/vol] glycerol and 1mM DTT). Samples were loaded onto a 15% SDS-PAGE gel to identify the fractions containing YtrA, which were then pooled and dialyzed into buffer (20mM Tris-HCl [pH 8.0], 1mM EDTA, and 1mM DTT) containing 50% glycerol before storage at –20^{o}C.

Electrophoretic mobiolity shift assay (EMSA). PCR fragments containing either P_{ytrA} (274 bp; primers #3700 #3701), P_{ywoB} (192 bp; primers #3734, #3735) and the control non-specific P_{yoeB} (106 bp) were amplified and end-labeled with T4 polynucleotide kinase (PNK; New England BioLabs) and [γ-^{32}P]ATP. Increasing amounts of YtrA (0-160 nM) were incubated at room temperature for 15 min with the labeled promoters in a total volume of 10 µl binding buffer (20 mM Tris-HCl [pH 8.0], 5% [vol/vol] glycerol, 50 mM NaCl, 50 µg/ml bovine serum albumin [BSA] and 5 µg/ml sheared salmon sperm DNA). Samples were loaded on a 6% polyacrylamide gel prepared and run in 90mM Tris-Borate buffer with 2mM EDTA. The gel was dried and imaged on a Storm 840 PhosphorImage scanner (Molecular Dynamics) after overnight exposure of a PhosphorImage screen.

3. Results and Discussion

The *ytrA* and *ywoB* promoters are induced by glycopeptide antibiotics. Previous transcriptome analyses have demonstrated that the *ytr* operon is induced by the glycopeptide antibiotic vancomycin (6) and the lipoglycodepsipeptide antibiotic ramoplanin (our unpublished data). Additional studies (13) have shown that this operon is also strongly induced by another glycopeptide antibiotic, ristocetin, but interestingly, not by other cell-wall targeting antibiotics (such as D-cycloserine, phosphomycin or oxacillin) or by antibiotics with other modes of action. This promoted the classification of P_{ytrA} as a reporter indicative of compounds with a glycopeptide mode of action (12).

To further study the expression patterns of the *ytr* operon I performed hierarchical clustering analysis using transcriptional profiles generated in our lab (ramoplanin and moenomycin) and by Hutter *et al*. (13) (Fig. 3.1). As expected, genes in the *ytr* operon clustered tightly, demonstrating strong induction by

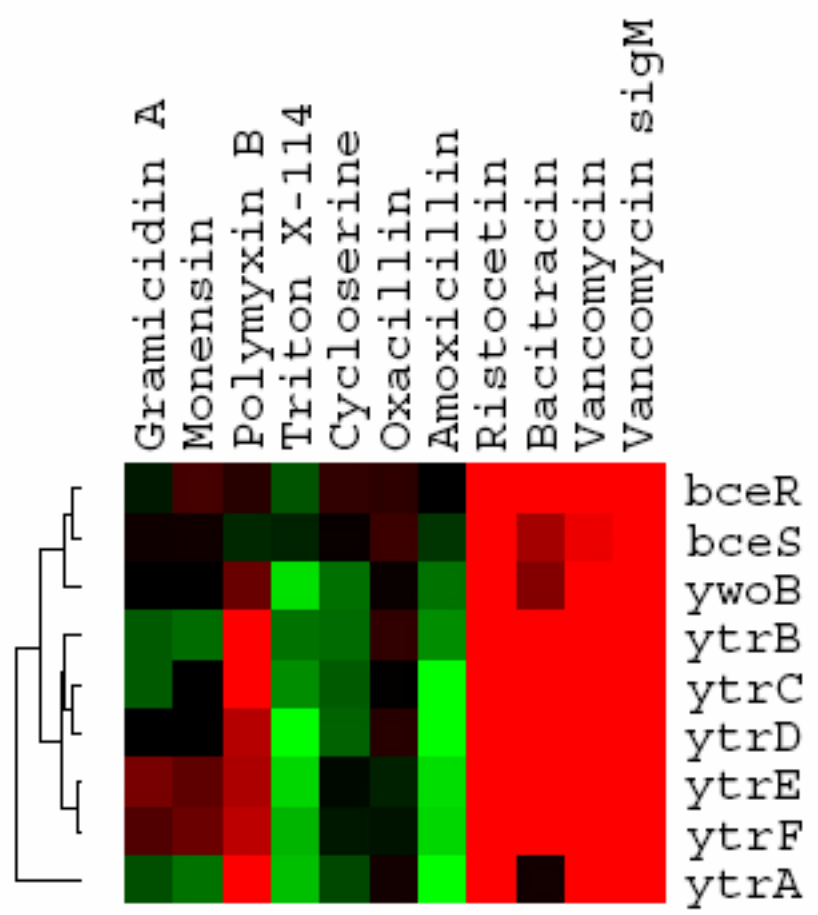
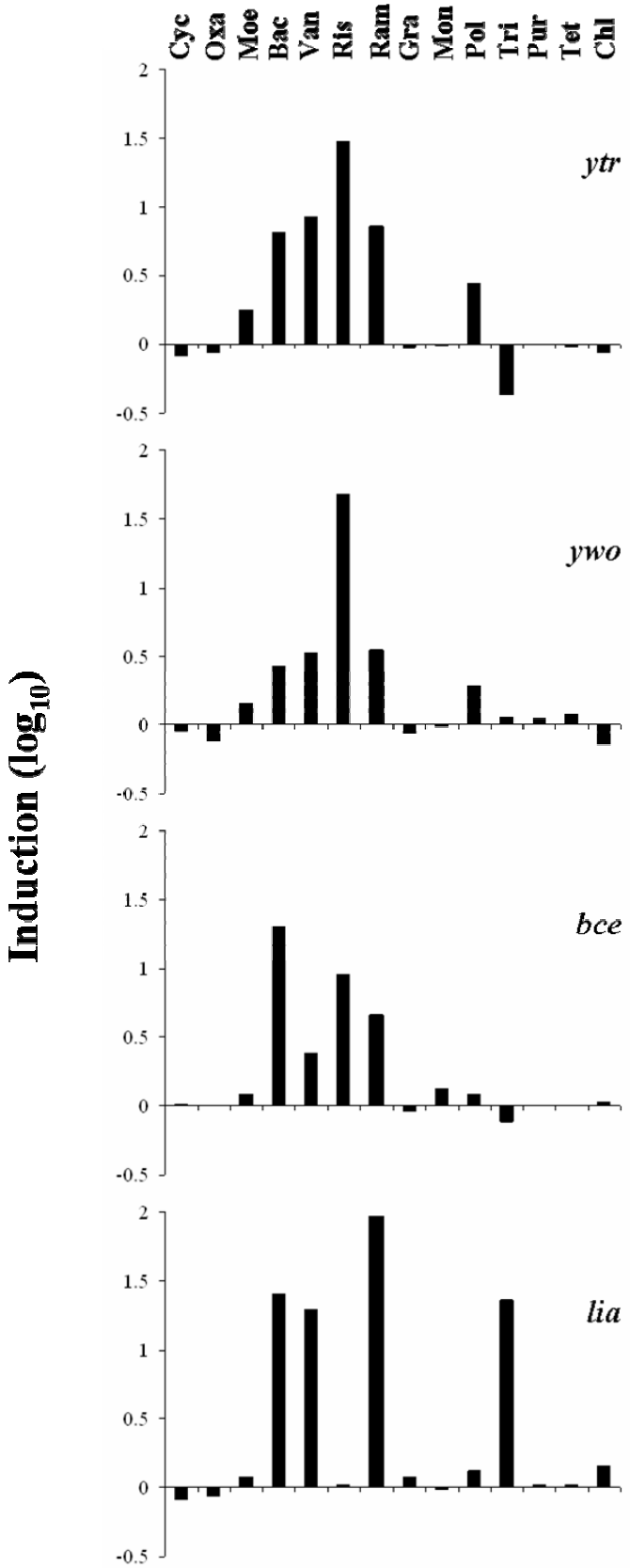


Figure 3.1. Hierarchical clustering analysis of gene expression in response to antibiotic stress. Antibiotic treatments indicated above. Gene expression data were clustered based on expression level. Red indicates induction, and green repression under the specified treatment condition.

Figure 3.2. Characteristic induction (log) profiles of the *ytr*, *ywo*, *bce* and *lia* operons. The expression level (fold induction) of all the genes in each of the operons was averaged for each of the antibiotic treatments. Datasets used include D-cycloserine (Cyc), oxacillin (Oxa), moenomycin (Moe), bacitracin (Bac), vancomycin (Van), ristocetin (Ris), ramoplanin (Ram), gramicidin A (Gra), monensin (Mon), polymyxin B (Pol), Triton –X- 114 (Tri), puromycin (Pur), tetracycline (Tet), and chloramphenicol (Chl). Transcriptome expression data were derived from unpublished results from our lab or a previously published study (13).


Induction (log10)
Cyc
Oxa
Moe
Bac
Van
Ris
Ram
Gra
Mon
Pol
Tri
Pur
Tet
Chl
ytr
ywo
bce
lia
2
1.5
1
0.5
0
-0.5

vancomycin, ristocetin and ramoplanin. Additional genes demonstrated similar specific induction profiles: *bceRS*, encoded immediately downstream of *ytrF*, and *ywoB* (Fig. 3.2). Indeed *ywoB* had been previously proposed as an additional reporter for glycopeptide antibiotic compounds (12). In comparison, the *liaIHGFSR* operon, also known to be strongly induced by cell wall-targeting antibiotics such as vancomycin (6) and bacitracin (14), demonstrates a different induction profile (Fig. 3.2), suggesting that the *ytr* and *ywo* operons might be under similar transcriptional control.

Transcriptional fusions for P_{ytrA} and P_{ywoB} were induced in response to treatment with vancomycin and ramoplanin, but not bacitracin, (Fig. 3.3), albeit to a much lower level than that observed in a *ytrA* null background, suggesting that YtrA might still be partially bound to these promoters. Although I cannot exclude that YtrA may be sensing the antibiotics directly, it would seem more likely that it is sensing a signal caused by damage to the cell wall and/or membrane, since the antibiotics inducing these promoters affect different targets and are unlikely to enter the cell due to their size.

To clarify the possible involvement of other regulators in the antibiotic induction of *ytrA*, I created a transcriptional fusion containing only a putative ECF σ^W promoter sequence located 73-bp downstream of the transcriptional start site identified (Fig 3.4). This fusion was not induced in an *rsiW* null background (data not shown), in which σ^W would be overexpresssed, indicating that its involvement in the regulation of *ytrA* is unlikely.

YtrA binds specifically to the *ytrA* and *ywoB* promoters. Yoshida *et al*. mapped the transcriptional start site and identified a σ^A-dependent promoter sequence (TTGACT-17-TATTGT) 244-bp upstream of the *ytrA* translation start site, and

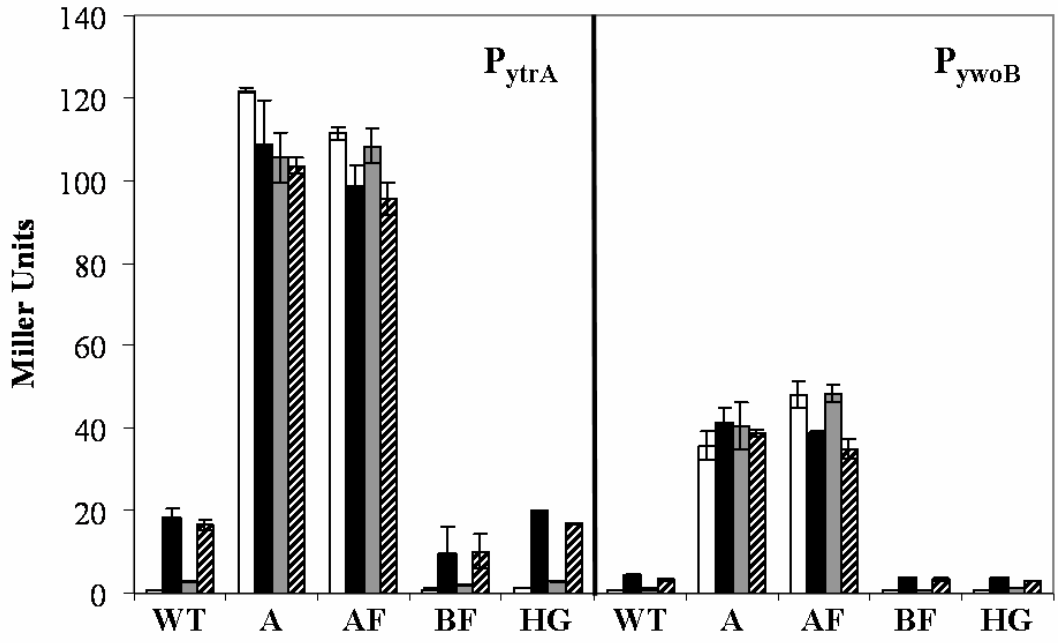


Figure 3.3. P_{ytrA} and P_{ywoB} are derepressed in a *ytrA* null background, and are induced by glycopeptide antibiotics. Induction of P_{ytrA} and P_{ywoB} was measured by β-galactosidase assays. Treatments included: untreated control (white bars), vancomycin (2 mg/ml; black bars), bacitracin (20 mg/ml; grey bars) and ramoplanin (10 mg/ ml; hatched bars). β-galactosidase results are the average (± SD) of at least four independent cultures. Labels on bottom indicate the background strain: WT; wild-type CU1065, A; *ytrA* in-frame deletion, AF; *ytrABCDEF::kan*, BF; *ywoBCDEF::MLS* and HG; *ywoHG::spc.*

proposed a 31-bp inverted repeat overlapping the transcriptional start site as a possible binding site for YtrA (Fig. 3.4). I used 5' RACE-PCR to map the *ywoB* start of transcription (33-bp upstream of the translation start site), and identified a σ^A-dependent promoter sequence, which contains a shortened spacer sequence (TTGACA-15-TATACT; Fig. 3.4). Further inspection of this promoter region revealed an inverted repeat sequence similar to that in P_{ytrA} overlapping the transcriptional start site (Fig. 3.4-3.5).

In order to establish whether YtrA acts as a transcriptional regulator of both the *ytr* and *ywo* operons I constructed transcriptional fusions for P_{ytrA} and P_{ywoB} and introduced them into WT, *ytrA* in-frame deletion, *ytrABCDEF::kan*, *ywoBCDEF::MLS* and *ywoGH::spc* backgrounds. Both P_{ytrA} and P_{ywoB} were completely derepressed in a *ytrA* in-frame deletion or *ytrABCDEF::kan* background (Fig 3.3), suggesting that YtrA acts as a transcriptional repressor of both these promoters.

To determine whether additional loci are controlled by YtrA I conducted a pattern search using the P_{ytrA} inverted-repeat motif identified the binding site in the *ywoB* promoter in addition to somewhat less conserved motifs containing varying spacing upstream of four additional candidate promoters (Fig. 3.5). Although these genes do not cluster with either *ytr* or *ywo* genes in our hierarchical clustering analysis, it does not rule out the possibility that these genes might be regulated by YtrA or other GntR-type regulators.

To address whether the regulatory effect observed *in vivo* involved a direct interaction of YtrA with the *ytrA* and *ywoB* promoters, YtrA was overproduced in *E. coli* under the control of T7 RNA polymerase and purified to ~95%. Purified YtrA bound and shifted the promoters of both *ytrA* and *ywoB* at 5nM (Fig. 3.6), but did not

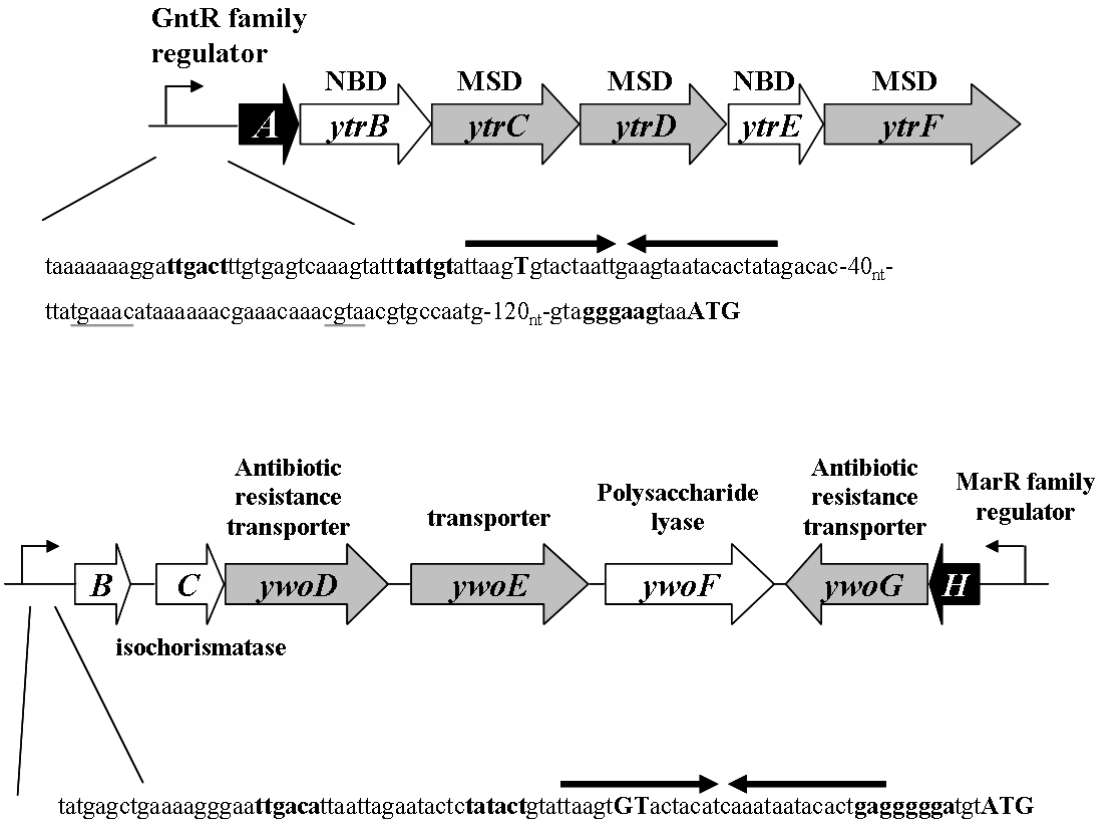


Figure 3.4. Arrangement of *ytrABCDEF* and *ywoBCDEFGH* operons. The schematic is drawn to scale and predicted functions are shown above genes. NBD; nucleotide-binding domain. MSD; membrane spanning domain. Promoter sequences are detailed below schematic. -35, -10 and RBS elements are in bold, +1 of transcription and translation are in capital letters. The proposed σ^W promoter elements are indicated by grey underline. YtrA binding site is indicated by black arrows.

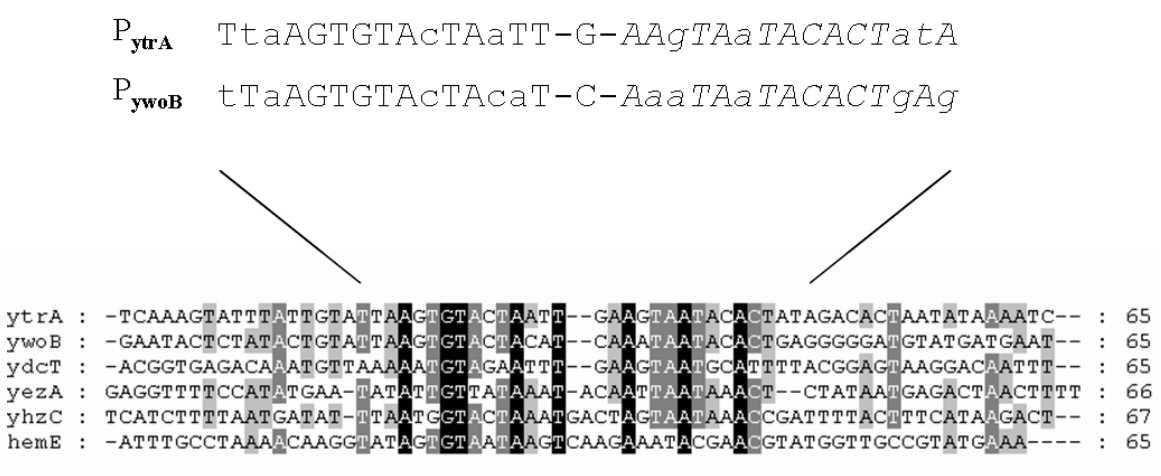


Figure 3.5. Alignment of Putative YtrA binding site. A binding site consensus search was performed using the SubtiList graphical interface (http://genolist.pasteur.fr/SubtiList/) for the inverted repeat motif found in the P_{ytrA} promoter. Alignment was performed using ClustalW and shaded using Genedoc program (http://www.psc.edu/biomed/genedoc). The conserved motif is detailed above the alignment. Italicized letters represent the second half of the inverted repeat sequence, capital letters represent conserved nucleotides within the binding site.

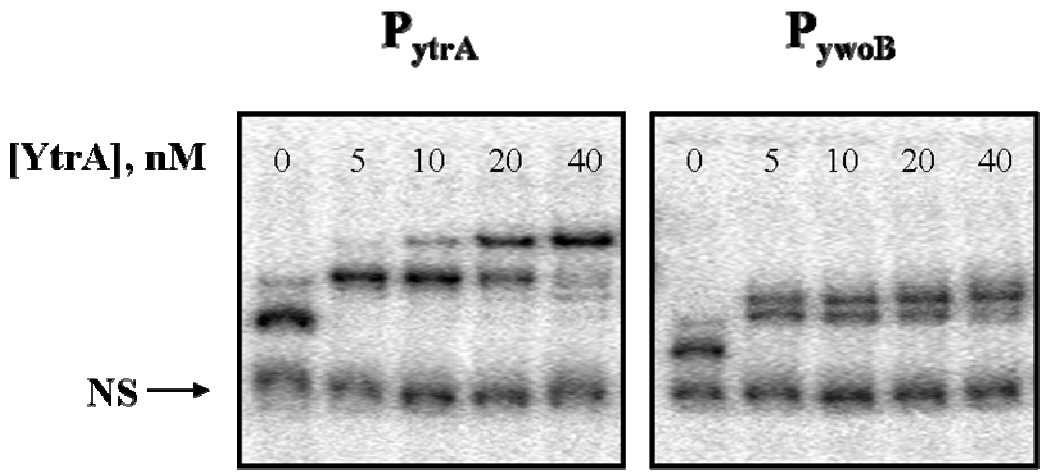


Figure 3.6. Specific binding of YtrA to the *ytrA* and *ywoB* regulatory regions. Promoter region were end-labled using PNK and [γ-32P]ATP. Constant amounts of the specific *ytrA* or *ywoB* promoters were incubated with increasing amounts of YtrA protein and constant amounts of the non-specific *yoeB* promoter (NS).

bind to the control DNA fragment. Interestingly, increasing the concentration of YtrA (20nM for P_{ytrA} and 40nM for P_{ywoB}) resulted in a super shift of the promoter fragments, suggesting the possibility of multiple binding sites, and that no additional co-factors are required for YtrA binding.

***ywoBCDEF* contributes to reduced lysis in response to bacitraicin.** To test the involvement of the *ytr* and *ywo* operons in antibiotic resistance I constructed null mutants of the *ytrABCDEF* and *ywoBCDEF* operons by allelic replacement with a kanamycin or MLS resistance gene using LFH-PCR (25). In addition an in-frame markerless deletion of *ytrA* was constructed using the pMAD vector (2). Double mutants were constructed by further rounds of transformation. I next tested the sensitivity of these mutants to different classes of cell wall antibiotics.

Surprisingly, none of the strains tested demonstrated increased or reduced sensitivity to vancomycin. However, exposure of logarithmically growing cultures of either the *ytrABCDEF::kan* or the *ytrA* in-frame deletion to the Lipid-II inhibitor bacitracin resulted in reduced lysis (as measured by a drop in OD_{600}) compared to that of the WT strain (Fig. 3.7). Intrigued by this result, I wished to ascertain whether this was due to the derepression of the *ywoBCDEF* operon. Indeed, treatment of either the *ytrABCDEF::kan ywoBCDEF::MLS* or the *ytrA* in-frame *ywoBCDEF::MLS* with bacitracin resulted in WT levels lysis (Fig. 3.7). This reduced lysis in response to bacitracin due to overproduction of *ywoBCDEF* is especially intriguing due to the close proximity of *ytr* and *ywo* operons to the specific *B. subtilis* bacitracin resistance mechanisms *bceRSAB* (formerly *ytsABCD*) and *bcrC* (formerly *ywoA*). *bcrC* encodes for a undecaprenyl pyrophosphate phosphatase (3, 5), a secondary bacitracin-resistance determinant, controlled by the extracytoplasmic function (ECF) sigma factors, σ^X and σ^M (5). *bceRSAB* are situated immediately downstream of the *ytrABCDEF* operon. *bceRS encode* for a two-component regulatory system which

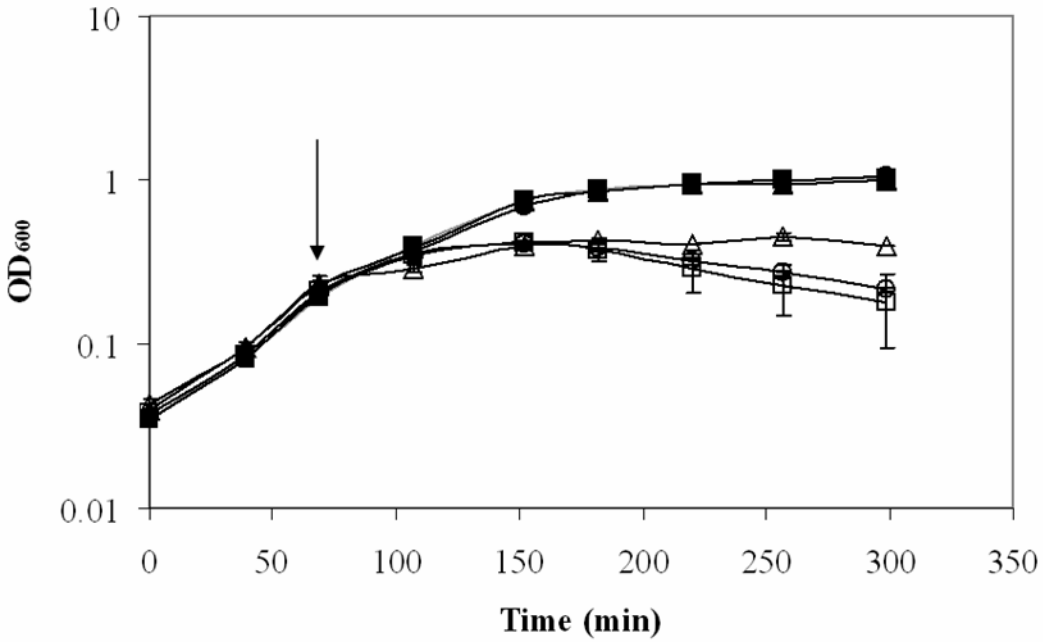


Figure 3.7. The *ywoBCDEF* operon contributes to reduced lysis in response to bacitracin. *B. subtilis* wild-type (squares) and the isogenic *ytrABCDEF* null mutant (triangles) and *ytrABCDEF ywoBCDEF* double null mutant (circles) were grown in LB were either untreated (filled symbols) or treated at OD_{600} ~ 0.25 (unfilled symbols) with 200 mg/ml bacitracin. Arrow indicates the time at which bacitracin was added. Results are representative of three independent experiments (average ± SD).

controls the expression of the BceAB ATP-binding cassette (ABC) transporter (16). The BceAB transporter system is responsible for exporting the majority of bacitracin in the cell, and inactivation of it results in a drastic decrease in resistance (4). However, an alternative model suggests that BceAB acts as a bacitracin importer, and that BceS is induced during bacitracin import (20). The tight clustering of the *ytr* and *bce* operons could perhaps indicate a regulatory connection between the two systems, or might be the result of readthrough off P_{ytrA}.

In silico analysis of the YtrA regulon. The *ytr* operon arrangement (Fig. 3.4) is conserved in the *B. subtilis* close relatives *Bacillus licheniformis* (Bli), *Bacillus amyloliquefaciens* (Bam), as well as the more distant *Bacillus pumilus* (Bpu). In contrast, the operon structure of *ywoBCDEF* is not conserved. BLAST searches reveal that there is no apparent homolog of YwoB in either Bli or Bam, and only in Bam do *ywoCDE* and *ywoGH* appear in the same genetic context.

YtrA is a representative of a subfamily of GntR regulators that possess a reduced C-terminal domain, and are predicted to bind an inverted-repeat operator sequence in an anti-parallel dimer configuration (21). YtrA regulates the *ytrABCDEF* operon proposed to encode for an ABC transport system involved in acetoin utilization but not its production (27).

Based in part on the observation that *ytrABCDEF* is expressed as a single transcript during stationary phase, Yoshida *et al*. suggested a model whereby YtrB and YtrE are situated in the inner side of the cytoplasmic membrane and act as the nucleotide-binding domain (NBD) providing energy for transport through ATP hydrolysis, YtrC and YtrD are the membrane-spanning domains (MSD) comprising the permease, while YtrF is proposed to act as the periplasmic substrate-binding lipoprotein. Together these proteins form an integrated transport system for the import of acetoin, although acetoin was not found to induce its expression (27).

However, an analysis of *B. subtilis* ABC transport systems (19) classified YtrBCD and YtrEF as separate ABC export systems, containing two (YtrCD) or one (YtrF) MSD. In addition, YtrF is predicted to be a cytoplasmic membrane integral membrane protein containing at least three transmembrane domains.

Further analysis of the *B. subtilis* genome reveal another GntR-type regulator exhibiting a shortened C-terminal domain, *yhcF*. *yhcF* is located upstream of *yhcGHI*. YhcG and YhcH have been classified as ATPases related to the NBDs of antibiotic resistance systems, with YhcG clustering in the same sub-group as YtrB (21). *yhcI* encodes for a permease similar to a lantibiotic immunity protein in Bli, and a bacitracin transport permease in *Bacillus thuringiensis*. Interestingly, the *yhcGHI* operon was strongly induced by the cationic antimicrobial peptide LL-37 (18)

Concluding remarks. The proximity of these regulators to ABC transporters, clustering and induction by antibiotics suggest they form a new group of transcriptional regulators controlling the expression of multi-drug efflux systems.

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CHAPTER FOUR

Phenotypic and transcriptomic characterization of *Bacillus subtilis* mutants with grossly altered membrane composition

1. Summary

The *Bacillus subtilis* membrane contains diacylglycerol-based lipids with at least five distinct headgroups that impart distinct physical and chemical properties to the lipid bilayer. Here, I describe the phenotypic characterization of mutant strains lacking one or more of the following lipids: glycolipids (*ugtP* mutants), phosphatidylethanolamine (*pssA* and *psd* mutants), lysylphosphatidylglycerol (*mprF*), and cardiolipin (*ywnE* and *ywjE*). Alterations of membrane lipid headgroup composition are generally well tolerated by the cell and even severe alterations lead to only modest effects on growth proficiency. Mutants with decreased levels of positively charged lipids display an increased sensitivity to a cationic antimicrobial compounds and cells lacking glycolipids are sensitive to the lantibiotic sublancin and are defective in swarming motility. A quadruple mutant strain (*ugtP pssA mprF ywnE*), with a membrane comprised predominantly of phosphatidylglycerol, is viable and grows at near wild-type rates, although it forms long, coiled filaments. Transcriptome comparisons identify numerous regulons with altered expression in *ugtP* mutant cells, the *pssA mprF ywnE* triple mutant, and the *uptP pssA mprF ywnE* quadruple mutant. These effects include a general decrease in expression of the SigD and FapR regulons, and increased expression of cell envelope stress responses mediated by σ^M and the YvrGHb two-component system.

2. Introduction

The cytoplasmic membrane forms an essential permeability barrier and is a defining feature of life. In most bacteria, the membrane is a lipid bilayer composed of fatty esters linked to *sn*-glycerol-3-phosphate (G3P) (45). These complex lipids vary not only in the length and modifications of the acylated fatty acid groups but also in the composition of their headgroups, which differ significantly in charge and propensity to form non-bilayer structures within the membrane. The membrane of the soil bacterium *Bacillus subtilis* is a complex structure comprised mainly of the anionic phospholipid phosphatidylglycerol (PhG) and the zwitterionic phosphatidylethanolamine (PhE). Other components include a relatively large amount (~30%) of neutral glycolipids (GL), a variable amount of positively charged lysylphosphatidylglycerol (LPG), and a small amount of anionic cardiolipin (CL) (see Fig. 1.3).

The complex composition of the membrane is presumed to be important since the physical properties of membrane lipids influence many cell processes including division (42), DNA replication (21), and protein transport (12). For example, the anionic lipids PhG and CL mediate the cycling of the DNA replication protein DnaA from the ADP-DnaA to ATP-DnaA (active) state, and inhibit the binding of DnaA to *oriC* via sequestration (6). *E. coli* cells with reduced amounts of anionic phospholipids accumulate outer membrane proteins in the cytoplasm and some proteins insert incorrectly into the membrane (59). Furthermore, CL and PhE facilitate the formation of non-bilayer structures (12) important in cell division and sporulation (34). Recent evidence suggests that these various lipid species may assort into spatially distinct lipid micro-domains (55), which in turn may affect the localization of membrane proteins (34). In *B. subtilis* CL-rich and PhE-rich domains are localized to the septal regions and the poles (25, 40), corresponding with the FtsZ-dependent subcellular

localization of the lipid biosynthesis enzymes PssA, YwnE, and PgsA (phosphatidylglycerophosphate synthase) (40).

In *B. subtilis*, membrane lipids are synthesized from the common precursor phosphatidic acid (PA) (Fig. 1.3). In the case of PhE, PA is converted to CDP-diacylglycerol (CDP-DAG) which is condensed with serine and then rapidly decarboxylated to generate PhE. Condensation of CDP-DAG with glycerol-3-phosphate followed by removal of the phosphate leads to PhG, the only essential complex lipid in *B. subtilis*. PhG can be further modified to form two minor complex lipids CL and LPG. CL is formed by the condensation of two PhG molecules by cardiolipin synthase (CLS). *B. subtilis* contains two CLS enzymes: the major form (YwnE; also called ClsA (25)) is expressed during vegetative growth, while the minor (YwjE) is involved in sporulation (25). LPG is formed when MprF transfers a lysyl group from lysyl-tRNA^{Lys} to PhG. Finally, GL are created by dephosphorylation of PA to diacylglycerol which is then modified by the transfer of one or two glucose molecules from UDP-glucose by UgtP (24). Fatty acid synthesis and desaturation are controlled by the FapR (51), and DesRK (13) regulatory systems and both chain length and desaturation may be regulated by various stress conditions (30, 31, 37, 48). In contrast, little is known about how membrane headgroup composition is regulated, although the extracytoplasmic-function (ECF) σ factor σ^X has been shown to contribute to expression of PhE biosynthesis genes (10).

Here, I report the characterization of a series of isogenic *B. subtilis* strains with altered membrane composition. Mutant strains were characterized for growth, antibiotic resistance, morphology, and alterations in global gene expression patterns. My results suggest that the cell can tolerate even large changes in membrane composition. Remarkably, *B. subtilis* retains viability and even rapid growth when the membrane is comprised predominantly, if not exclusively, of PhG.

3. Materials and Methods

Bacterial strains and growth conditions. All *B. subtilis* strains used were derivatives of either CU1065 (W168 *trpC2 attSPβ*) or the related strain NCIB3610 (Table 4.1). *Escherichia coli* strain DH5α was used for standard cloning procedures. Bacteria were grown in Luria-Bertani (LB) medium, LB supplemented with 25 mM $MgSO_4$ or modified minimal medium (8) at 37°C with vigorous shaking. Antibiotics were added to the growth medium when appropriate: 100 μg/ml ampicillin for *E. coli*, and 1 μg/ml erythromycin plus 25 μg/ml of lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 μg/ml chloramphenicol, 10 μg/ml kanamycin, 20 μg/ml tetracycline and 100 μg/ml spectinomycin for *B. subtilis*. Sensitivity to nisin was measured by growing the different strains in LB and then adding the indicated concentration during early exponential growth (OD_{600} ~ 0.25). Sensitivity to sublancin was tested via spot-on-lawn assay. Lawns were created by inoculating 100 μl of mid exponential cultures into 2 ml of 0.7% LB agar. This was poured into one well of an 8 well rectangular multidish (26 mm x 33 mm; Nunc). Once set, the plates were dried for 30 min in a laminar flow hood and 5 μl of strain JH642 and HB6164 (JH642 *sunA::kan*; see Table 4.1), grown to an OD_{600} of 0.6, were spotted in the center of the well. Plates were incubated overnight at 37°C in an airtight container with moist paper towels to prevent drying.

Construction of null mutants. Chromosomal deletions were created in CU1065 background using long-flanking homology PCR (LFH-PCR) as described (33) with the following changes: flanking fragments were amplified using *Pfu* DNA Polymerase (Stratagene) and the flanking fragments and antibiotic resistance marker were joined using Expand Long Template PCR System (Roche). The Xylose inducible mutants were created by LFH using a spectinomycin resistance gene fused with the P_{xylA}

region amplified from pTn7SX (7). Detailed protocol available at http://www.micro.cornell.edu/faculty/helmann/supplemental%20index.htm
Primer sequences used for mutant construction are detailed in Table 4.1.
The null mutants in the NCIB3610 background were created by SPP1-mediated transduction from the CU1065 strains harboring the mutation of interest as previously described (27). Transductants were tested for their ability to grow on the appropriate antibiotic and in minimal medium without the addition of tryptophan.

Lipid extraction and thin layer chromatography. 10 ml of mid exponential cultures were centrifuged for 10 min at 4,500 x g and lipids were extracted from the pellet via a modified Bligh-Dyer method (5,28). Briefly, the cell pellet was resuspended in 100 µl ddH_2O with the addition of perchloric acid to a final concentration of 1M. Cell suspension was then incubated at 0^oC for 30 min, after which lipids were extracted by the addition of 1 ml methanol/chloroform/water (12:6:2 v/v) followed by incubation for 50 min on ice. Phase separation was achieved by the sequential addition of 0.3 ml water and 0.3 ml chloroform, after which suspensions were incubated overnight at –20^oC, and then centrifuged for 5 min at 720 x g at 4^oC. The organic phase was then removed and dried under nitrogen. The lipids were resuspended in 20 µl of chloroform/methanol (2:1 v/v), spotted to silica gel 60 plates (VWR) and separated using the solvent mixture chloroform/methanol/water (65:25:4 v/v). Phospholipids were detected using molybdenum blue spray reagent (Sigma-Aldrich). PhG and PhE standards obtained from Sigma-Aldrich.

Microscopy. Phase-contrast and fluorescence microscopy were performed using an Olympus BX61 epifluorescence microscope, with 40x UPlanFl (N.A. 0.75) and 100x UPlanApo (N.A. 1.35) objectives. The microscope is equipped with filter cubes for viewing fluorescein, 7-Aminoactinomycin D/Mitotracker Red, and DAPI/Hoechst

fluorescence. Images were acquired using a Cooke SensiCam and Slidebook software (Intelligent Imaging Inc). Figures were assembled using Adobe Photoshop.

Swarming motility assay. LB plates containing 0.7% agar were dried in a laminar flow hood for 30 min and then spotted in the center with 5 μl of mid-exponential cultures grown in LB medium. The plates were then dried for another 15 min and incubated overnight at 37°C. Figures were assembled using Adobe Photoshop.

Construction and analysis of P_{sigM} transcriptional fusion. DNA fragments of the *sigM* regulatory region were PCR amplified using primers: #2866 and #436 (Table 4.1). The fragment were digested with HindIII and BamHI, and cloned into vector pDG1661 which contains a promoterless *lacZ* gene (20), resulting in plasmid pLS30. The sequences of the insert were verified by DNA sequencing (Cornell DNA sequencing facility). *B. subtilis* CU1065 and HB5346 (*ugtP::MLS*) were transformed to Cm^r with the ScaI-linearized plasmid which integrated into the *amyE* locus, creating strains HB5423 and HB5426, respectively. For quantitative measurements of β-galactosidase activity, strains HB5423 and HB5426 were grown in LB at 37°C with vigorous shaking, and samples were collected at different growth stages as determined by OD_{600}. To test promoter induction, strains HB5423 and HB5426 were grown in LB until an OD_{600} ~ 0.3, and then the cultures were split into aliquots, which were either unchallenged or challenged with vancomycin (2 μg/ml) for 30 min. β-galactosidase activity was measured according to the method of Miller (36) except that cells were lysed by the addition of lysozyme to a final concentration of 20 μg/ml followed by a 30 min incubation at 37°C.

RNA isolation and Microarray analysis. Strains CU1065, HB5346 (*ugtP*, abbreviated as U), HB5437 (*mprF pssA ywnE* triple mutant; abbreviated as T), and HB5391 (*ugtP mprF pssA ywnE* quadruple mutant; abbreviated as Q) were inoculated into LB and grown at 37°C with vigorous shaking until an OD_{600} ~0.4, and RNA

isolation and was performed using the RNeasy mini kit (Qiagen). RNA was subsequently DNase treated with TURBO DNA-free™ (Ambion) and precipitated overnight. The RNA was dissolved in RNAse free water and quantified using a NanoDrop spectrophotometer (Nanodrop Tech. Inc., Wilmington, DE). RNA was isolated from three biological replicates.

cDNA synthesis was performed using the SuperScript™ Plus Indirect cDNA labeling System (Invitrogen) as per the manufacturer's instructions using 20 μg of total RNA, then purified using the Qiagen MinElute kit (Qiagen, Maryland) and quantified with NanoDrop. Total cDNA was labeled overnight with Alexa Fluor 555 or Alexa Fluor 647 (Invitrogen) then purified using the Qiagen MinElute kit (Qiagen, Maryland) and quantified with NanoDrop. Equal amounts (100-150 pmol) of labeled cDNA (WT/U, WT/T, WT/Q) were combined to a final volume of 15 μl, and 1 μl salmon sperm DNA (10 mg/ml, Invitrogen) plus 16 μl 2X hybridization buffer (50% formamide, 10X SSC, 0.1% SDS) were added. cDNA mix was denatured at 95°C and hybridized 16-18 hours at 42°C to DNA microarray slides which had been prehybridized for at least 30 min at 42°C in 1% bovine serum albumin, 5X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), washed in water and dried. Following hybridization the slides were washed sequentially in: 2X SSC + 0.1% SDS for 5 min at 42°C, 2X SSC + 0.1% SDS for 5 min at room temperature, 2X SSC for 5 min at room temperature, 0.2X SSC for 5 min at room temperature, and finally dipped in water and spun until dry. Arrays were scanned using a GenePix™ 4000B array scanner (Axon Instruments, Inc.). Our arrays are based on a *B. subtilis* oligonucleotide library manufactured by Sigma Genosys consisting of 4,128 oligonucleotides (65-mers) representing 4,106 *B. subtilis* genes, 10 control oligonucleotides (from *E. coli* and Brome mosaic virus), and 12 random oligonucleotides. A single oligonucleotide was designed to represent each of the *B.*

subtilis genes as annotated in the genome data release R16.1 (26 April 2001) at the SubtiList website (http://genolist.pasteur.fr/SubtiList/). The arrays were printed onto poly-L-Lysine coated Corning CMT-Gap slides at the W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University. Each array contains 8,447 features corresponding to duplicates of each ORF-specific oligonucleotide, additional oligonucleotides of control genes, and 50% DMSO blank controls.

Raw data files were produced from the scanned images using the GenePix Pro 4.0 software package (GPR files), and the red/green fluorescence intensity values were normalized such that the ratio of medians of all features was equal to 1. The normalized data was exported to Excel for analysis. The datasets were filtered to remove those genes that were not expressed at levels significantly above background in either condition (sum of mean fluorescence intensity <30). For analysis, we filtered to identify those genes that were altered at least 1.5-fold in signal intensity in at least 2 of the 3 biological replicates (and excluding those genes with an opposing change in the third replicate). Microarray datasets and related files are available at NCBI GEO accession number (pending).

Table 4.1 Bacterial strains and primers used in this study

Name	genotype	Created / source
CU1065	W168 *trpC2 attSPβ*	Lab strain
NCIB3610	Wild type isolate	BGSC # 3A1
JH642	*pheA1 trpC2*	BGSC # 1A96
HB6164	JH642 *sunA::kan*	(9)
HB5337	CU1065 *mprF::kan*	LFH-PCR → CU1065
HB5343	CU1065 *psd::MLS*	LFH-PCR → CU1065
HB5346	CU1065 *ugtP::MLS*	LFH-PCR → CU1065
HB5347	CU1065 *ywnE::tet*	LFH-PCR → CU1065
HB5348	CU1065 *ywjE::kan*	LFH-PCR → CU1065
HB5361	CU1065 *pssA::spc*	LFH-PCR → CU1065
HB5362	CU1065 *ywnE::cat*	LFH-PCR → CU1065
HB5445	CU1065 *ywjE::cat*	LFH-PCR → CU1065
HB5344	CU1065 *psd::MLS mprF::kan*	HB5337 chr DNA → HB5343
HB5349	CU1065 *ywjE::kan ugtP::MLS*	LFH-PCR → HB5348
HB5350	CU1065 *mprF::kan ugtP::MLS*	LFH-PCR → HB5337
HB5387	CU1065 *ywnE::cat mprF::kan*	LFH-PCR → HB5362
HB5388	CU1065 *mprF::kan pssA::spc*	LFH-PCR → HB5337
HB5438	CU1065 *ugtP::MLS pssA::spc*	LFH-PCR → HB5346
HB5439	CU1065 *ugtP::MLS ywnE::cat*	LFH-PCR → HB5346
HB5440	CU1065 *pssA::spc ywnE::cat*	LFH-PCR → HB5361
HB5441	CU1065 *ywnE::cat ywjE::kan*	HB5348 chr DNA → HB5362
HB5442	CU1065 *ywnE::cat psd::MLS*	HB5343 chr DNA → HB5362
HB5443	CU1065 *pssA::spc ywjE::kan*	HB5348 chr DNA → HB5361
HB5389	CU1065 *ugtP::MLS mprF::kan pssA::spc*	LFH-PCR → HB5350
HB5390	CU1065 *ugtP::MLS mprF::kan ywnE::cat*	LFH-PCR → HB5350
HB5436	CU1065 *ugtP::MLS pssA::spc ywnE::cat*	LFH-PCR → HB5438
HB5437	CU1065 *mprF::kan pssA::spc ywnE::cat*	LFH-PCR → HB5388
HB5391	CU1065 *ugtP::MLS pssA::spc ywnE::cat mprF::kan*	LFH-PCR → HB5436
HB5481	CU1065 *ugtP::MLS pssA::spc mprF::kan ywnE::tet*	LFH-PCR → HB5389
HB5482	CU1065 *ugtP::MLS pssA::spc mprF::kan ywnE::tet ywjE::cat*	HB55445 chr DNA → HB5481
HB5406	CU1065 *pssA::spc*-P_{xylA}	LFH-PCR → CU1065
HB5407	CU1065 *ybfM::spc*-P_{xylA}	LFH-PCR → CU1065
HB5461	NCIB3610 *pssA::spc*	transduction → NCIB3610
HB5462	NCIB3610 *ugtP::MLS*	transduction → NCIB3610
HB5463	NCIB3610 *mprF::kan*	transduction → NCIB3610
HB5464	NCIB3610 *ywnE::cat*	transduction → NCIB3610
HB5465	NCIB3610 *ywjE::cat*	transduction → NCIB3610
HB5466	NCIB3610 *ugtP::MLS pssA::spc*	transduction → HB5462
HB5467	NCIB3610 *ugtP::MLS mprF::kan*	transduction → HB5462
HB5468	NCIB3610 *ywnE::cat ugtP::MLS*	transduction → HB5464
HB5469	NCIB3610 *ugtP::MLS ywjE::cat*	transduction → HB5462
HB5470	NCIB3610 *pssA::spc mprF::kan*	transduction → HB5461
HB5471	NCIB3610 *ywnE::cat pssA::spc*	transduction → HB5464
HB5472	NCIB3610 *pssA::spc ywjE::cat*	transduction → HB5461
HB5473	NCIB3610 *ywnE::cat mprF::kan*	transduction → HB5464
HB5474	NCIB3610 *mprF::kan ywjE::cat*	transduction → HB5463
HB5475	NCIB3610 *ugtP::MLS mprF::kan pssA::spc*	transduction → HB5467
HB5476	NCIB3610 *ugtP::MLS pssA::spc ywnE::cat*	transduction → HB5466
HB5477	NCIB3610 *ugtP::MLS mprF::kan ywnE::cat*	transduction → HB5467
HB5478	NCIB3610 *mprF::kan pssA::spc ywnE::cat*	transduction → HB5470
HB5479	NCIB3610 *ugtP::MLS mprF::kan pssA::spc ywnE::cat*	transduction → HB5475

Table 4.1 (Continued)

Oligonucleotide primers		
#	**Name**	**Sequence***
1761	yfiW up fwd	tccaagcgcctgaatcagcc
1762	yfiW up rev (kan)	CCTATCACCTCAAATGGTTCGCTGtgatgaccatgagcgtacg
1763	yfiX do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGccatgttcctcgttacacg
1764	yfiX do rev	gagccgtaaagtcatggaagg
1913	psd up fwd	ccgatccttctggcaatcgg
1914	psd up rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCgacagacgatgattcgtcagc
1915	psd do fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCcaagtcggagaactgatagg
1916	psd do rev	gacatcttctgtgtgccatacagcg
2092	ugtP up fwd	gacgagctatactacgtgcc
2093	ugtP up rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCttggctacctgcacatgtcc
2094	ugtP do fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCggaatcagaaatgatgaccg
2095	ugtP do rev	tgccgcttatccgtaaagcc
2100	ywnE up fwd	gctcttcctttatttgtcgtgcg
2101	ywnE up rev (tet)	GAGAACAACCTGCACCATTGCAAGAccaagatgcactggcatcgc
2102	ywnE do fwd (tet)	GGGATCAACTTTGGGAGAGAGTTCctatgaggagtatctgcagc
2103	ywnE do rev	tttgtacagtgacgcagacc
2367	ywnE up rev (cat)	CTTGATAATAAGGGTAACTATTGCCccaagatgcactggcatcgc
2368	ywnE do fwd (cat)	GGGTAACTAGCCTCGCCGGTCCACGctatgaggagtatctgcagc
2104	ywjE up fwd	agaaagcccatgctcatgcc
2105	ywjE up rev (kan)	CCTATCACCTCAAATGGTTCGCTGgatatcaagcagaatcaaggc
2106	ywjE do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGtggaggatttcagcaagcg
2107	ywjE do rev	Agacaaacggaacatcctcc
3880	ywjE up rev (cat)	CTTGATAATAAGGGTAACTATTGCCgatatcaagcagaatcaaggc
3881	ywjE do fwd (cat)	GGGTAACTAGCCTCGCCGGTCCACGtggaggatttcagcaagcg
2369	pssA up fwd	cttgagacggcacatctggc
2370	pssA up rev (spc)	CGTTACGTTATTAGCGAGCCAGTCcctatcgtaatcatacaggg
2371	pssA do fwd (spc)	CAATAAACCCTTGCCCTCGCTACGcagaaaacctggagtctggg
2372	pssA do rev	gaaccacaccgtcaacaggg
2449	pssA up rev (spc xylA)	CAATAAACCCTTGCCCTCGCTACGcctatcgtaatcatacaggg
2450	pssA do fwd (spc xylA SD)	CCAACCAGATAAGTGCGAGCCAGTCAAGAGGATCTGCatggaattagttcagcagc
2451	ybfM up rev (spc xylA)	CAATAAACCCTTGCCCTCGCTACGcgtaatccgctatgagctgc
2452	ybfM do fwd (spc xylA SD)	CCAACCAGATAAGTGCGAGCCAGTCAAGAGGATCTGCatgtttaatacggctgtaaag
2866	PsigM fwd (H)	ccgtaagctttgggtactatatagtaatggtg
436	SigM-r3	cgggatccagtaagtcttcagcaagatg

* Capped letters indicate sequences complementary to antibiotic resistance genes. Underlined sequences indicate restriction sit

4. Results and Discussion

The *Bacillus subtilis* cytoplasmic membrane can be dramatically simplified.

Bacterial cytoplasmic membranes display a great variety of lipid compositions. PhE is a dominant inner membrane component in *E. coli* (75% of total; (12)), whereas in *Staphylococcus aureus* membranes are predominantly formed from PhG (~90% of total; (39)). In *Mycobacterium tuberculosis*, membranes are dominated by phosphatidylinositol mannosides (~70%; (50)). In many other cases, no single lipid type dominates and in all systems there are at least three or more different types of lipid headgroup represented. These observations are consistent with the hypothesis that membrane function requires lipid components with different physio-chemical properties.

Here, I set out to characterize a set of strains with altered membrane composition using *B. subtilis* as a model system. I inactivated genes involved in the biosynthesis of complex lipids including genes necessary for the synthesis of PhE (*pssA;* P), GL (*ugtP;* U*),* LPG *(mprF;* M*),* and CL (*ywnE;* Y) by allelic replacement with antibiotic resistance cassettes (Table 4.1). I also constructed mutants of *psd* (S) and *ywjE* (J). The *psd* mutant accumulates phosphatidylserine (PS), but is unable to synthesize PhE. The *ywjE* mutant is missing a minor CLS thought to be present during sporulation (25). Allelic replacements were verified by PCR, and the absence PhE and LPG of was verified by thin-layer-chromatography (TLC) (Fig. 4.1 and data not shown). By additional rounds of transformation I created a suite of mutants comprised of various combinations of these mutations with increasingly simplified membrane composition. Surprisingly I was able to create a viable quadruple mutant strain (Q; *ugtP pssA mprF ywnE*) with membranes containing predominantly, if not exclusively, phosphatidylglycerol (PhG) (Table 4.1). The only other DAG-based lipids that might be present are PA and DAG itself. Previously, DAG was reported to be

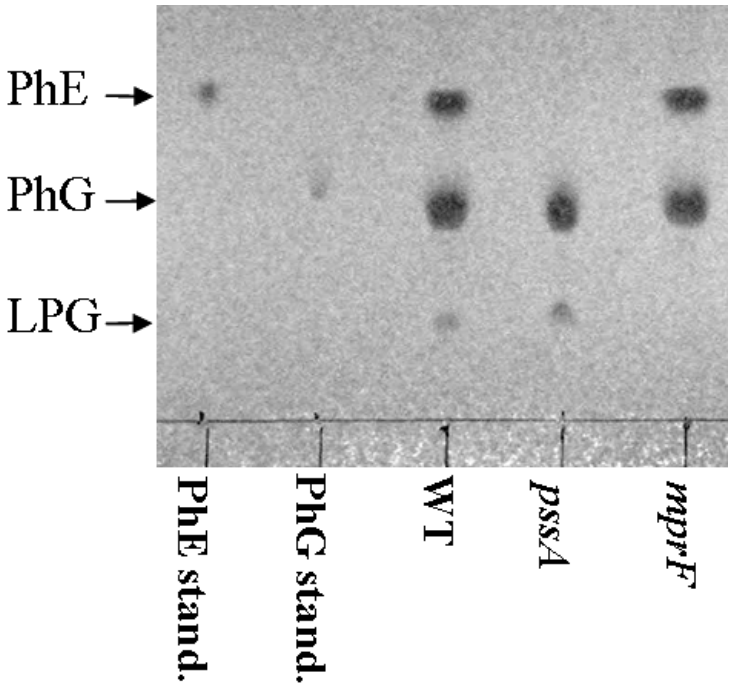


Figure 4.1. TLC analysis of membrane mutants. Membrane lipids were extracted from exponential cultures of WT or membrane mutants grown in LB using a modified Bligh-Dyer method (5). Extracted lipids were spotted to silica TLC plate and detected with molybdenum blue (Sigma) and compared to standards as indicated.

present in low levels in *B. subtilis* membranes (4). DAG is formed as a precursor to GL and is also generated as an intermediate during the synthesis of lipoteichoic acids (23).

I monitored growth rates for each of our strains using a BioScreen automated growth curve (optical density) analysis system. All mutant strains were able to grow in a variety of liquid and solid media including LB, LB + 2% glucose, Difco Sporulation Medium (DSM), minimal competence medium (MC), as well as a defined minimal medium (MM). However, some of the strains had increased doubling and/or lag times. In LB medium, increased doubling times were observed for those strains lacking the neutral lipid GL (*ugtP* mutant; U; Table 4.2). Comparison of the U single and double mutants lacking GL indicates that the loss of UgtP is primarily responsible for the growth defects in LB (Table 4.2). Surprisingly, the quadruple mutant (UPMY) exhibits a doubling time similar to that of the double mutants. However the lag time of this strain, as well as all strains lacking *ugtP*, is considerably longer than that of WT (data not shown). It is important to note, however, that UgtP plays several roles in the cell. In addition to the synthesis of GL, UgtP is involved in generating anchor lipids for lipoteichoic acids (24), and functions as a sensor to couple cell division to the availability of nutrients (60).

In minimal medium, in which cells grow considerably slower, the only strain to demonstrate a significantly increased doubling time was the UPMY quadruple mutant (74 vs. 48 min for WT). Growth rates in DSM were similar to those in LB (data not shown). All strains were capable of sporulating: phase bright spores were observed after growth in DSM for 24 hours and all strains formed pigmented colonies when streaked on DSM plates.

The viability and robust growth of strains with greatly simplified membrane composition is surprising in light of previous studies of *E. coli* mutants. For example,

Table 4.2. Effects of membrane headgroup alteration on cell growth

Strain[a]	Membrane lipid (charge)					Doubling time[b]	
	PhG -1	CL -2	PhE 0	GL 0	LPG +1	LB	MM
WT						34 ± 1	48 ± 3
P[*]			■			37 ± 2	49 ± 3
U				■		50 ± 3	51 ± 4
M[*]					■	38 ± 3	45 ± 2
Y		■				36 ± 4	50 ± 1
S			■			37 ± 2	52 ±2
J		▒				38 ± 0	49 ± 2
UP			■	■		37 ± 5	50 ± 3
UM				■	■	44 ± 7	62 ± 8
UY		■		■		47 ± 8	51 ± 2
UJ		▒		■		40 ± 2	51 ± 2
PM			■		■	40 ± 4	44 ± 5
PY[*]		■	■			41± 5	50 ± 2
PJ		▒	■			39 ± 1	55 ± 2
MY[*]		■			■	40 ± 4	50 ± 3
MS			■		■	38± 2	47 ± 2
YJ		■				41 ± 1	49 ± 2
YS		■	■			39 ± 3	52 ± 4
UPM			■	■	■	37 ± 2	55 ± 6
UPY		■	■	■		53 ± 9	50 ± 4
UMY		■		■	■	42 ± 9	49 ± 5
MPY (T)		■	■		■	37 ± 3	46 ± 7
UPMY (Q)		■	■	■	■	40 ±7	74 ± 9

LB: Luria-Bertani medium, MM: defined minimal medium.

[*] Lipid composition verified by TLC.

[a] **P** : *pssA::spc*, **U** : *ugtP::MLS*, **M** : *mprF::kan*, **Y** : *ywnE::cat*, **S** : *psd::MLS*, **J** : *ywjE::kan*

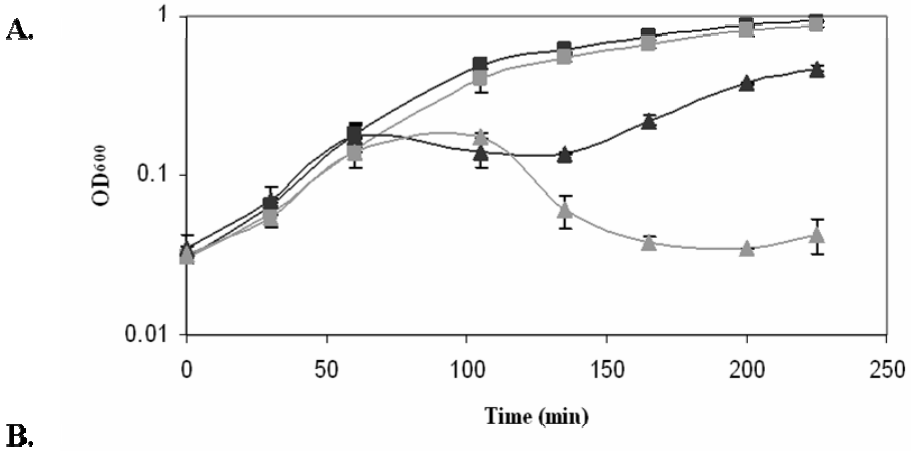
White boxes: lipid is present in the membrane, black boxes: lipid is absent, grey boxes: CL may be partially lacking, at least under some growth conditions, due to loss of the minor CLS encoded by *ywjE*.

[b] Doubling time calculated for maximum specific growth rate.

an *E. coli pssA* null mutant has severe growth defects and requires divalent metal ion supplementation for growth (14), and a mutant lacking both PhE and CL is non viable (41). These defects are thought to result from a requirement for the formation of non-bilayer structures within the membrane (facilitated by PhE) as well as the proper localization of membrane proteins (2, 35). These differences may result, in part, from the fact that *B. subtilis* only contains a single membrane whereas *E. coli* requires both an inner and outer membrane.

***mprF* and *ugtP* mutants exhibit increased sensitivity to cationic antimicrobial peptides.** Modification of the membrane has been proposed as a strategy for protection against cationic antimicrobial peptides (56). I tested my collection of strains for their sensitivity to a variety of cell envelope active compounds including vancomycin, nisin, sublancin, and duramycin. In the presence of the glycopeptide antibiotic vancomycin most strains grew to an OD_{600} comparable to that of WT. However, all *ugtP* mutant strains were vancomycin sensitive (data not shown). Conversely, in the presence of the cationic lantibiotic nisin *mprF* mutants were sensitive (Fig. 4.2A). LPG can constitute up to 20% of the *B. subtilis* membrane (15), and has been shown to impart resistance to cationic antimicrobials including nisin, vancomycin and human defensins in *S. aureus* (49, 56).

Next I tested sensitivity to the bacteriocin sublancin produced by *B. subtilis* strains harboring the SPβ prophage. my mutant strains were constructed in *B. subtilis* CU1065 which lacks the SPβ prophage, and is therefore sublancin sensitive. To test sublancin senstivity, lawns were spotted with *B. subtilis* JH642 (a sublancin producer) or an isogenic strain unable to produce sublancin (*sunA::kan* mutant). Increased zones of inhibition were observed for all *ugtP* mutants spotted with JH642 but not with the *sunA* mutant (Fig. 4.2B). Sensitivity was not further increased in the double, triple or quadruple *ugtP* lacking mutants.



B.

CU1065	*mprF::kan*	*ugtP::MLS*	UPM	UPY	UMY
MPY	UPMY	UP	UM	UY	UJ

Figure 4.2. Variation of cytoplasmic membrane composition results in altered antibiotic sensitivity. (A) Deletion of *mprF* leads to increased sensitivity to nisin. WT (black symbols) and *mprF::kan* cells (grey symbols) were grown in LB to early log and then either left untreated (squares) or treated with 10 μg/ml nisin (triangles). (B) Strains lacking *ugtP* demonstrate increased sensitivity to sublancin. Lawns of various strains spotted with the sublancin producing *B. subtilis* JH642 (left spot), or a JH642 strain containing a *sunA* deletion.

Duramycin is a lanthionine containing antimicrobial peptide (or lantibiotic) produced by *Streptoverticillium cinnamoneum*. Previous studies have reported that strains selected for increased resistance to duramycin demonstrated decreased levels of PhE and increased levels of CL (16), and have predicted a mode of action in which duramycin binds to PhE and then inserts into the membrane creating pores (22). Consistent with this expectation, *pssA* and *psd* mutant strains exhibited greatly increased resistance to duramycin relative to the WT strain (data not shown). No other single mutation conferred this resistance, and double and triple mutants lacking either *pssA* or *psd* showed similar resistance levels to that of the single mutants. Unexpectedly, the UPMY quadruple mutant was more sensitive to duramycin than other PhE-lacking mutants, but still more resistant than WT. This suggests that the requirement for PhE in the biological action of duramycin may be bypassed in strains with grossly perturbed membrane structure.

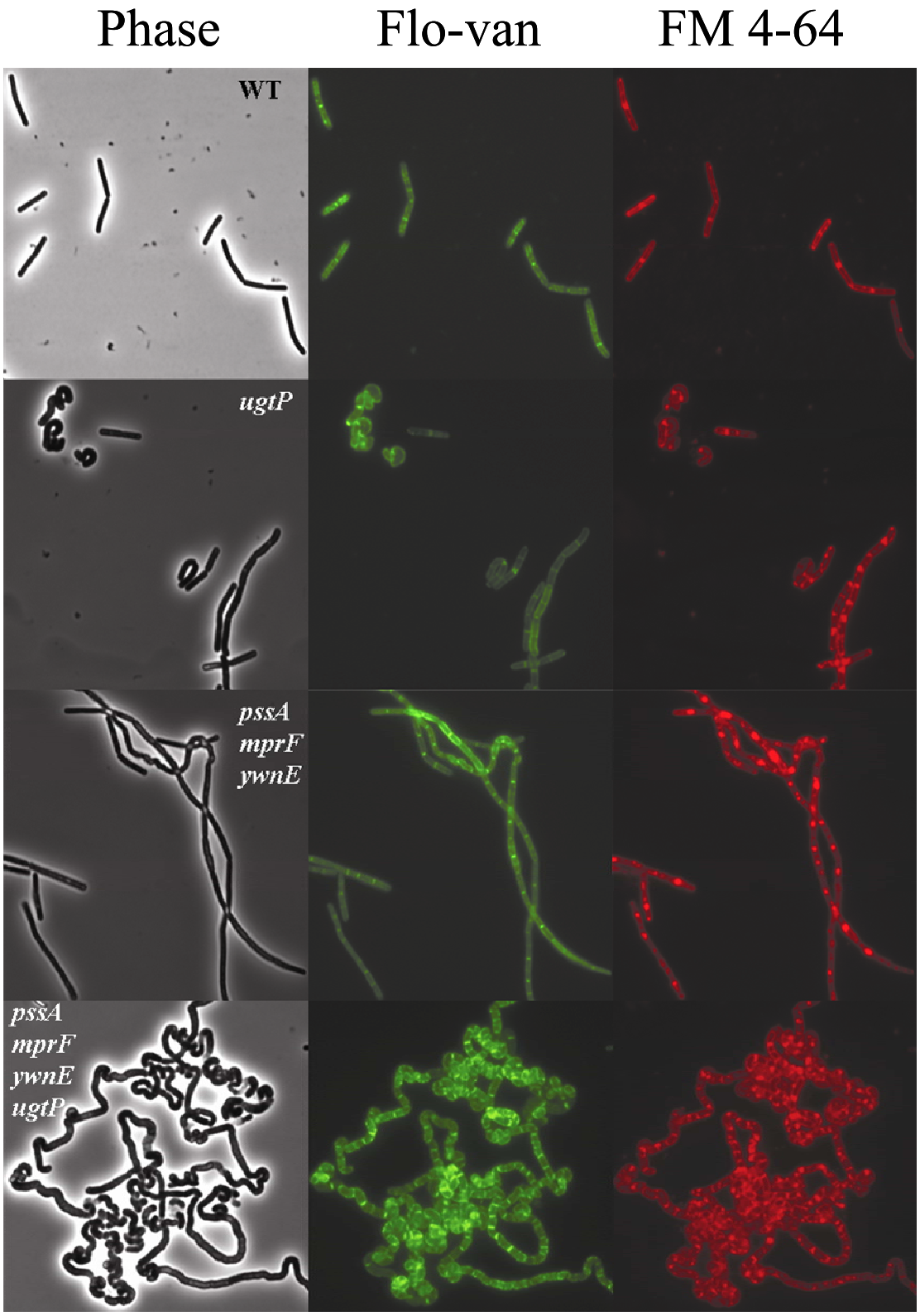
In *B. subtilis*, the PhE biosynthetic genes (*pssA, psd*) are part of a tri-cistronic operon *pssA ybfM psd*. In order to study the possible role of *ybfM* in PhE biosynthesis and duramycin sensitivity, I constructed a strain in which *ybfM* was replaced by a spectinomycin resistance cassette, and *psd* was placed under the control of a xylose inducible promoter (*ybfM::spc*-P_{xylA}). In the presence of xylose strain *ybfM::spc*-P_{xylA} was as sensitive to duramycin as WT, whereas in the presence of glucose it was as resistant as a *pssA* null mutant (data not shown). This suggests that *ybfM* is not required for the synthesis or localization of PhE. In other organisms containing homologs of these proteins, the tricistronic operon structure is not conserved, and often *pssA* and *psd* are even situated in separate operons.

Cells containing simplified cytoplasmic membranes exhibit aberrant morphologies. Microscopic observation indicates that mutants with altered membrane composition have distinctive changes in cell morphology. During mid-exponential

growth in LB, the *ugtP* mutant was the only single mutant to demonstrate a change in cell shape: cells were shorter and tended to curl at the ends (Fig. 4.3). Double mutants lacking *ugtP* appeared mostly as curling short chains of cells or clumps of curled cells while triple mutants demonstrated lengthened filaments of curled cells (data not shown). The most dramatic effect is observed in the quadruple mutant in which the filamentation and curling is even more pronounced (Fig. 4.3). Double mutants that still contain *ugtP* resembled WT cells, with a small amount of cells in longer chains (data not shown), while the MPY triple mutant did not curl but was highly filamentous (Fig. 4.3). At later growth stages in LB the filamentation phenotype is lost and cells appear as either single cells or short chains (data not shown). When the MPY and UPMY mutants were grown in LB supplemented with Mg^{2+}, known to stabilize the effects of PhE loss in *E.coli* (12), the filamentation was reduced. Staining of these filamentous cells with the non-specific membrane stain FM 4-64 and fluorescently-labeled vancomycin (which targets uncrosslinked peptidoglycan containing pentapeptide sidechains) clearly demonstrates that these are septated filaments (Fig. 4.3). Fluorescently-labeled vancomycin stains the septal regions and also in a characteristic helical pattern along the axis of the cell as also seen for WT cells (58) (Fig. 4.3 and data not shown), suggesting that peptidoglycan synthesis and incorporation remains mostly unimpaired even in the quadruple mutant. Staining with FM 4-64 showed a similar accumulation at the septal region, with no obvious aberrant membrane formations such as involutions or blebs.

In general, the most dramatic morphological changes were observed in those cells lacking GL. *ugtP* codes for a multifunctional UDP-glucose:diacylglycerol glucosyltransferase that is required for the (i) formation of GL (24, 47), (ii) synthesis of diglucosyldiacylglycerol, the membrane anchor for lipotechoic acids (24, 29), and

Figure 4.3. Alteration of cytoplasmic membrane leads to aberrant morphology. Exponential phase cultures grown in LB were treated with both the non-specific membrane dye FM 4-64 and fluorescently-labeled vancomycin.


Phase
Flo-van
FM 4-64
WT
ugtP
pssA
mprF
ywnE
pssA
mprF
ywnE
ugtP

(iii) coupling nutrient availability to cell division in *B.subtilis* (60). When nutrients are available, UgtP is expressed at high levels, evenly distributed in the cytoplasm and concentrated at the septum, co-localizing and interacting with FtsZ (60). UgtP is proposed to inhibit the assembly of FtsZ rings by sequestering the FtsZ subunits, resulting in larger cells at the time of division (54,60). Upon carbon depletion localization of UgtP to the septum is abolished resulting in smaller cells. This dual role of UgtP in envelope biosynthesis and regulation of cell size could explain the severity of the defects seen in *ugtP* null mutants.

Cells containing simplified cytoplasmic membranes are altered for swarming motility. In order to study the effect of alteration of cytoplasmic membrane composition on motility and biofilm formation I transferred my mutant collection into a NCIB 3610 background which, unlike most *B. subtilis* 168 laboratory strains, exhibits a robust swarming phenotype. In this background, the *ugtP* mutant strains exhibited decreased swarming while, curiously, some of the single mutants displayed enhanced swarming (Fig. 4.4). This enhanced swarming is reduced only in the PY and MY double mutants as well as the MPY triple mutant. This does not seem to be due to a specific lack of CL since the Y single mutant did not show a similar decrease. Surprisingly, all strains tested were able to form characteristic pellicules on the air-liquid interface of MSgg medium. However, the biofilms of strains lacking *ugtP* were somewhat flatter and more matte, while that of the MPY triple mutant resembled WT (data not shown).

Transcriptome comparisons. I conducted DNA microarray experiments to compare the transcriptional profile of the *ugtP* single mutant, MPY triple mutant, and UMPY quadruple to that of WT cells in LB medium at mid-log stage. These mutations are highly pleiotropic and we identified a large number of genes that were reproducibly altered in expression levels relative to WT. These included genes

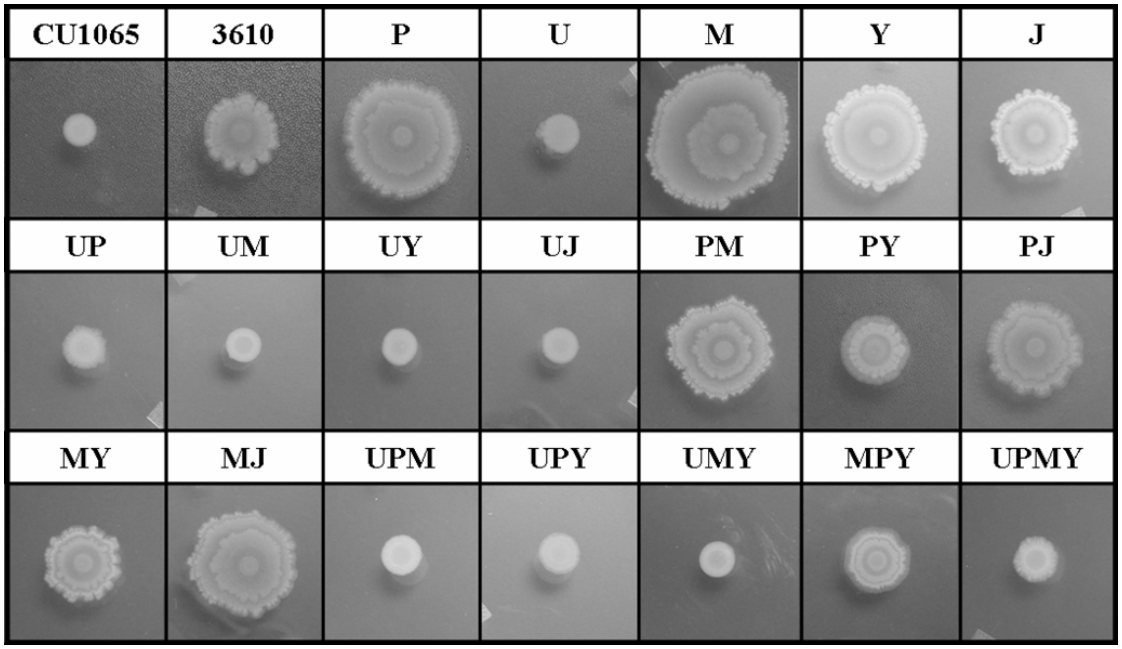


Figure 4.4. Deletion of *ugtP* results in a swarming defect. Membrane mutants in the NCIB3610 background were grown to mid exponential phase in LB and then spotted to 0.7% LB-agar plates. Plates were incubated overnight.

involved in carbon and protein metabolism, cell wall and membrane biosynthesis, DNA metabolism and repair, motility and chemotaxis, and production of secondary metabolites, as well as a large number of uncharacterized membrane proteins. In addition, the expression of many known or putative ABC transporter and other transport permeases were affected, including multidrug-efflux pumps and antibiotic resistance proteins. Alterations of membrane composition also affected the level of expression of numerous known and predicted transcriptional regulators. In general, these changes were most dramatic for the *ugtP* mutant and the UMPY quadruple mutant, with generally more modest changes noted in the MPY triple mutant. These results suggest that cell envelope perturbations caused by the absence of UgtP are potentially the most deleterious to the cell and result in a shift in the transcriptional profile.

To determine the likely origins of these large shifts in transcriptional pattern, I have linked many of the genes affected (either positively or negatively) by membrane alteration to known regulons. As summarized in Fig. 4.5, large fractions of several known regulons were altered in their expression in one or more of the tested mutant backgrounds. For example, genes regulated by σ^D (involved in motility, chemotaxis, and autolysin synthesis; 32) were expressed at lower levels in all three mutant backgrounds. In contrast, numerous members of the large DegU and σ^B (general stress response) regulons were elevated in expression in these strains. There was also a general increase in the expression of genes under the control of the sporulation specific sigma factors σ^E, σ^F, σ^G and σ^K in all backgrounds (Fig. 4.5). This suggests that sporulation may have initiated earlier and asynchronously within this growing population whereas sporulation genes are generally not expressed during logarithmic growth in WT.

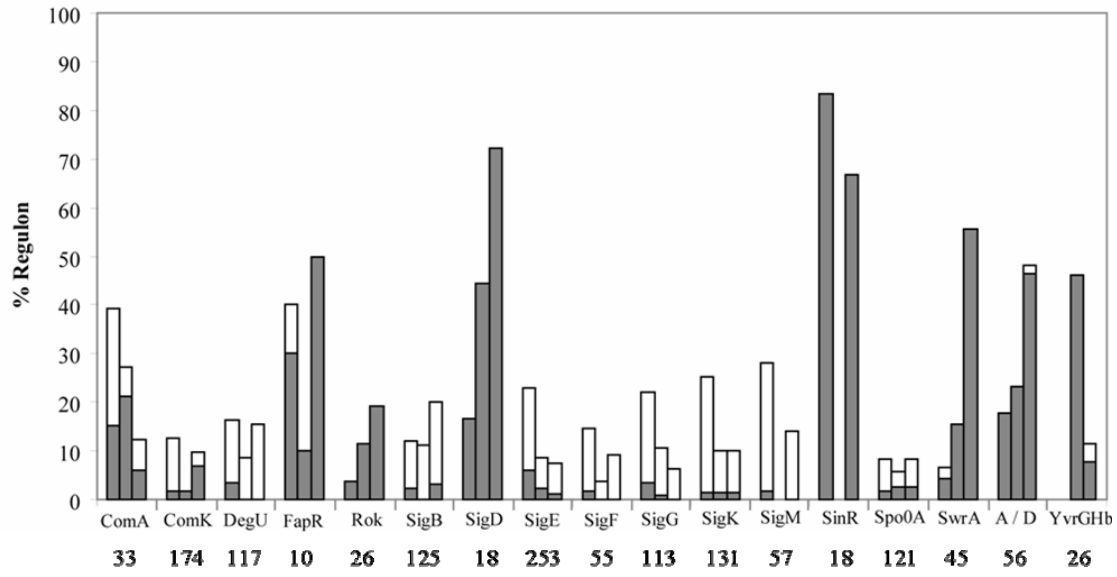


Figure 4.5. Analysis of regulons demonstrating differential expression patterns. Microarray experiments comparing WT with either *ugtP*, MPY or UMPY mutants strains. RNA extracted from mid-log cells grown in LB. Results represent average of three separate experiments. Each three bars correspond to a specific regulon, the order of experiments is WT vs. *ugtP*, WT vs. MPY, WT vs. UPMY. The height of the bar represents the overall percentage of the regulon with altered expression.
Grey sections represents % of regulon that was downregulated at least 1.5 fold, white sections represents % of regulon that was upregulated at least 1.5 fold The number of genes in each regulon is beneath the regulon name (adapted from 44, 3, 51, 1, 46, 53, 57, 18, 19, 17, 11, 38, 26 and 52).

In several cases, the role of UgtP appeared to be most important since the transcriptome changes were most dramatic for the U and UMPY mutations with relatively little effect noted in the MPY triple mutant. For example, genes involved in biosynthesis of fatty acids and under the negative control of FapR were strongly downregulated in both the U and UMPY backgrounds but only slightly altered in the MPY triple mutant. Genes negatively regulated by SinR, involved in the production of exopolysaccharides and biofilm formation (11), were also downregulated only in a U and UMPY background. In addition, the σ^M cell envelope stress regulon (17) was induced only in the U and UMPY backgrounds. This induction was verified by β-galactosidase assays showing that in a *ugtP* null background the expression level of the P_{sigM} promoter was higher throughout all growth phases (Fig. 4.6A), although it could still be additionally induced by treatment with vancomycin (Fig. 4.6B). Interestingly, the expression of the regulons of ECF sigma factors σ^W (which provides intrinsic resistance to antimicrobial compounds; 9) and σ^X (which regulates modification of the cell envelope and resistance to cationic antimicrobial peptides (10) were unaltered.

A contrasting example is provided by analysis of genes assigned to the YvrGHb TCS regulon. YvrGHb is reported to regulate cell surface homeostatic functions (52). This regulon was expressed at a reduced level in the MPY triple mutant but affected to a lesser extent in the quadruple mutant.

Among the most highly induced genes in the UMPY and MPY mutant backgrounds were the *yrkNM* operon encoding for a GCN5-related N-acetyltransferase and an unknown protein respectively, and *yrkO* which encodes for a putative transporter. *yrkN* and *yrkO* are thought to be positively regulated by the divergently encoded response regulator YrkP, which was suggested to regulate membrane function

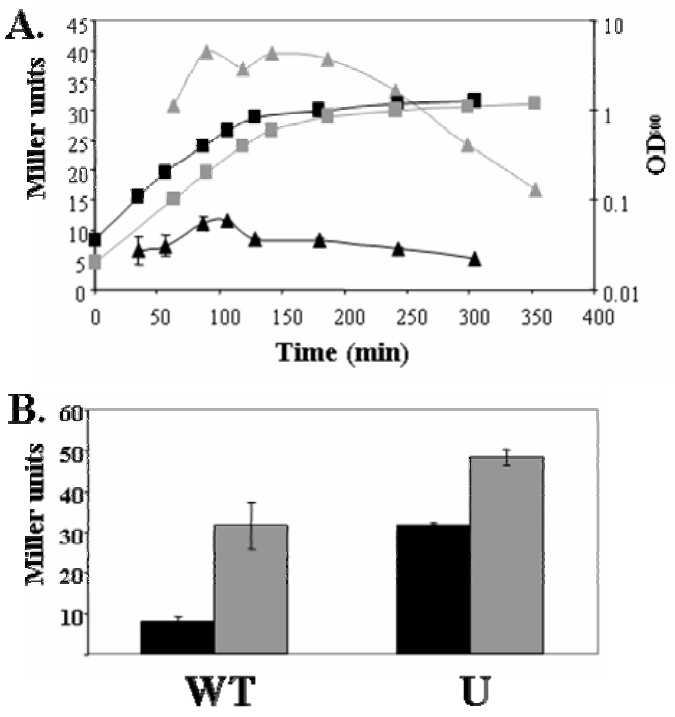


Figure 4.6. Activity of the σ^M promoter is increased in a *ugtP::MLS* background. (A) WT (black symbols) and *ugtP::MLS* (grey symbols) cells containing a P_{sigM}-*lacZ* promoter fusion were grown in LB. Samples were taken periodically and OD_{600} (squares) and LacZ activity (triangles) were monitored. (B) P_{sigM} can still be induced in a *ugtP::MLS* background. Mid-log cells either untreated (black) or treated with 2 μg/ml vancomycin for 30 min.

(43). However, the expression of the *ykcBC* operon, also thought to be regulated by YrkP (43), remained unchanged in all three backgrounds tested.

Concluding remarks. We report the construction of a large set of isogenic strains with alterations in membrane lipid composition. Perhaps the most surprising finding from this work is that the membrane of *B. subtilis* can be greatly simplified by removal of GL, PhE, LPG, and CL and the cells grow robustly. These cells are morphologically abnormal and exhibit a combination of cell separation and cell shape defects: they grow as long and curled filaments. In this initial survey, we have highlighted the effects of grossly perturbing membrane composition on antibiotic sensitivity, swarming motility, and global transcription patterns. In many of our phenotypic assays, the loss of *ugtP* appears to have a dominant effect, with changes in the *ugtP* single mutant often more dramatic than in the MPY triple mutant. Additional studies are clearly required to further understand these complex phenotypes, and to determine if the *ugtP* phenotypes are due to the loss of GL or in addition or instead to the important role of this protein as a metabolic sensor.

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