MEMBRANE STRESS RESISTANCE MECHANISMS IN *BACILLUS SUBTILIS*

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MEMBRANE STRESS RESISTANCE MECHANISMS IN *BACILLUS SUBTILIS*

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Bacteria exist in environments that can inflict a variety of stresses upon the cell, many of which target the cell membrane. As a result, bacterial survival often depends upon the ability of cells to adjust the cell membrane in response to environmental stress. This process is controlled by the cell envelope stress response (CESR), the signal transducing regulatory systems that allow cells to sense and respond to conditions that perturb the cell wall or membrane. In *Bacillus subtilis*, a major component of CESR is controlled by extracytoplasmic function sigma (ECF σ) factors. Numerous studies have associated ECF σ factors with membrane stress adaptations, but the specific details concerning the effects of particular σ factors on membrane composition and the underlying mechanisms involved are largely unknown. Here, we investigate these details using *B. subtilis* as a model system. The majority of this work consists of two main projects. In one project, I characterized a novel homeoviscous adaptation in which an ECF σ promoter modifies fatty acid composition by regulating the membrane biosynthesis genes *fabHa* and *fabF*. The altered expression of these genes leads to a greater proportion of straight chain fatty acids in the membrane and an increase in average fatty acid chain length. Such changes in the lipid profile of *B. subtilis* reduce membrane fluidity thereby conferring resistance against detergents and antimicrobial compounds produced by competing

Bacillus strains. The second project focuses on ECF σ factor-mediated lantibiotic resistance mechanisms in *B. subtilis*. I've identified six distinct lantibiotic resistance loci activated by ECF σ factors. These loci include genes encoding phage shock proteins, tellurite resistance related proteins, signal peptide peptidase, and proteins that synthesize and modify teichoic acids. My work has made substantial progress on defining the resistance mechanisms associated with these genes.

BIOGRAPHICAL SKETCH

Anthony Kingston was born on July 14th, 1986 in Rochester, NY. His parents, Bill Kingston and Maryann Romagnano were instrumental in his birth. He grew up with his older brother Bill, his younger brother Andrew, and his younger sister Alison. He had a relatively carefree childhood in the unremarkable town of Brighton and likes video games.

From 2000 to 2004, Anthony attended Brighton High School where he discovered his interest in the sciences. He pursued this interest at Skidmore College where he majored in biochemistry. His work with Michelle Frey on an aminopeptidase of *Vibrio cholerae* cemented his desire to continue working in this field. After graduating magna cum laude with a B. A. in biochemistry, he enrolled in the biochemistry molecular and cell biology department at Cornell University as a graduate student. He eventually joined John Helmann's lab where he studied cell envelope stress resistance mechanisms in *B. subtilis*. He enjoyed his time in Ithaca and is looking forward to the adventures his future life will bring.

To my parents, siblings, and Leah

ACKNOWLEDGMENTS

My achievements at Cornell would not have been possible without the people who helped me along the way. I have been extremely fortunate to have John Helmann as an advisor and mentor. He is always there to provide advice when needed, but gives me the freedom to think critically and learn on my own. His enthusiasm for science and compassion for students has taught me what it means to be a true scientist. I would also like to thank my other committee members, Eric Alani and Stephen Winans for their advice and encouragement. Finally, the BMCB and microbiology departments have my appreciation for supporting me and my peers throughout my time here.

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CHAPTER 1

INTRODUCTION

1.1: The cell envelope of *Bacillus subtilis*

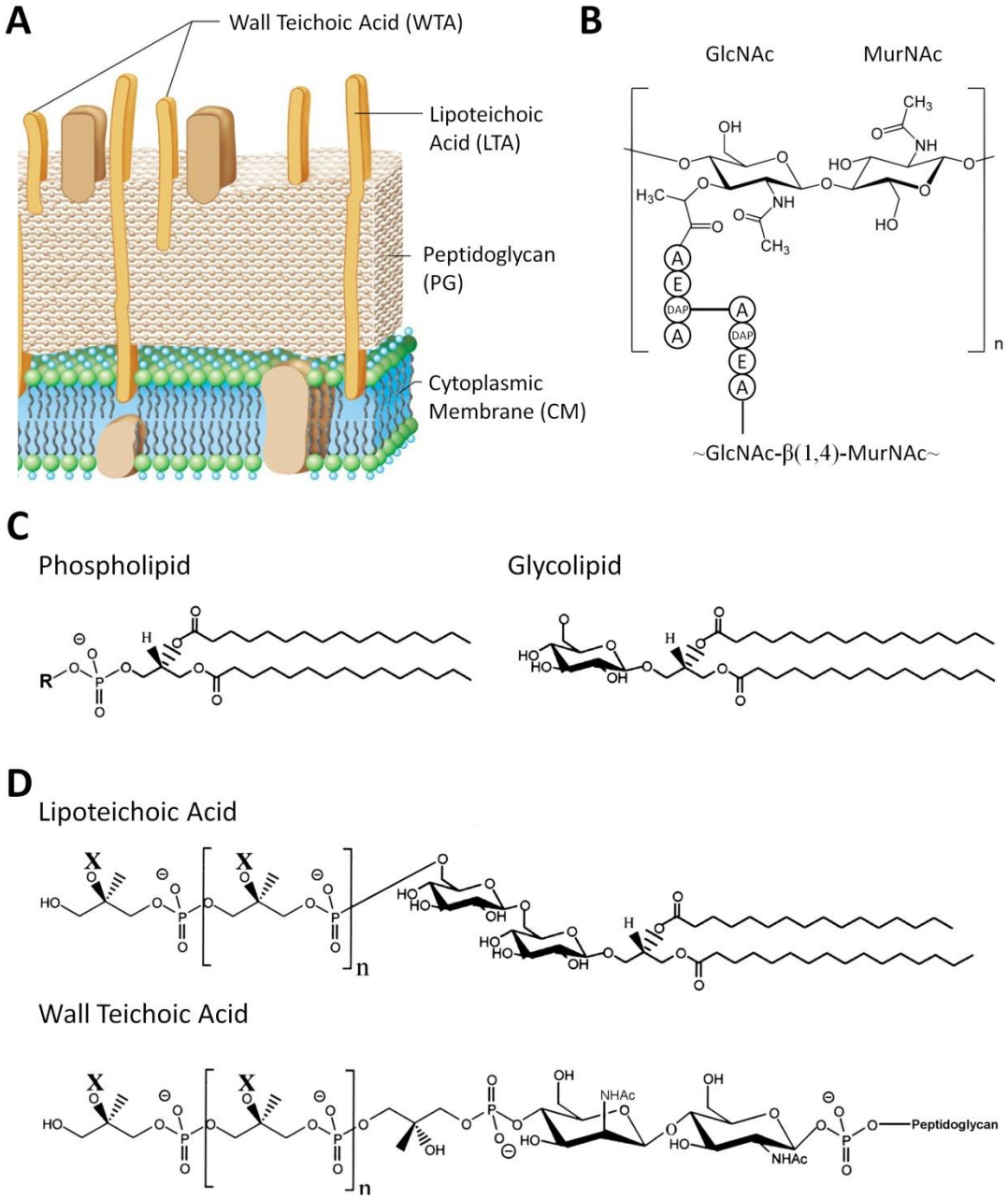
Most prokaryotes are enclosed in a cell envelope that serves as the primary line of defense against environmental stress (96). However, environments can abruptly change the stresses that they inflict upon the cell and bacteria must have mechanisms in place to adapt to these changes. The signal transducing regulatory systems that allow cells to sense and respond to conditions that perturb the cell wall or membrane are collectively known as the cell envelope stress response (CESR) (51). This chapter will summarize CESR mechanisms and relevant background information in *Bacillus subtilis*, a model Gram positive soil bacterium. As with most Gram positive bacteria, the cell envelope of *B. subtilis* consists of a phospholipid bilayer surrounded by a thick peptidoglycan cell wall interspersed with teichoic acids (Figure 1.1).

1.1.1: The cell membrane

The inner layer of the cell envelope is a protein-embedded lipid bilayer known as the plasma membrane (16). It completely surrounds the cytoplasm and primarily functions as a selectively permeable barrier responsible for regulating what enters and exits the cell. The plasma membrane is also involved in signal transduction, protein transport, division, and other essential processes.

Most bacteria use the type II fatty acid synthase system (FAS II) to begin synthesizing membrane lipids. This pathway consists of an initiation phase followed

Figure 1.1: The Gram positive bacterial cell envelope. A. Overall organization of the cell envelope. B, C, & D. Detailed structures of B. peptidoglycan, C. membrane lipids, and D teichoic acids. GlcNAc and MurNAc refer to the repeating subunits of peptidoglycan, N-acetylglucosamine and N-acetylmuramic acid respectively. The R group of phospholipids represents H for phosphatidic acid (PA), CH_2-CHOH-CH_2OH for phosphatidylglycerol (PPG), CH_2-CHOH-CH_2-Lys for lysyl-phosphatidylglycerol (lys-PPG), and CH_2-CH_2-NH_3^+ for phosphatidylethanolamine (PE). The glycolipid shown has a single glucosyl residue, but di-, and tri-glucosyl residues are possible. The X group of newly synthesized teichoic acids is H, but can be modified to D-alanyl or α-glycosyl for wall teichoic acid (WTA) and to D-alanyl, α-GlcNAc, or α-Galactosyl for lipoteichoic acid (LTA). Adapted from (68) and (81).


A
Wall Teichoic Acid (WTA)
Lipoteichoic Acid (LTA)
Peptidoglycan (PG)
Cytoplasmic Membrane (CM)
B
GlcNAc
MurNAc
~GlcNAc-β(1,4)-MurNAc~
C
Phospholipid
Glycolipid
D
Lipoteichoic Acid
Wall Teichoic Acid
Peptidoglycan

by progressive elongation cycles (76, 104). The initiation phase is characterized by a key step in which a FabH enzyme catalyzes the condensation of an acyl-CoA with malonyl-ACP to form a ketoacyl-ACP. This ketoacyl-ACP serves as the first substrate for the elongation phase, a cyclic series of reactions that add two carbons to the growing FA chain for every round of elongation (104). In *B. subtilis*, each elongation cycle is initiated by FabF which condenses the growing ketoacyl-ACP product with malonyl-ACP until the fatty acid reaches the desired length, usually between 14 and 18 carbons long (74). The long chain acyl-ACP products are then transferred to the membrane where acyltransferases convert them to phosphatidic acid (PA). PA is a phosphoglycerol containing two fatty acid chains that serves as a central intermediate in lipid synthesis.

In *B. subtilis*, PA matures into either the phospholipids, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), lysylphosphatidylglycerol (LPG), and cardiolipin (CL), or into various glycolipids (GL) (74). These lipid components are primarily differentiated by the structure and charge of their head groups. LPG maintains a net positive charge, PE and GL are neutral, and PG and CL have anionic character (25). In addition, CL is formed by the condensation of two PG molecules and is therefore much bulkier than the other lipids. The relative proportions of these different lipids are tightly regulated to maintain the optimum net charge and fluidity of the membrane relative to the extracellular environment (109). Lipid content is ~50% PE, 30% GL, 16% PG, 2.4% LPG, and 0.8% CL in actively growing cells (76), but PG and CL levels increase (to ~45% & 25%) and the amount of PE reduces (to 20%) as they approach stationary phase (60). Presumably, these adjustments are a

response to changing environmental conditions associated with stationary phase (e.g. nutrient limitation).

The plasma membrane displays substantial heterogeneity, with specific proteins and lipids aggregating in lipid domains (22, 61). These domains are known as lipid rafts and have been shown to play roles in sporulation, biofilm formation, signal transduction, and other cell processes. A key factor in the formation of these lipid rafts are the NfeD proteins (30). Bioinformatic analysis has shown that NfeD homologs are present in most bacterial genomes and typically associate with a member of the SPFH (stomatin-prohibitin-flotillin-HflC/K) protein family. This family includes flotillins which are central to the formation of lipid rafts in eukaryotes (88). *B. subtilis* contains two NfeD proteins, YuaF and YqeZ, which are co-transcribed with corresponding flotillin homologs, FloT (YuaG) and YqfA, respectively. Previous studies have shown that both FloT and YqfA localize to discrete foci in the membrane and contribute to lipid raft formation and function (22, 61).

1.1.2: The cell wall

The Gram positive cell wall is characterized by a thick outer layer of peptidoglycan consisting of N-acetylglucosamine (GlcNAc) N-acetylmuramic acid (MurNAc) disaccharide repeats (96). MurNAc is attached to a short peptide chain that can cross-link to the peptide chain of another peptidoglycan subunit to form a connected network of glycan strands across the entire cell. This network is essential for maintaining cell shape and preventing lysis due to the osmotic pressure of the cytoplasm.

Synthesis of the cell wall begins inside the cell. The MurA-F ligases initially produce uridine diphosphate N-acetylmuramic acid (UDP-MurNAc) pentapeptide (73). MraY then attaches this molecule to undecaprenyl phosphate carrier lipid (UP) to form lipid I (UPP-MurNAc). A GlcNAc molecule is subsequently transferred to lipid I by MurG to form lipid II which contains a complete PG subunit attached to undecaprenyl pyrophosphate (UPP). Lipid II translocates to the outer membrane where the PG subunit is exposed to high molecular weight penicillin binding proteins that have both transglycolase and transpeptidase activities. Transglycosylases incorporate the GlcNAc-MurNAc-pentapeptide from lipid II onto a peptidoglycan strand and transpeptidases cross-link the stem peptides. Meanwhile, UPP is recycled back to UP and transported to the cytoplasm. As peptidoglycan is synthesized, it is simultaneously broken down by peptidoglycan hydrolases called autolysins (28). This dynamic feature allows for cell growth, sporulation, motility, and other essential cell processes.

In vegetative *B.subtilis* cells, the cell wall is approximately 10-20 layers thick and an individual PG polymer contains an average of 97 disaccharides (28). Each peptide side chain consists of L-Ala-D-Glu-mDAP-D-Ala-D-Ala with cross linking occuring between the 3rd (mDAP) and 4th (D-Ala) amino acids of adjacent side chains. About 30% of PG side chains are cross-linked in non-stressed *B. subtilis* cells, but this percentage can vary depending on the growth conditions.

1.1.3: Teichoic acids

In *B. subtilis* and most other Gram positive bacteria, a major proportion of the cell envelope consists of teichoic acids (TAs). TAs are glycopolymers that extend into

and beyond the cell wall (81). They can either be covalently attached to glycolipids that insert into the membrane (lipoteichoic acid or LTA) or to peptidoglycan (wall teichoic acid or WTA). *B. subtilis* also produces teichuronic acid under low-phosphate conditions, but since this polymer is a response to nutrient limitation rather than envelope stress, it will not be discussed further (102). LTA and WTA are involved in many critical cell functions including cation homeostasis, division, host infection, autolysis, biofilm formation, and antibiotic resistance (102). They are also a major determinant of the elasticity, porosity, shape, and strength of the cell wall (81). Neither WTA nor LTA alone are essential to *B. subtilis*, but a strain lacking both is not viable and LTA- or WTA-depleted cells are growth deficient. Although LTA and WTA have similar functions, they independently contribute to the cell envelope and are synthesized by completely different pathways.

In *B. subtilis*, WTA synthesis is initiated in the cytoplasm by TagO which transfers a GlcNAc phosphate to membrane-anchored undecaprenyl phosphate (99). This molecule is converted into a mature TA by the TagABEF enzymes with TagF solely responsible for attaching glycerol phosphates to the linkage unit to form the polymer. The TagGH ABC transporter then exports the mature TA from the cell. When the exported TA reaches the cell wall, one of three LCP transferases (TagT, TagU, or TagV) covalently attach the phosphate group of the GlcNac residue to peptidoglycan to complete WTA synthesis (53).

The synthesis of LTA begins when PgcA (phosphoglucomutase) and GtaB (α-glucose-1-phosphate uridyltransferase) convert glucose-6-phosphate to UDP-glucose (86). The glucose moieties from two UDP-glucose molecules are then transferred to

diacylglycerol (DAG) by the glycosyltransferase UgtP to form the glycolipid Glc_2-DAG. This glycolipid is transferred to the outer leaflet of the cytoplasmic membrane where it serves as a foundation for the polyglycerolphosphate chain. LTA synthases are then responsible for creating this chain. *B. subtilis* has 3 functional LTA synthases, LtaS, LtaSa, and YqgS (107). All of these Mn^{2+}-dependent enzymes cleave the glycerolphosphate head group from phosphatidylglycerol and attach it to the growing LTA chain. LtaS is regarded as the primary LTA synthase responsible for housekeeping functions and the bulk of LTA synthesis, LtaSa is considered the stress induced LTA synthase because its located downstream of a σ^M dependent promoter, and YqgS is thought to play a role in sporulation (94). *B. subtilis* may utilize multiple LTA synthases to optimize LTA for various growth or stress conditions. For example, LtaSa synthesizes longer LTAs than those produced by LtaS or YqgS which may help make the cell envelope less permeable to antimicrobial agents (107). *B. subtilis* also has an LTA primase, YvgJ, which can only transfer the initial glycerol-phosphate subunit onto the membrane-embedded glycolipid (107). However, this priming activity is not necessary for the LTA synthases to function correctly.

Once synthesized, TAs can be further modified by substituting the hydroxyl groups of polyglycerol phosphate monomers with various functional groups. The most well-studied example of this is the D-alanylation of LTA and WTA through the activity of the Dlt proteins (82). Most Gram positive bacteria D-alanylate a significant proportion of TAs and can vary the degree of D-alanylation to modify the charge of the cell envelope (81). Other modifications include substitutions of α-GlcNAc or α-galactosyl in LTAs and α-glycosyl in WTAs.

1.2: Stresses that target the cell envelope

This dissertation references a variety of stresses that target the cell envelope. Some inhibit the cell wall, others disrupt the phospholipid membrane, and many have multiple functions. The cell envelope active agents that are relevant to later chapters will be described in more detail here.

1.2.1: Cell wall active agents

The cell wall is a prime target for antibiotics because it is highly conserved among bacteria but not present in animals. Most cell wall antibiotics inhibit peptidoglycan synthesis (55). Such inhibition does not harm the cell directly, but growth, division, and autolysins will weaken a cell wall in this state which can lead to lysis from osmotic pressure (89). From the discovery of penicillin, our history of antibiotic use has involved cell wall active agents and a majority of the antibiotics in use today target the cell wall (95). Structures of all the compounds discussed below are shown in Figure 1.2.

β -lactam antibiotics

β-lactam antibiotics, such as the cephalosporins and penicillins are characterized by the presence of a β-lactam ring (67). This ring structurally mimics the D-ala-D-ala residues of PG side chains. The PBPs that normally interact with the D-ala-D-ala residues will form a complex with these antibiotics. Complex formation is irreversible and inactivates the PBPs, thereby reducing the amount of PG cross-linking by transpeptidases.

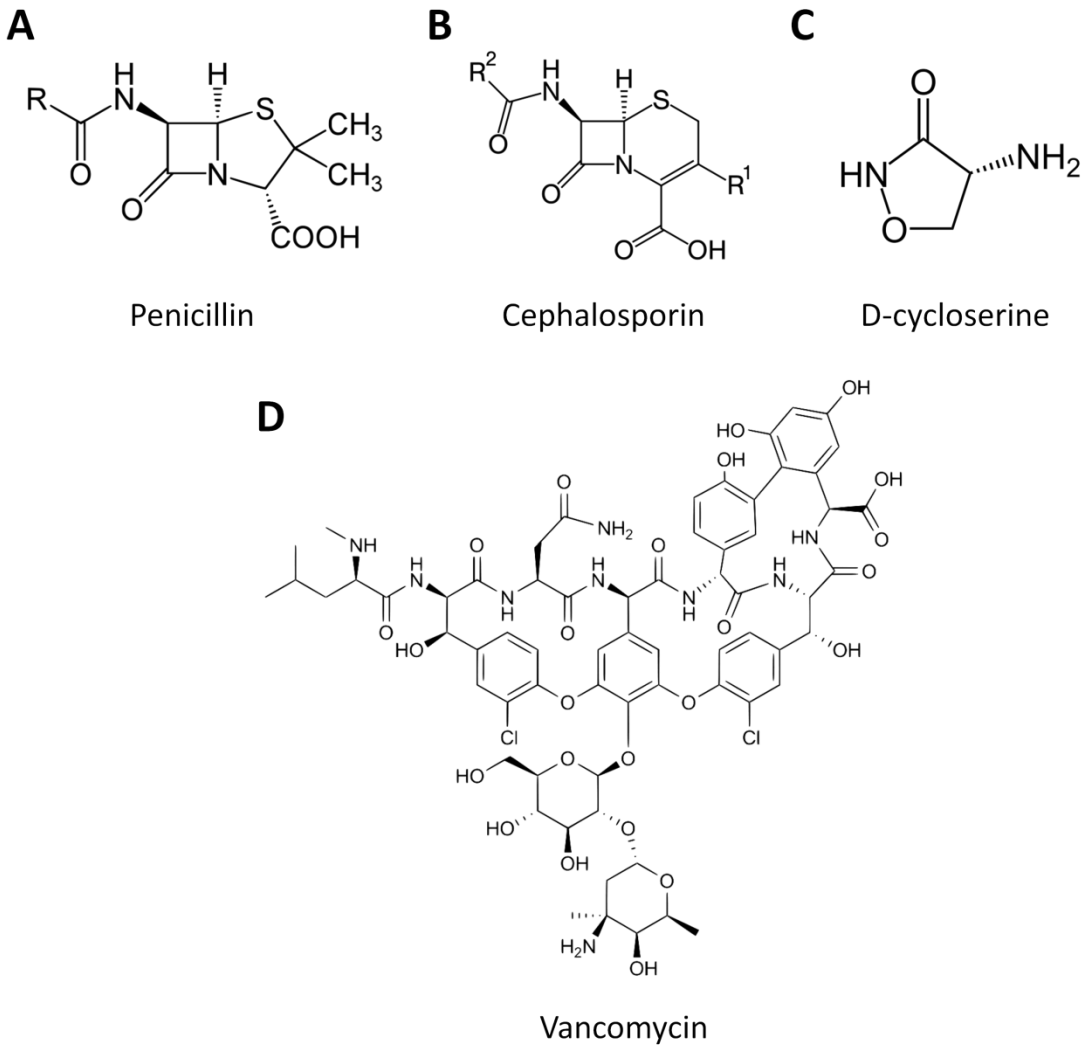


Figure 1.2: Structures of cell wall antibiotics A. penicillin, B. cephalosporin, C. D-cycloserine, and D. vancomycin. For penicillin and cephalosporin, only the core structure is shown with R, R^1, and R^2 representing variable function groups.

Vancomycin

Vancomycin is a glycopeptide antibiotic active against a broad range of Gram positive bacteria. It disrupts cells by binding to the terminal D-ala-D-ala resides of uncrosslinked side chains on lipid II (87). This sterically hinders both the transglycosylation and transpeptidation activities of PBPs necessary for peptidoglycan synthesis.

D-cycloserine

D-cycloserine is a D-alanine analog and therefore competitively inhibits D-alanine racemace and D-alanine-D-alanine ligase. Both of these enzymes are necessary for synthesizing the terminal D-ala-D-ala resides on peptidoglycan side chains (26). Since the side chains are synthesized in the cytoplasm, D-cycloserine must permeate the cell envelope before it can disrupt the cell.

Despite the effectiveness of these antibiotics, their widespread use has caused many pathogenic bacteria to evolve into strains that are resistant to cell wall active agents (80). As a result, many researchers are searching for new antibiotics that inhibit other aspects of bacterial physiology.

1.2.2: Membrane active agents

The cell membrane is an attractive antibiotic target for several reasons. Since bacterial cell membranes are substantially different than eukaryotic membranes (35), the potential for host toxicity with a membrane antibiotic is relatively low. The

essential nature of the cell membrane also hinders the development of resistance (48). In addition, membrane stresses do not typically require cell growth to be active and can be effective at treating slow growing persistent infections like *Mycobacterium tuberculosis* (48). Structures of the membrane active agents discussed below are shown in Figure 1.3.

Detergents

Detergents are a universal source of membrane stress. Their amphiphilic nature allows them to integrate into the outer leaflet of the plasma membrane (40). This creates a positive curvature in the membrane which reduces membrane order, density, and thickness. These effects lead to a loss of membrane stability which can result in increased permeability or pore formation. At higher concentrations, detergents will completely solubilize the membrane into mixed micelles (42, 59). Common detergents include triton X-100, sodium dodecyl sulfate, and amitriptyline. In addition, many organisms employ detergents or detergent-like compounds as antibacterial agents. The bile salts produced by mammals and lipopeptides produced by bacteria are two prominent examples.

Daptomycin

Daptomycin is a lipopeptide consisting of a 10 cyclic amino acids and three exocyclic residues connected to a fatty acid moiety (3). It has a net charge of -3, but complexes with Ca^{2+} which increases its attraction toward the anionic cell envelope. When daptomycin encounters the cell envelope, its fatty acid side chain inserts into

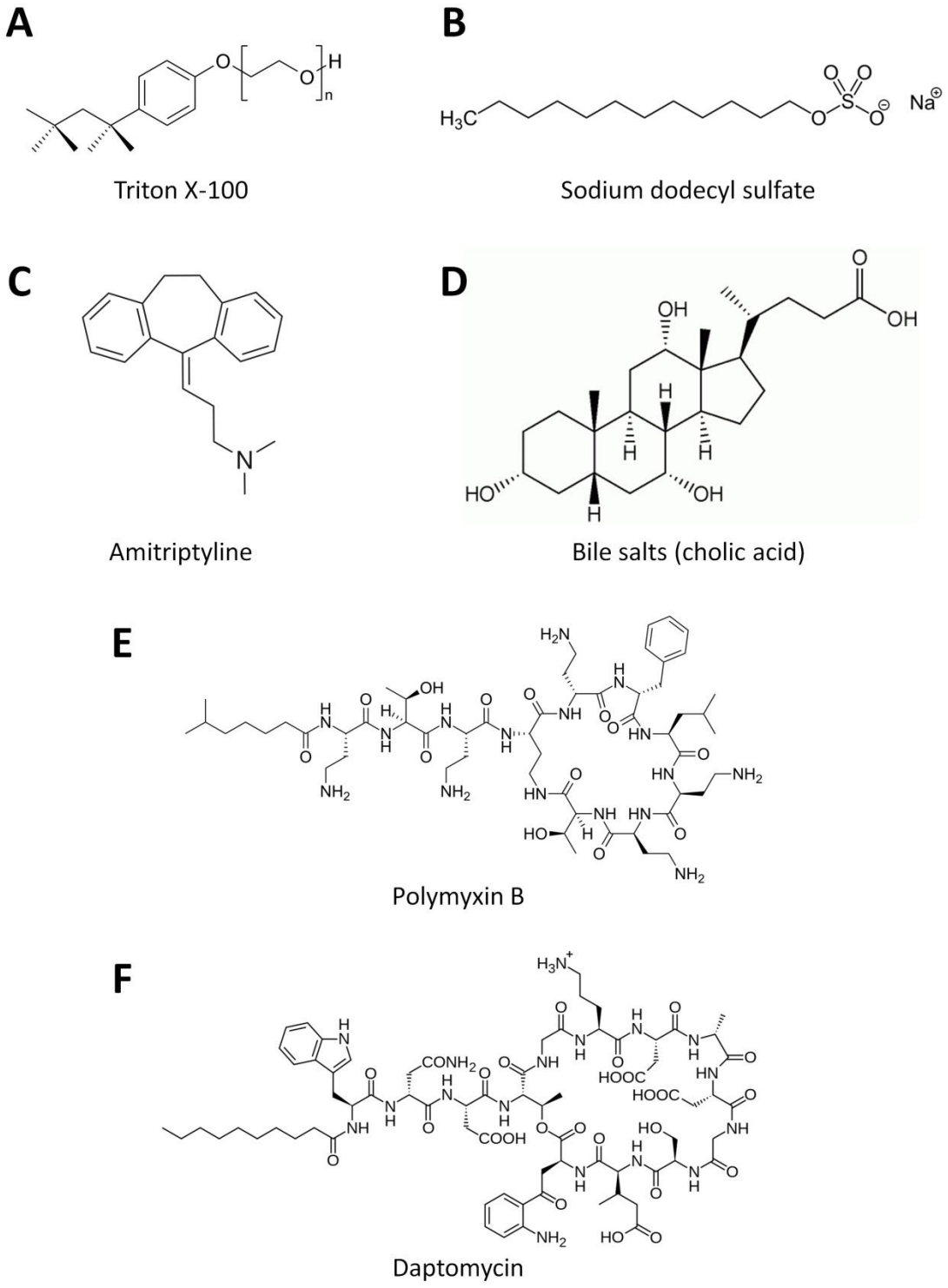


Figure 1.3: Structures of membrane active agents A. triton X-100, B. sodium dodecyl sulfate (SDS), C. amitryptline, D. bile salts, E. polymyxin B, and F. daptomycin.

the plasma membrane leading to pore formation, depolarization and eventual cell death. It is believed to interact preferentially with PG over other membrane lipids (34).

Polymyxins

The polymyxins (eg. polymyxin B and colistin) are similar to daptomycin in that they consist of a peptide ring connected to a tripeptide side chain with a fatty acid tail but differ from daptomycin in that they are cationic (net charge = +5) (108). Although polymyxins are primarily used against Gram negative bacteria, they are active against *B. subtilis* (71). When Polymyxins interact with a bacterium, they damage the cell in two distinct ways. The peptide ring binds divalent cations that would otherwise aid in stabilizing the cell envelope and the fatty acid chain forms pores in the membrane.

1.2.3: Antimicrobial peptides

Antimicrobial peptides are a diverse group of molecules that contain an amino acid backbone and function as potent antibiotics. These compounds are synthesized by all forms of life as defensive mechanisms against pathogens or competing microbes (35). They exhibit diverse modes of action ranging from disruption the cell envelope (48, 95) to inhibition of cytoplasmic components (35). Despite this diversity, antimicrobial peptides tend to be cationic, which attracts them to the negatively charged bacterial cell envelope, and amphiphilic, which allows them to associate with membranes (35). The prevalence and broad spectrum activity of antimicrobial peptides

has led to considerable interest in developing them for clinical and industrial applications.

Many of these antimicrobial compounds are bacteriocins, peptides produced by one bacteria that target another bacteria (15). They can include everything from small lipopeptides like polymyxin and daptomycin to large heat-labile proteins like peptidoglycan hydrolysases (15). A sub-group of bacteriocins called lantibiotics (class I bacteriocins) are the focus of Chapter 3 and will be reviewed in detail below.

Lantibiotics

The term lantibiotic refers to antimicrobial peptides that contain thioether-bridged amino acids called lanthionines. These lanthionine residues arise from the posttranslational dehydration of serines and/or threonines followed by cyclization with a cystine residue which introduces intramolecular rings within a peptide. Lantibiotics are ribosomally synthesized by Gram positive bacteria and are primarily involved in interspecies competition against other Gram positive microbes (84). They have become promising candidates for clinical applications because they are effective against numerous pathogens (6, 18, 27, 66) and share similarities with defensins and other human antimicrobial peptides (83, 91, 97).

The most widely used and extensively studied lantibiotic is nisin, which is produced by *Lactococcus lactis*. It is 34 amino acids long, has a charge of +5, and contains 5 lanthionine rings (Figure 1.4, Table 1.1). This lantibiotic is active against a broad spectrum of Gram positive bacteria and has been used as a food preservative for almost 60 years (15, 19). The extensive history of nisin has led researchers to

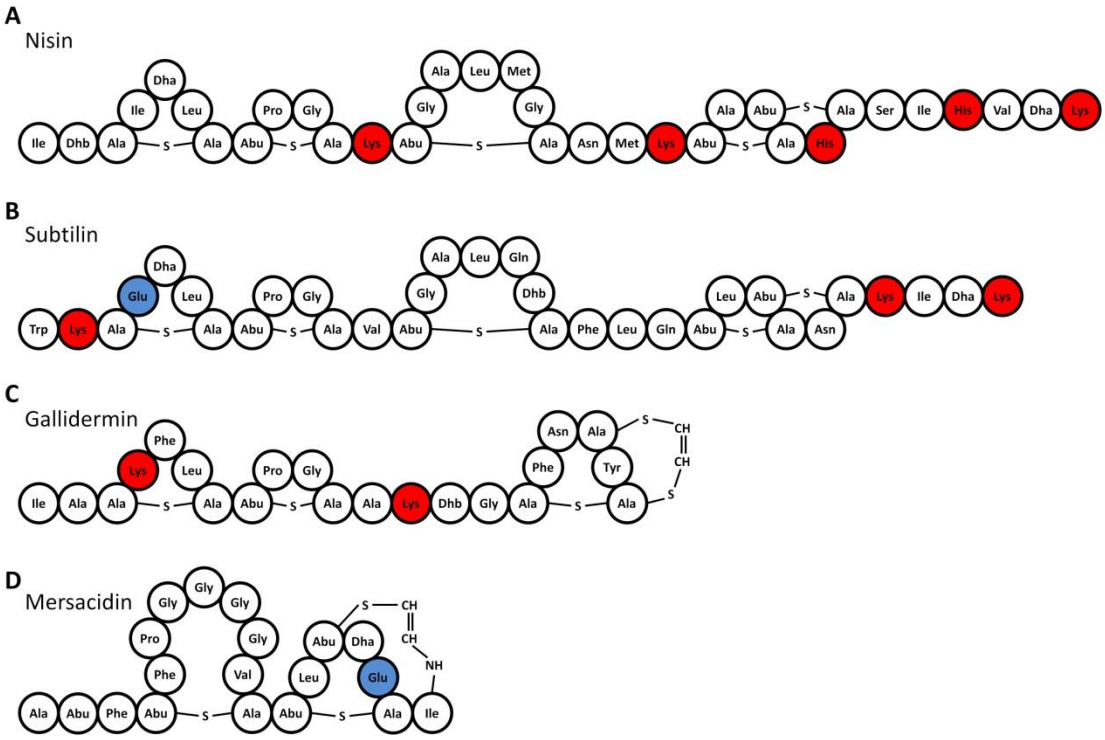


Figure 1.4: Structures of the lantibiotics A. nisin, B. subtilin, C. gallidermin, and D. mersacidin. Abu refers to -aminobutyric acid, Dha refers to 2,3-didehydroalanine, and Dhb refers to 2,3-didehydrobutyrine. Red residues are basic, blue residues are acidic.

Table 1.1 Characteristics of select lantibiotics

Lantibiotic	Producing Strain	Type	Charge	Lipid II Binding	Pore forming
Nisin	*Lactococcus lactis*	A (I)	+5	Yes	Yes
Subtilin	*B. spizizenii ATCC 6633*	A (I)	+2	Yes	Yes
Gallidermin	*Staphylococcus gallinarum*	A (I)	+2	Yes	No[a]
Mersacidin	*Bacillus sp. HIL Y-85*	B	-1[b]	Yes	No

[a.] Although gallidermin does not readily form pores in the membrane, it still inserts its C-terminal end into the membrane

[b.] Mersacidin complexes with Ca^{2+}, so its functional net charge is higher than its actual charge.

acknowledge that it is a prototypical lantibiotic that serves as a model antimicrobial peptide (4).

Nisin is an effective lantibiotic because it employs a potent mechanism of action. The cationic residues of nisin initially attract this molecule towards the anionic cell membrane. Upon reaching the membrane, the N-terminal region of nisin functions as a pyrophosphate cage that binds to lipid II with high affinity (39). The lanthionine rings are essential for forming this lipid II binding motif (45). Since lipid II is required for peptidoglycan synthesis, nisin binding inhibits cell wall synthesis. While the N-terminal region of nisin is bound to lipid II, its C-terminal end inserts into the membrane. At high enough concentrations of nisin, multiple nisin and lipid II molecules will combine into a complex that forms pores in the membrane (106). A flexible hinge region in the middle of the peptide allows both membrane insertion and lipid II binding to occur at the same time (4). Since nisin damages cells in two distinct ways, by inhibiting cell wall synthesis and disrupting cell membrane, it is referred to as having a dual mechanism of action.

Most lantibiotics share all or part of this dual mechanism of action. Those that bind lipid II and form pores in the membrane are usually type A (I) lantibiotics like subtilin, ericin, gallidermin, epidermin and entianin (106). This class of lantibiotic consists of linear peptides synthesized by separate dehydratase and cyclase enzymes. Most other lantibiotics are classified as type B lantibiotics. They are globular peptides, dehydrated and cyclized by a single enzyme, that typically bind lipid II, but do not integrate into membranes (49). Mersacidin and actagardine fall under this category.

However, not all lantibiotics function similarly to nisin. Some, like duramycin, do not bind to lipid II, and others, like SapB from *Streptomyces coelicolor*, lack antibacterial activity and perform alternative functions (4).

The lantibiotics nisin, subtilin, gallidermin, and mersacidin are all involved in Chapter 3 of this thesis. The structures of these lantibiotics are shown in Figure 1.4, and Table 1.1 compiles their relevant characteristics.

1.3: The cell envelope stress responses of *B. subtilis*

B. subtilis encounters a diverse array of antimicrobial compounds produced by competing microbes in nature. Since these compounds often target components of the cell envelope, *B. subtilis* has evolved numerous resistance mechanisms against cell envelope stress and even low levels of cell envelope active compounds can induce complex CESR (51). In *B. subtilis*, these CESR consists primarily of two component regulatory systems (TCS) and extracytoplasmic function sigma (ECF σ) factors (Figure 1.5) (51).

1.3.1: Two component systems

TCS are phosphotransfer signal transduction pathways that consist of a membrane-bound histidine kinase (HK) and its corresponding response regulator (RR) (98). The HK typically contains an extracellular N-terminal sensing domain that can detect environmental stimuli. Once a stimulus is detected, the cytoplasmic C-terminal domain phosphorylates a specific RR. The phosphorylated RR then modifies the

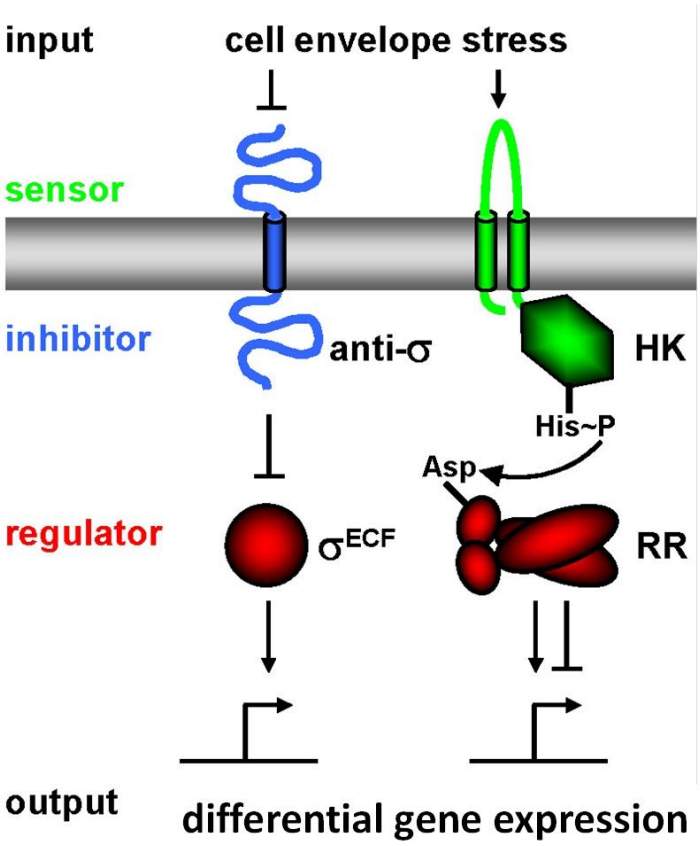


Figure 1.5. Model of *B. subtilis* CESR regulated by ECF σ factors (left) and TCS pathways (right). (HK = histidine kinase; RR = response regulator). Adapted from (51).

expression of specific genes. The simplicity of this system has allowed bacteria to evolve numerous TCS to respond to a variety of stresses (105).

Although *B. subtilis* has at least 35 putative TCSs (57), only the LiaRS TCS will be discussed in detail (Chapter 3). In this system, the HK LiaS detects stresses that interfere with the undecaprenol cycle (bacitracin, nisin, vancomycin, etc) and phosphorylates the RR LiaR (72). LiaR will then activate the expression of the *liaIHGFSR* and the *yhcYZyhdA* operons by binding to a conserved palindromic DNA sequence (52). In addition, LiaF negatively regulates this system. Since TCS are not a focus of this thesis, the rest of this section will review ECF σ factors.

1.3.2: Extracytoplasmic function sigma factors

Sigma (σ) factors are dissociable subunits of the bacterial RNA polymerase holoenzyme that are required for transcription initiation. Their primary role in transcription initiation is to recognize the -10 and -35 elements of DNA promoters (5, 78). All bacteria contain at least one primary sigma factor responsible for expressing the genes that maintain housekeeping functions (σ^A in *B. subtilis*). In addition, many bacteria utilize alternative σ factors to activate a specific set of genes in response to particular stimuli.

This dissertation focuses on a subfamily of alternative σ factors called the extracytoplasmic function (ECF) σ factors. The term "extracytoplasmic function" is used to describe this group of σ factors because they all activate the expression of genes associated with the cell envelope (44). Most ECF σ factors are inactive under

normal conditions due to sequestration by corresponding membrane-bound anti-σ factors. However, specific environmental stresses can trigger a CESR in which conformational changes or regulated intramembrane proteolysis of the anti-σ factor result in the release of the ECF σ factor (41). Once released, the σ factor is free to activate the transcription of genes that will presumably resist the stress that originally activated it.

The *B. subtilis* genome contains seven known ECF σ factors: σ^M, σ^W, σ^V, σ^X, σ^Y, σ^Z, and σ^{YlaC}. Of these seven, the most active and best characterized are σ^M, σ^W, and σ^X. Together, these three σ factors protect against numerous environmental stresses including antibiotics and detergents that interfere with cell envelope synthesis or function (44). σ^M, σ^W, and σ^X exhibit a moderate level of basal activity under normal growing conditions and can be induced by a variety of cell envelope stresses. Some stresses only activate one σ factor (moenomycin & σ^M) (92), while other stresses can stimulate a strong response from all three ECF σ factors (Triton X-100, vancomycin) (13, 44). Their known regulons, or the genes activated by a specific ECF σ factor, consist of ~30-70 genes each (10, 12, 24, 110), but the three σ factors only regulate a total of ~120 known genes because they exhibit a significant amount of regulatory overlap. Certain genes are activated by multiple ECF σ factors, and resistance to some cell envelope stresses like D-cycloserine, polymyxin B, and nisin can only be completely abrogated by deleting multiple σ factors from *B. subtilis* (71).

Despite this overlap, each of these σ factors have specialized functions. σ^M activates a numerous genes involved in cell envelope synthesis and confers resistance

to cell wall antibiotics like moenomycin, aztreonam, and cefuroxime (24, 65). The σ^W regulon includes genes encoding proteins that can modify the cell membrane and defend against antimicrobial compounds produced by other bacteria like fosfomycin and sublancin (7, 12, 43). Finally, many of the genes activated by σ^X alter cell surface properties to prevent antimicrobial compounds from penetrating the cell envelope (10).

The other four ECF sigma factors, σ^V, σ^Y, σ^Z, and σ^{YlaC} are thought to play relatively minor roles in the CESR of *B. subtilis*. They are inactive under normal conditions and deleting them has little effect on cell envelope stress sensitivity (64, 71). The most well understood of these four is σ^V which is primarily activated by, and provides resistance to, lysozyme (31). Previous studies have implicated σ^Y in preventing the loss of the SPβ prophage (75) and σ^{YlaC} in resistance against reactive oxygen species (90). In addition, σ^Y is induced by nitrogen starvation and may affect sporulation. The inducers and functions of σ^Z are unknown, and it is the only ECF σ factor in *B. subtilis* that lacks a corresponding anti-σ factor and cannot autoregulate its own expression.

Although many of the genes activated by each ECF σ factor have been identified, we may be unaware of additional ECF σ-activated genes that play critical roles in CESR. One strategy for uncovering novel ECF σ-regulated genes is a promoter consensus search. As mentioned previously, ECF σ factors activate transcription by binding to specific recognition elements called promoters (71). Computational analysis of each known promoter for the three most active ECF σ

factors (σ^M, σ^W, and σ^X) has given a consensus sequence that exhibits high conservation in both the -35 and -10 elements (Figure 1.6). We can search the *B. subtilis* genome for this sequence to identify novel ECF σ activated loci. In fact, one of the candidate ECF σ binding sites identified by this type of search is P_5, a novel promoter within *fabHA* and upstream of *fabF* that will be analyzed in Chapter 2.

1.3.3: Membrane Stress adaptations

One of the major goals of this work is to elucidate resistance pathways that adapt *B. subtilis* to cell membrane stress. This aspect of CESR is often overlooked because most of the antibiotics currently used to combat pathogens target the cell wall (95). However, bacteria naturally encounter many cell envelope active compounds that affect the biophysical properties of the phospholipid bilayer and have evolved numerous mechanisms to acclimatize to these stresses (109). The study of these mechanisms is becoming increasingly important as we begin to rely more on developing novel membrane targeting antibiotics (48).

Many membrane-oriented forms of CESR directly modify the plasma membrane to aid in resistance (109). Some modifications affect its net charge while others adjust its fluidity (21, 88). These adjustments are critical for maintaining the desired biophysical properties of the plasma membrane such as permeability of the lipid bilayer, protein mobility, protein-protein interactions and active transport processes (62). Changes in membrane composition are often exerted at the level of membrane synthesis (63). For example, *B. subtilis* has two FabH isozymes, that differ in their substrate specificities (14). Chapter 2 of this thesis explains how these

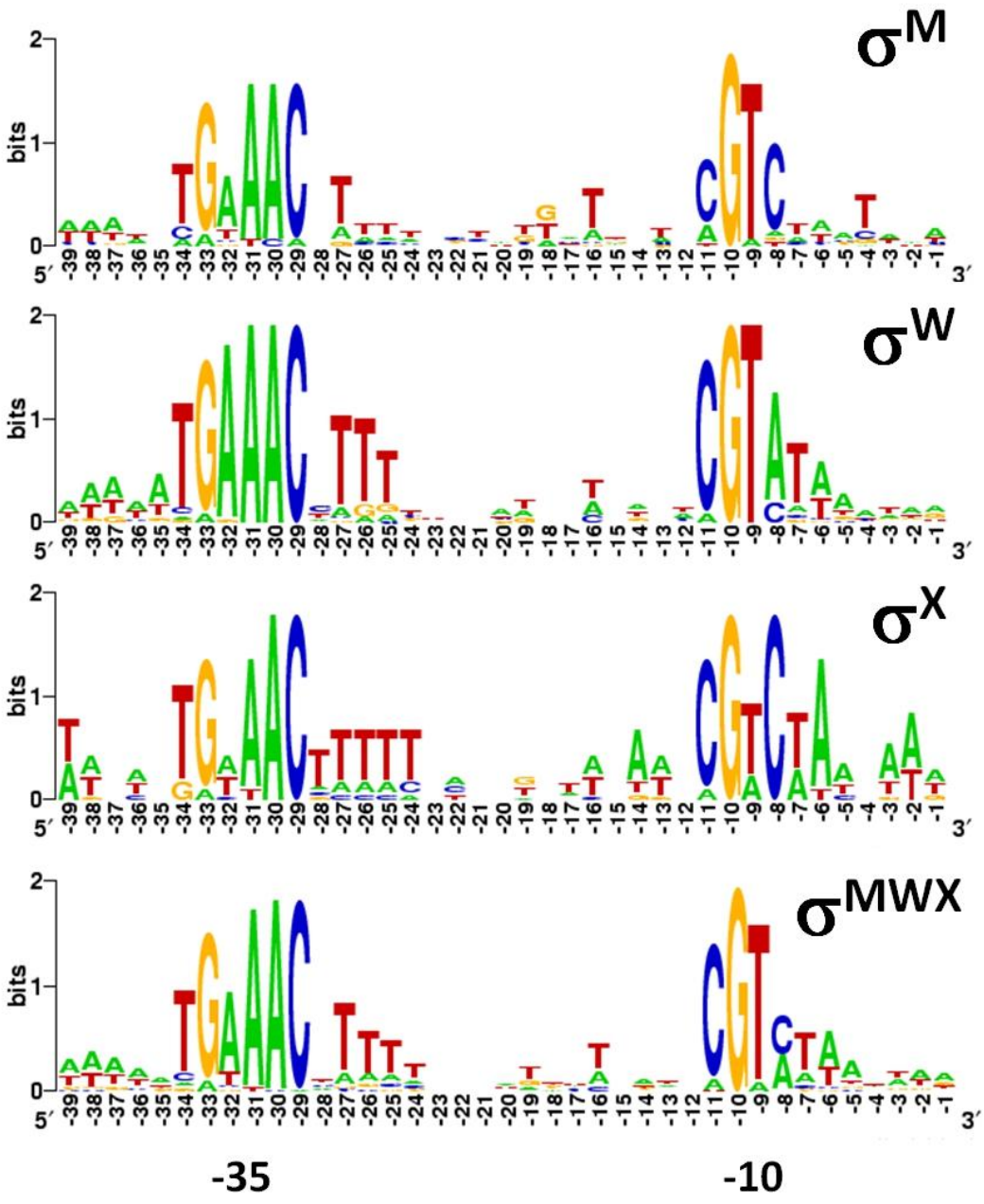


Figure 1.6: Consensus promoter sequences recognized by σ^{M}, σ^{W}, and σ^{X} individually, and a σ^{MWX} consensus sequence recognized all three of these ECF σ factors. The -10 and -35 elements are indicated. The degree of sequence conservation is represented by the height of the nucleotide(s) at that position. Created using the Weblogo service (http://weblogo.berkeley.edu/logo.cgi) and adapted from (71).

differences are exploited by *B. subtilis* to adjust the iso:anteiso ratio of lipids in the membrane. Bacteria can also influence membrane composition by modifying existing phospholipids which is more efficient and faster than de novo synthesis (17). A common example of this type of CESR in *B. subtilis* is the DesRK two component system which contributes to the universally conserved tendency of bacteria to maintain a constant membrane fluidity throughout changes in temperature (2, 63, 109). A decrease in temperature will lower membrane fluidity causing *B. subtilis* to trigger the activation of the DesK histidine kinase. This kinase will phosphorylate its cognate response regulator DesR which then activates the expression of *desA* encoding an acyl-desaturase that introduces a double bond into the fatty acid chains of existing phospholipids (69). Unsaturated FAs have greater steric hindrance than saturated FAs which help raise membrane fluidity to normal levels.

Proteins can also contribute to membrane stress resistance through interactions with the phospholipid bilayer. This kind of interaction is evident in the phage shock proteins of Gram negative bacteria. Psp systems are activated by agents that dissipate the proton motive force such as ethanol, temperature shifts, and organic solvents (18). Activated phage shock proteins then bind to the inner membrane and help reestablish proton motive force by blocking leakage (50). The keystone of this stress response is PspA which forms an oligomeric ring hypothesized to act as a scaffold that enhances the rigidity of the membrane. Another example of proteins that can influence the biophysical properties of the membrane through direct interaction is bacterial flotillins and NfeD proteins. As explained in section 1.1, these enzymes functionally organize the bacterial membrane and have been shown to affect membrane fluidity (58), protein

secretion (61), cell shape (20), and antibiotic resistance (7).

In *B. subtilis*, ECF σ factors and membrane stress adaptations are fundamentally connected. A central tenant of this connection is that a significant portion of the signaling pathways that activate ECF σ factors occur in the membrane. Anti-σ factors are membrane bound, as are many of the enzymes involved in releasing a sequestered σ factor, such as the proteases PrsW and RasP that activate σ^W through regulated intramembrane proteolysis of its anti-σ factor RsiW (41). This association with the membrane suggests that ECF σ factor activity can be affected by membrane perturbations or changes in lipid composition. Indeed, Both σ^M and σ^V are activated when the phosphatidylglycerol content of the membrane is reduced (38) confirming a connection between lipid composition and ECF σ factor activity. This may explain why membrane stresses like triton X-100, polymyxin B, and daptomycin stimulate an ECF σ response as well (43, 71, 103).

ECF σ factors are also known to activate genes involved in membrane synthesis and modification. The σ^M regulated *ytpAB* operon encodes the phosphatidylglycerol hydrolysis enzyme YtpA (24, 100), and the tetraprenyl-β-curcumene synthase YtpB which synthesizes unique membrane lipids known as sesquarterpenes (93). Both of these proteins have the capacity to alter membrane composition, but their roles are largely unexplored. σ^W regulates many proteins with membrane associated functions including the phage shock proteins PspA and YvlC (a PspC homolog), membrane proteases SppA YqeZ, and YjoB, fatty acid synthase enzymes FabHA and FabF (12), NfeD proteins (YuaF and YqeZ) (61) and flotillin

homologs (FloT and YqfA). Several studies have already hinted at some of the functions of these enzymes. The σ^W regulon limits membrane proteins overproduction which could be related its control over the membrane proteases SppA, YqeZ, and YjoB (111), σ^W-dependent regulation the Fab proteins, NfeD proteins, and flotillins has been shown to influence membrane fluidity (46, 54), and the Psp proteins may enhance membrane stability as evidenced by the contribution of PspA to resistance against the pore forming antibiotic daptomycin (33). Finally, PE synthesis genes *pssA, ybfM,* and *psd* are upregulated by σ^X which may represent a resistance mechanism that reduces the net negative charge of the bilayer to repel cationic antimicrobial compounds (10). Some of these membrane-associated genes have already been shown to contribute to cell envelope stress resistance. FloT resists cefuroxime (58), FloA protects against sublancin (7), and YtpB affects bacitracin sensitivity (personal observation). Furthermore, Chapters 2 and 3 attribute detergent and lantibiotic resistance mechanisms to several of these genes and future studies will likely uncover additional roles for them in mediating membrane stress.

1.3.4: Cell wall and teichoic acid stress adaptations

Cell envelope stress adaptations are not limited to those affecting the cell membrane. ECF σ factors activate many genes encoding proteins with cell wall- or TA-associated functions. Although these resistance pathways are not a primary focus of this work, they still merit discussion because modifications to the cell wall or TAs can indirectly affect the cell membrane and its ability to resist stress.

Numerous studies have shown that ECF σ factors and TAs are strongly

connected. LTA depletion in *B. subtilis* produces a strong ECF σ response with σ^M and σ^X induced under mild LTA depletion followed by induction of σ^W and σ^{ylaC} and, subsequently, σ^V and σ^Y as depletion becomes more severe (37). A similar response to WTA depletion in *Staphylococcus aureus* has been observed as well (8). In *B. subtilis*, several ECF σ factor-mediated stress responses alter TA synthesis or directly modify TAs. σ^X activates the *dlt* operon which increases the net charge of TAs via D-alanination. The alternative LTA synthase LtaSa is activated by σ^M (24) and is capable of producing LTA polymers that are different from those produced by the primary LTA synthase (107). Additionally, *tagT* (formerly, *ywtF*) and *tagU* (formerly, *lytR*), which encode two of the three enzymes in *B. subtilis* responsible for attaching WTAs to peptidoglycan (53), are partially regulated by σ^M and σ^X respectively (24, 47). Changes in TAs may prevent membrane stressors from reaching the bilayer. Activation of the *dlt* operon has been shown to repel membrane stressing cationic antimicrobial peptides in multiple Gram positive bacteria including *B. subtilis* (10), *Clostridium difficile* (74), *Lactococcus lactis* (56), *Staphylococcus aureus* (83). In *Streptococcus bovis*, a nisin resistant strain was found to produce longer and denser LTAs than WT cells (70). It should also be noted that LTAs have the potential to affect bilayer properties because they are embedded in the membrane and LTA synthesis consumes phosphatidylglycerol from the cell membrane thereby contributing to fatty acid turnover (109).

Direct connections between cell wall stress adaptations and membrane integrity are less clear, but both cell envelope components are dependent upon each other. A stable membrane requires a healthy cell wall since the cell membrane will

lyse without its support, and several essential steps in cell wall synthesis require a functional membrane. In addition, some stresses target both the cell wall and the cell membrane, and both targets must be taken into account to fully understand the CESR of *B. subtilis* to these stresses.

ECF σ factors are integral in mediating resistance against many cell envelope stresses. Most common cell wall antibiotics stimulate an ECF σ response which upregulates a number of cell wall associated genes. σ^M activates the cell shape determining proteins MreBCD & RodA, the MurB & MurF ligases, Ddl (D-alanine-D-alanine ligase), and the MinCD & DivIB proteins involved in septum formation while σ^X and σ^W induce the penicillin binding proteins PbpX and PbpE, respectively (10, 12, 24). Some ECF σ factor dependent proteins confer resistance against specific cell wall stresses. The UPP phosphatase BcrC (σ^M) resists bacitracin (11), bacillithiol-S-transferase FosB (σ^W) inactivates fosfomycin (9), and MurNAc specific O-acetyltransferase OatA (σ^V) reduces the affinity of lysozyme for peptidoglycan (31). ECF σ factors confer resistance against many other cell envelope stresses as well including moenomycin, azetreonam, D-cycloserine, cephalosporins, and penicillins, but precise resistance mechanisms for these stresses have yet to be defined (64, 72).

1.4: Assays to analyze membranes

Proper analysis of membrane stress responses requires specialized assays that can evaluate various properties of the cell membrane. Assays of this nature that will be employed in subsequent chapters are described in this section.

1.4.1 Fluorescence anisotropy

Fluorescence anisotropy is frequently used to analyze membrane fluidity. In this assay, a fluorescent probe is excited with polarized light and the intensities of polarized and depolarized light that are emitted from the molecule are measured (1). The key principle behind fluorescence anisotropy is that during the period between the excitation and emission of polarized light, a molecule can rotate to emit depolarized light (101). The faster that the probe rotates, the greater the proportion of emitted light that will be depolarized (29). To assess membrane fluidity with this technique, a fluorescent probe like 1,6-diphenyl-1,3,5-hexatriene (DPH) is used (79). This probe localizes to the hydrophobic core of the lipid bilayer and orients itself parallel to fatty acid side chains (101). In this orientation, the molecule's rotational freedom and hence depolarization levels are affected by changes in membrane dynamics (21, 85, 101) which can be observed with fluorescence anisotropy.

1.4.2: FAME and ESI-MS

Membrane composition can be accurately quantified with a combination of fatty acid methyl ester (FAME) analysis and electrospray ionization tandem mass spectrometry (ESI-MS). FAME analysis consists of the esterification of lipids, and their subsequent separation, identification, and quantification of esterified fatty acids by gas chromatography/mass spectrometry (23, 32, 77). It specifically provides information on iso:anteiso ratios, fatty acid chain lengths, and the degree of saturation within a membrane. However, FAME analysis is limited in that it does not distinguish

between different phospholipid and glycolipid species. To identify relative proportions of these species, ESI-MS is employed. ESI-MS is a common technique in which an electric field ionizes larger molecules with minimal fragmentation and subsequent mass spectrometry or tandem mass spectrometry provides enough information to identify them (36).

1.5: Content of this dissertation

This dissertation investigates CESR pathways with an emphasis on ECF σ factor mediated responses to membrane stress. The majority of the work presented here will be split up into two main chapters.

Chapter 2 focuses on P_5, a σ^W-dependent promoter located within *fabHa* and upstream of *fabF*. Activation of this internal promoter downregulates FabHa and upregulates FabF. Reduced FabHa levels increase the utilization of the FabHb paralog leading to a greater proportion of straight chain fatty acids in the membrane. Higher FabF production improves the probability that the synthesis of a fatty acid will include additional elongation cycles which raises the average fatty acid chain length. These changes in fatty acids reduce membrane fluidity which resists detergents and antimicrobial compounds produced by other bacteria.

Chapter 3 identifies and analyzes the ECF σ factor dependent lantibiotic resistance mechanisms in *B. subtilis*. Using nisin as a model, we showed that σ^M, σ^W, and σ^X all contribute to innate lantibiotic resistance. σ^X activates the *dlt* operon which D-alanylates TAs and σ^M upregulates *ltaSa* which increases the size and density of LTA. Both of these teichoic acid alterations decrease access of lantibiotics to its lipid

II target. The σ^W-regulated lantibiotic resistance genes principally protect against membrane permeabilization and include a signal peptide peptidase (SppA), phage shock proteins (PspA and YvlC), and proteins related to tellurite resistance (YceGHI).

These two chapters identify and characterize multiple physiologically relevant membrane stress resistance mechanisms in *Bacillus subtilis* that are mediated by ECF σ factors. In addition, the appendices cover several experiments that analyze ECF σ factor-dependent mechanisms that modify the plasma membrane but don't fit into a full story. Appendix A shows that the *yuaFGI* operon encoding both the NfeD protein YuaF and the flotillin YuaG (FloT) can reduce membrane fluidity when activated by σ^W, Appendix B presents evidence suggesting that the phospholipase YtpA resists daptomycin by reducing PG levels, and Appendix C identifies resistance mechanisms associated with the tetraprenyl-β-curcumene synthase YtpB. Overall, this work advances our knowledge of how Gram positive bacteria mediate cell envelope stress which adds to our understanding of interspecies competition and may contribute to the development of novel antibiotics.

REFERENCES

1. **Adler, M., and T. Tritton.** 1988. Fluorescence depolarization measurements on oriented membranes. Biophys J **53:**989-1005.

2. **Aguilar, P. S., and D. de Mendoza.** 2006. Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. Mol Microbiol **62:**1507-1514.

3. **Baltz, R. H.** 2009. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. Curr Opin Chem Biol **13:**144–151.

4. **Bierbaum, G., and H.-G. Sahl.** 2009. Lantibiotics: Mode of Action, Biosynthesis and Bioengineering. Curr Pharm Biotechno **10:**2-18.

5. **Borukhov, S., and K. Severinov.** 2002. Role of the RNA polymerase sigma subunit in transcription initiation. Res Microbiol **153:**557-562.

6. **Breukink, E., and B. d. Kruijff.** 2006. Lipid II as a target for antibiotics. Nat Rev Drug Discov **5:**321-332.

7. **Butcher, B. G., and J. D. Helmann.** 2006. Identification of *Bacillus subtilis* σ^W-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli. Mol Microbiol **60:**765-782.

8. **Campbell, J., A. K. Singh, J. G. Swoboda, M. S. Gilmore, B. J. Wilkinson, and S. Walkera.** 2012. An antibiotic that inhibits a late step in wall teichoic acid biosynthesis induces the cell wall stress stimulon in *Staphylococcus aureus*. Antimicrob Agents Ch **56:**1810–1820.

9. **Cao, M., B. A. Bernat, Z. Wang, R. N. Armstrong, and J. D. Helmann.** 2001. FosB, a cysteine-dependent fosfomycin resistance protein under the control of σ^W, an extracytoplasmic-function σ factor in *Bacillus subtilis*. J Bacteriol **183:**2380-2383.

10. **Cao, M., and J. D. Helmann.** 2004. The *Bacillus subtilis* extracytoplasmic-function σ^X factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. J Bacteriol **186:**1136-1146.

11. **Cao, M., and J. D. Helmann.** 2002. Regulation of the *Bacillus subtilis* bcrC bacitracin resistance gene by two extracytoplasmic function σ factors. J Bacteriol **184:**6123-6129.

12. **Cao, M., P. A. Kobel, M. M. Morshedi, M. Fang, and W. Wu.** 2002. Defining the *Bacillus subtilis* σ^W regulon: A comparative analsis of promoter consensus search, run-off transcription/macroarrray analysis (ROMA), and

transcriptional profiling approaches. J Mol Biol **315:**443-457.

13. **Cao, M., T. Wang, R. Ye, and J. D. Helmann.** 2002. Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ^W and σ^M regulons. Mol Microbiol **45:**1267-1276.

14. **Choi, K. H., R. J. Heath, and C. O. Rock.** 2000. β-ketyacyl-acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. J Bacteriol **182:**365-370.

15. **Cotter, P. D., C. Hill, and R. P. Ross.** 2005. Bacteriocins: Developing innate immunity for food. Nat Rev Microbiol **3:**777-788.

16. **Cronan, J. E.** 2003. Bacterial membrane lipids: Where do we stand? Annu Rev Microbiol **57:**203-223.

17. **Cronan, J. E.** 2002. Phospholipid modifications in bacteria. Curr Opin Microbiol **5:**202-205.

18. **Dawson, M. J., and R. W. Scott.** 2012. New horizons for host defense peptides and lantibiotics. Curr Opin Pharmacol **12:**545–550.

19. **Delves-Broughton, J.** 2005. Nisin as a food preservative. Food Aust **57:**525-527.

20. **Dempwolff, F., H. M. Wischhusen, M. Specht, and P. L. Graumann.** 2012. The deletion of bacterial dynamin and flotillin genes results in pleiotrophic effects on cell division, cell growth and in cell shape maintenance. BMC Microbiol **12:**298.

21. **Denich, T. J., L. A. Beaudette, H. Lee, and J. T. Trevors.** 2003. Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Meth **52:**149-182.

22. **Donovan, C., and M. Bramkamp.** 2009. Characterization and subcellular localization of a bacterial flotillin homologue. Microbiology **155:**1786-1799.

23. **Eder, K.** 1995. Gas chromatographic analysis of fatty acid methyl esters. J Chromatogr B **671:**113-131.

24. **Eiamphungporn, W., and J. D. Helmann.** 2008. The *Bacillus subtilis* σ^M regulon and its contribution to cell envelope stress responses. Mol Microbiol **67:**830-848.

25. **Fang, J., and M. J. Barcelona.** 1998. Structural determination and

quantitative analysis of bacterial phospholipids using liquid chromatography/ electrospray ionization / mass spectrometry. J Microbiol Meth **33:**23-35.

26. **Feng, Z., and R. G. Barletta.** 2003. Roles of *Mycobacterium smegmatis* D-alanine:D-alanine ligase and D-alanine racemase in the mechanisms of action of and resistance to the peptidoglycan inhibitor D-cycloserine. Antimicrob Agents Ch **47:**283-291.

27. **Field, D., M. Begley, P. M. O'Connor, K. M. Daly, F. Hugenholtz, P. D. Cotter, C. Hill, and R. P. Ross.** 2012. Bioengineered nisin A derivatives with enhanced activity against both Gram positive and Gram negative pathogens. PLOS One **7:**e46884.

28. **Foster, S. J., and D. L. Popham.** 2001. Structure and synthesis of cell wall, spore cortex, teichoic acids, S-layers, and capsules, p. 21-54. *In* J. A. Hoch, A. L. Sonenshein, and R. Losick (ed.), *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells. American Society for Microbiology, Washington D.C.

29. **Gradinaru, C. C., D. O. Marushchak, M. Samimab, and U. J. Krull.** 2010. Fluorescence anisotropy: from single molecules to live cells. Analyst **135:**452-459.

30. **Green, J. B., R. P. J. Lower, and J. P. W. Young.** 2009. The NfeD protein family and its conserved gene neighbours throughout prokaryotes: functional implications for stomatin-like proteins. J Mol Evol **69:**657–667.

31. **Guariglia-Oropeza, V., and J. D. Helmann.** 2011. Bacillus subtilis σ^V Confers Lysozyme Resistance by Activation of Two Cell Wall Modification Pathways, Peptidoglycan O-Acetylation and D-Alanylation of Teichoic Acids. J Bacteriol **193:**6223-6232.

32. **Haack, S. K., H. Garchow, D. A. Odelson, L. J. Forney, and M. J. Klug.** 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. Appl Environ Microbiol **60:**2483-2493.

33. **Hachmann, A. B., E. R. Angert, and J. D. Helmann.** 2009. Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. Antimicrob Agents Ch **53:**1598-1609.

34. **Hachmann, A. B., E. Sevim, A. Gaballa, D. L. Popham, H. Antelmann, and J. D. Helmann.** 2011. Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*.

35. **Hancock, R. E. W., and H.-G. Sahl.** 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol **24:**1551-

1557.

36. **Harkewicz, R., and E. A. Dennis.** 2011. Applications of mass spectrometry to lipids and membranes. Annu Rev Biochem **80:**301–325.

37. **Hashimoto, M., T. Seki, S. Matsuoka, H. Hara, K. Asai, Y. Sadaie, and K. Matsumoto.** 2012. Induction of extracytoplasmic function sigma factors in *Bacillus subtilis* cells with defects in lipoteichoic acid synthesis. Microbiol **159:**23-35.

38. **Hashimoto, M., H. Takahashi, Y. Hara, H. Hara, K. Asai, Y. Sadie, and K. Matsumoto.** 2009. Induction of extracytoplasmic function sigma factors in *Bacillus subtilis* cells with membranes of reduced phosphatidylglycerol content. Genes Genet Syst **84:**191-198.

39. **Hasper, H. E., N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. d. Kruijff, and E. Breukink.** 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science **313:**1636-1637.

40. **Heerklotz, H., T. Wieprecht, and J. Seelig.** 2004. Membrane perturbation by the lipopeptide surfactin and detergents as studied by deuterium NMR. J Phys Chem **108** 4909–4915.

41. **Heinrich, J., and T. Wiegert.** 2009. Regulated intramembrane proteolysis in the control of extracytoplasmic function sigma factors. Res Microbiol **160:**696-703.

42. **Helenius, A., and K. Simons.** 1975. Solubilization of membranes by detergents. BBA-Rev Biomembranes **451**.

43. **Helmann, J. D.** 2006. Deciphering a complex genetic regulatory network: The Bacillus subtilis σ^W protein and intrinsic resistance to antimicrobial compounds. . Sci Prog **89:**243-266.

44. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol **46:**47-110.

45. **Hsu, S.-T. D., E. Breukink, E. Tischenko, M. A. G. Lutters, B. d. Kruijff, R. Kaptein, A. M. J. J. Bonvin, and N. A. J. v. Nuland.** 2004. The nisin−lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nat Struct Mol Biol **11:**963 - 967.

46. **Huang, X., A. Gaballa, M. Cao, and J. D. Helmann.** 1999. Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function σ factor,

σ^W. Mol Microbiol **31:**361-371.

47. **Huang, X., and J. D. Helmann.** 1998. Identification of target promoters for the *Bacillus subtilis* sigma X factor using a consensus-directed search. J Mol Biol **279:**165-173.

48. **Hurdle, J. G., A. J. O'Neill, I. Chopra, and R. E. Lee.** 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol **9:**62-75.

49. **Islam, M. R., J.-i. Nagao, T. Zendo, and K. Sonomoto.** 2012. Antimicrobial mechanism of lantibiotics. Biochem Soc T **40:**1528-1533.

50. **Joly, N., C. Engl, G. Jovanovic, M. Huvet, T. Toni, X. Sheng, M. P. H. Stumpf, and M. Buck.** 2010. Managing membrane stress: the phage shock protein (Psp) response, from molecular mechanisms to physiology. FEMS Microbiol Rev **34:**797–827.

51. **Jordan, S., M. I. Hutchings, and T. Mascher.** 2008. Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol Rev **32:**107-146.

52. **Jordan, S., A. Junker, J. D. Helmann, and T. Mascher.** 2006. Regulation of LiaRS-dependent gene expression in *Bacillus subtilis*: identification of inhibitor proteins, regulator binding Sites, and target Genes of a conserved cell envelope stress-sensing two-component system. J Bacteriol **188:**5153-5166.

53. **Kawai, Y., J. Marles-Wright, R. M. Cleverley, R. Emmins, S. Ishikawa, M. Kuwano, N. Heinz, N. K. Bui, C. N. Hoyland, N. Ogasawara, R. J. Lewis, W. Vollmer, R. A. Daniel, and J. Errington.** 2011. A widespread family of bacterial cell wall assembly proteins. EMBO J **30:**4931-4941.

54. **Kingston, A. W., C. Subramanian, C. O. Rock, and J. D. Helmann.** 2011. A σ^W-dependent stress response in Bacillus subtilis that reduces membrane fluidity. Mol Micro **81:**69-79.

55. **Kohanski, M. A., D. J. Dwyer, and J. J. Collins.** 2010. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol **8:**423-435.

56. **Kramer, N. E., H. E. Hasper, P. T. C. v. d. Bogaard, S. Morath, B. d. Kruijff, T. Hartung, E. J. Smid, E. Breukink, J. Kok, and O. P. Kuipers.** 2008. Increased D-alanylation of lipoteichoic acid and a thickened septum are main determinants in the nisin resistance mechanism of *Lactococcus lactis*. Microbiology **154:**1755-1762.

57. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V.**

Azevedo, M. G. Bertero, P. Bessières, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S.-K. Choi, J.-J. Codani, I. F. Connerton, N. J. Cummings, R. A. Daniel, F. Denizot, K. M. Devine, A. Düsterhöft, S. D. Ehrlich, P. T. Emmerson, K. D. Entian, J. Errington, C. Fabret, E. Ferrari, D. Foulger, C. Fritz, M. Fujita, Y. Fujita, S. Fuma, A. Galizzi, N. Galleron, S.-Y. Ghim, P. Glaser, A. Goffeau, E. J. Golightly, G. Grandi, G. Guiseppi, B. J. Guy, K. Haga, J. Haiech, C. R. Harwood, A. Hénaut, H. Hilbert, S. Holsappel, S. Hosono, M.-F. Hullo, M. Itaya, L. Jones, B. Joris, D. Karamata, Y. Kasahara, M. Klaerr-Blanchard, C. Klein, Y. Kobayashi, P. Koetter, G. Koningstein, S. Krogh, M. Kumano, K. Kurita, A. Lapidus, S. Lardinois, J. Lauber, V. Lazarevic, S.-M. Lee, A. Levine, H. Liu, S. Masuda, C. Mauël, C. Médigue, N. Medina, R. P. Mellado, M. Mizuno, D. Moestl, S. Nakai, M. Noback, D. Noone, M. O'Reilly, K. Ogawa, A. Ogiwara, B. Oudega, S.-H. Park, V. Parro, T. M. Pohl, D. Portetelle, S. Porwollik, A. M. Prescott, E. Presecan, P. Pujic, B. Purnelle, et al. 1997. The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature **290:**249-256.

58. **Lee, Y. H., A. W. Kingston, and J. D. Helmann.** 2012. Glutamate dehydrogenase affects resistance to cell wall antibiotics in *Bacillus subtilis*. J Bacteriol **194:**993–1001.

59. **Lingwood, D., and K. Simons.** 2007. Detergent resistance as a tool in membrane research. Nat Protoc **2:**2159-2165.

60. **López, C. S., A. F. Alice, H. Heras, E. A. Rivas, and C. Sánchez-Rivas.** 2006. Role of anionic phospholipids in the adaptation of *Bacillus subtilis* to high salinity. Microbiol **152:**605-616.

61. **López, D., and R. Kolter.** 2010. Functional microdomains in bacterial membranes. Genes Dev **24:**1893-1902.

62. **Losa, D. A., and N. Murata.** 2004. Membrane fluidity and its roles in the perception of environmental signals. Biochim Biophys Acta **1666:**142-157.

63. **Lu, Y. J., Y. M. Zhang, and C. O. Rock.** 2004. Product diversity and regulation of type II fatty acid synthases. Cell Biol **82:**145-155.

64. **Luo, Y., K. Asai, Y. Sadaie, and J. D. Helmann.** 2010. Transcriptomic and phenotypic characterization of a *Bacillus subtilis* strain without extractyoplasmic function σ factors. J Bacteriol **192:**5736-5745.

65. **Luo, Y., and J. D. Helmann.** 2012. Analysis of the role of *Bacillus subtilis* σ^M in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan

homeostasis. Mol Micro **83:**623-639.

66. **M.Willey, J., and W. A. v. d. Donk.** 2007. Lantibiotics: Peptides of Diverse Structure and Function. Annu Rev Microbiol **61:**477–501.

67. **Macheboeuf, P., C. Contreras-Martel, O. D. Viviana Job, and A. Dessen.** 2006. Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. FEMS Microbiol Rev **30:**673-691.

68. **Madigan, M. T., and J. M. Martinko.** 2006. Brock Biology of Microorganisms, 11 ed. Pearson Prentic Hall, Upper Saddle River.

69. **Mansilla, M. C., L. E. Cybulski, D. albanesi, and D. de Mendoza.** 2004. Control of membrane lipid fluidity by molecular thermosensors. J Bacteriol **186:**6681-6688.

70. **Mantovani, H. C., and J. B. Russell.** 2001. Nisin resistance of *Streptococcus bovis*. Appl Environ Microbiol **67:**808-813.

71. **Mascher, T., A. B. Hachmann, and J. D. Helmann.** 2007. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. J Bacteriol **189:**6919-6927.

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

73. **Matteï, P.-J., D. Neves, and A. Dessen.** 2010. Bridging cell wall biosynthesis and bacterial morphogenesis. Curr Opin Struc Biol **20:**749-755.

74. **McBride, S. M., and A. L. Sonenshein.** 2011. The *dlt* operon confers resistance to cationic antimicrobial peptides in *Clostridium difficile*. Microbiology **157:**1457-1465.

75. **Mendez, R., A. Gutierrez, J. Reyes, and L. Márquez-Magaña.** 2012. The extracytoplasmic function sigma factor SigY is important for efficient maintenance of the Spβ prophage that encodes sublancin in *Bacillus subtilis*. DNA Cell Biol **31:**946-955.

76. **Mendoza, D. d., P. Aguilar, and G. Schujman.** 2001. Biosynthesis and function of membrane lipids, p. 43-55. *In* J. A. Hoch, A. L. Sonenshein, and R. Losick (ed.), *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells. American Society for Microbiology, Washington D.C.

77. **Mohedano, M. L., K. Overweg, A. d. l. Fuente, M. Reuter, S. Altabe, F. Mulholland, D. de Mendoza, P. Lopez, and J. M. Wells.** 2005. Evidence

that the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of fatty acid biosynthesis genes and alters membrane composition J. Bacteriol **187:**2357-2367.

78. **Murakami, K. S., and S. A. Darst.** 2003. Bacterial RNA polymerases: the wholo story. Curr Opin Struc Biol **13:**31-39.

79. **Mykytczuk, N. C. S., J. T. Trevors, L. G. Leduc, and G. D. Ferroni.** 2007. Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. Prog Biophys Mol Bio **95:**60-82.

80. **Neu, H. C.** 1992. The crisis in antibiotic resistance. Science **257:**1064-1073.

81. **Neuhaus, F. C., and J. Baddiley.** 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in Gram-positive bacteria. Microbiol Mol biol R **67:**686–723.

82. **Perego, M., P. Glaser, A. Minutello, M. A. Strauch, K. Leopold, and W. Fischer.** 1995. Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis.* Identification of genes and regulation. J Biol Chem **270:**15598-15606.

83. **Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Gotz.** 1999. Inactivation of the *dlt* Operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem **274:**8405-8410.

84. **Peschel, A., and H.-G. Sahl.** 2006. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol **4:**529-536.

85. **Petrackova, D., J. Vecer, J. Svobodova, and P. Herman.** 2010. Long-Term Adaptation of *Bacillus subtilis* 168 to Extreme pH Affects Chemical and Physical Properties of the Cellular Membrane. J Membrane Biol **233:**73-83.

86. **Reichmann, N. T., and A. Gründling.** 2011. Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum *Firmicutes*. FEMS Microbiol Lett **319:**97–105.

87. **Reynolds, P. E.** 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur J Clin Microbiol **8:**943-950.

88. **Rivera-Milla, E., C. A. O. Stuermer, and E. Málaga-Trillo.** 2006. Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain. Cell Mol Life Sci **63:**343-357.

89. **Rogers, H. J., and C. W. Forsberg.** 1971. Role of autolysins in the killing of bacteria by some bactericidal antibiotics. J Bacteriol **108:**1235-1243.

90. **Ryu, H.-B., I. Shin, H.-S. Yim, and S.-O. Kang.** 2006. YlaC is an extracytoplasmic function (ECF) sigma factor contributing to hydrogen peroxide resistance in *Bacillus subtilis*. J Microbiol **44:**206-216.

91. **Sahl, H.-G., U. Pag, S. Bonness, S. Wagner, N. Antcheva, and A. Tossi.** 2005. Mammalian defensins: structures and mechanism of antibiotic activity. J Leukocyte Biol **77:**466-475.

92. **Salzberg, L. I., Y. Luo, A. B. Hachmann, T. Mascher, and J. D. Helmann.** 2011. The *Bacillus subtilis* GntR family repressor YtrA responds to cell wall antibiotics. J Bacteriol **193:**5793-5801.

93. **Sato, T., S. Yoshida, H. Hoshino, M. Tanno, M. Nakajima, and T. Hoshino.** 2011. Sesquarterpenes (C35 terpenes) biosynthesized via the cyclization of a linear C35 isoprenoid by a tetraprenyl-β-curcumene synthase and a tetraprenyl-β-curcumene cyclase: Identification of a new terpene cyclase. J Am Chem Soc **133:**9734–9737.

94. **Schirner, K., J. Marles-Wright, R. J. Lewis, and J. Errington.** 2009. Distinct and essential morphogenic functions for wall- and lipo-teichoic acids in *Bacillus subtilis*. EMBO J **28:**830–842.

95. **Schneider, T., and H.-G. Sahl.** 2010. An oldie but a goodie – cell wall biosynthesis as antibiotic target pathway. Int J Med Microbiol **300:**161–169.

96. **Silhavy, T. J., D. Kahne, and S. Walker.** 2010. The bacterial cell envelope. Cold Spring Harbor Perspect Biol **2:a000414**.

97. **Staron, A., D. E. Finkeisen, and T. Mascher.** 2011. Peptide antibiotic sensing and detoxification modules of *Bacillus subtilis*. Antimicrob Agents Ch **55:**515–525.

98. **Stock, A. M., and V. L. R. P. N. Goudreau.** 2000. Two-component signal transduction. Annu Rev Biochem **69:**183–215.

99. **Swoboda, J. G., J. Campbell, T. C. Meredith, and S. Walker.** 2010. Wall teichoic acid function, biosynthesis, and inhibition. ChemBioChem **11:**35-45.

100. **Tamehiro, N., Y. Okamoto-Hosoya, S. Okamoto, M. Ubukata, M. Hamada, H. Naganawa, and K. Ochi.** 2002. Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrob Agents Ch **46:**315-320.

101. **Trevors, J. T.** 2003. Fluorescent probes for bacterial cytoplasmic membrane research. J Biochem Biophys Meth **57:**87-103.

102. **Weidenmaier, C., and A. Peschel.** 2008. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat Rev Microbiol **6:**279-287.

103. **Wenzel, M., B. Kohl, D. Münch, N. Raatschen, H. B. Albada, L. Hamoen, N. Metzler-Nolte, H.-G. Sahl, and J. E. Bandow.** 2012. Proteomic response of *Bacillus subtilis* to lantibiotics reflects differences in interaction with the cytoplasmic membrane. Antimicrob Agents Ch **51:**5749-5757.

104. **White, S. W., J. Zheng, Y.-M. Zhang, and C. O. Rock.** 2005. The structural biology of type II fatty acid biosynthesis. Annu Rev Microbiol **74:**791-831.

105. **Whitworth, D. E., and P. J. A. Cock.** 2009. Evolution of prokaryotic two-component systems: insights from comparative genomics. Amino Acids **37:**459-466.

106. **Willey, J. M., and W. A. van der Donk.** 2007. Lantibiotics: peptides of diverse structure and function. Annu Rev Microbiol **61:**477–501.

107. **Wörmann, M. E., R. M. Corrigan, P. J. Simpson, S. J. Matthews, and A. Gründling.** 2011. Enzymatic activities and functional interdependencies of *Bacillus subtilis* lipoteichoic acid synthesis enzymes. Mol Micro **79:**566-583.

108. **Zavascki, A. P., L. Z. Goldani, J. Li, and R. L. Nation.** 2007. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. J Antimicrob Chemother **60:**1206–1215.

109. **Zhang, Y. M., and C. O. Rock.** 2008. Membrane lipid homesostasis in bacteria. Nat Rev Microbiol **6:**222-233.

110. **Zweers, J. C., P. Nicolas, T. Wiegert, J. M. v. D. mail, and E. L. Denham.** 2012. Definition of the σ^W regulon of *Bacillus subtilis* in the absence of stress. PLOS One **7:**e48471.

111. **Zweers, J. C., T. Wiegert, and J. M. V. Dijl.** 2009. Stress-responsive systems set specific limits to the overproduction of membrane proteins in *Bacillus subtilis*. Appl Environ Microbiol **75:**7356-7364.

CHAPTER 2

A σ^W-DEPENDENT STRESS RESPONSE IN *BACILLUS SUBTILIS* THAT REDUCES MEMBRANE FLUIDITY[1]

2.1 Summary

Bacteria respond to physical and chemical stresses that affect the integrity of the cell wall and membrane by activating an intricate cell envelope stress response. The ability of cells to regulate the biophysical properties of the membrane by adjusting fatty acid composition is known as homeoviscous adaptation. Here, we identify a homeoviscous adaptation mechanism in *Bacillus subtilis* regulated by the extracytoplasmic function σ factor σ^W. Cell envelope active compounds, including detergents, activate a sense-oriented, σ^W-dependent promoter within the first gene of the *fabHa fabF* operon. Activation leads to a decrease in the amount of FabHa coupled with an increase in FabF, the initiation and elongation condensing enzymes of fatty acid biosynthesis, respectively. Down-regulation of FabHa results in an increased reliance on the FabHb paralog, leading to a greater proportion of straight chain fatty acids in the membrane, and the up-regulation of FabF increases the average fatty acid chain length. The net effect is to reduce membrane fluidity. The inactivation of the σ^W-dependent promoter within *fabHa* increased sensitivity to detergents and to antimicrobial compounds produced by other *Bacillus* spp. Thus, the σ^W stress response provides a mechanism to conditionally decrease membrane fluidity through the opposed regulation of FabHa and FabF.

[1]The results of this study were published in Kingston AW, Subramanian C, Rock CO, and Helmann JD. Molecular Microbiology. 2011 Jul; 81(1):69-79. C.S. and C.O.R performed the lipid analysis. All other experiments were performed by A.W.K. A.W.K and J.D.H wrote the manuscript.

2.2 Introduction

Bacillus subtilis, like other soil microbes, produces a wide variety of secondary metabolites, many of which have antibacterial activity. Many of these compounds affect the integrity of the cell envelope and elicit specific stress responses. *B. subtilis* is a model system for studying cell envelope stress responses in Gram positive bacteria mediated by a complex network of two-component regulatory systems (TCS) and extracytoplasmic function (ECF) σ factors (22). *B. subtilis* encodes seven ECF σ factors, several of which are induced by, and confer resistance to, antibiotics targeting the cell envelope (19). Many cell envelope active compounds are detergents or otherwise affect the biophysical properties of the phospholipid bilayer. As a result, cells have evolved the ability to modify membrane lipid composition to acclimatize to membrane stress (46). Some modifications affect the net membrane charge, while others adjust fluidity by changing the fatty acid (FA) composition (13, 34). Adjustments in membrane fluidity, known as homeoviscous adaptation, are critical for maintaining the desired biophysical properties such as permeability of the lipid bilayer, protein mobility, protein-protein interactions and active transport processes (26). A clear example of homeoviscus adaptation is the response of *B. subtilis* to cold-temperature stress (1, 27, 46). The decrease in membrane fluidity associated with a rapid decrease in temperature triggers the activation of the DesK histidine kinase that phosphorylates its cognate response regulator (DesR), which activates the expression

of *des* encoding an acyl-desaturase that increases membrane fluidity by introducing a double bond into the FA chains of existing phospholipids (12) .

Several ECF σ factors of *B. subtilis* are involved in stress responses elicited by compounds that affect membrane integrity and/or fluidity. A strain lacking all seven ECF σ factors displays increased susceptibility to antibiotics and detergents that affect the cell membrane (28). The σ^X regulon includes the phosphatidylethanolamine synthesis genes *pssA* and *psd* (8) while the σ^M regulon includes the phosphatidylglycerol hydrolysis enzyme YtpA (14, 41) and numerous proteins involved in cell wall synthesis and cell division (14). Both the σ^M and σ^V stress responses are activated when the phosphatidylglycerol content of the membrane is reduced (18). The σ^W regulon includes numerous membrane-localized proteins (20) and is activated under conditions of membrane stress, such as the presence of detergents or when membrane proteins are overproduced (14).

Here, we report a σ^W-dependent pathway that contributes to homeoviscous adaptation in *B. subtilis* by modifying the membrane phospholipid structure. In contrast with the DesRK pathway, which responds to conditions that decrease membrane fluidity, σ^W responds to compounds that increase membrane fluidity. Activation of a σ^W-dependent promoter within the *fabHa fabF* operon downregulates FabHa and upregulates FabF leading to a higher proportion of straight chain FA and a longer average chain length for membrane phospholipids. These membrane compositional changes reduce bilayer fluidity and increase resistance to detergents and antimicrobial compounds produced by other *Bacillus* species.

2.3 Materials and methods

Strains, plasmids, and growth conditions. All *B. subtilis* strains, plasmids, and oligonucleotides (oligos) used in this study are listed Table S2. Bacteria were grown in liquid Luria-Bertani (LB) medium at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bacto agar (Difco) with appropriate selection. Plasmids were amplified in *Escherichia coli* DH5α before transformation of *B. subtilis* strains. Ampicillin (amp; 100 μg ml^{-1}) was used to select *E. coli* transformants. For *B. subtilis*, antibiotics used for selection were: spectinomycin (spec; 100 μg ml^{-1}), kanamycin (kan; 15 μg ml^{-1}), chloramphenicol (cat; 10 μg ml^{-1}), and macrolide-lincosoamide-streptogramin B (MLS; contains 1 μg ml^{-1} erythromycin and 25 μg ml^{-1} lincomycin).

Genetic techniques. Chromosomal and plasmid DNA transformations were performed as described previously (17). Unless otherwise stated, all PCR products were generated using W168 chromosomal DNA as a template and all strains were verified by sequence analysis (Cornell University Life Sciences Core Laboratories Center).

To create HB13069 (*P_5-lacZ*), a DNA fragment containing P_5 was PCR-amplified with primers 4577 and 4852 and cloned into pDG1661 (16). The resulting plasmid (pTK022) was linearized by digestion with ScaI and integrated into the *amyE* locus. To create HB13001 (*P_{fabHaF}-P_5-lacZ*), the same protocol was used except that the DNA fragment was synthesized using primers 4576 and 4577 and the resulting plasmid was pTK001.

To create HB13054 (*thrC::P_{fabHaF}-fabHa-FLAG)*, the *fabHa* ORF and the *P_{fabHaF}* promoter were PCR-amplified (primers 4778 and 4780) to generate a product

with a BamH1 site upstream of P_{fabHaF} and a *flag* tag followed by an EcoRI site at the 3' end of *fabHa*. The PCR fragment was cloned into pDG1664 (16) and integrated into W168 at *thrC*. To create HB13058 (*thrC::*P_{fabHaF}*-fabHa-fabF-FLAG)*, the same procedure was used with primers 4778 and 4781.

The Quikchange site-directed mutagenesis kit (Stratagene) and primers 4883 and 4884 were used to change the -10 element of P_5 from CGTA in pTK001, pTK013, pTK015, pTK022, and pTK043 to CGGA (designated P_5*) in plasmids pTK033, pTK045, pTK046 pTK031, and pTK044, respectively. To incorporate P_5* at the genomic locus in *B. subtilis*, a 525 bp fragment containing P_5, P_{fabHaF}, and the FapR binding site was PCR-amplified (primers 4576 and 4577) and cloned into pMUTIN4 (42) to create pTK043. P_5* was generated with site-directed mutagenesis as described above to create pTK044. Uncleaved pTK044 was inserted into W168 at the *fabHa* locus through Campbell integration to create HB13118 in which P_5 is changed to P_5* at the genomic locus. Note that the upstream regulatory elements remain unchanged. Since this integration vector also incorporated ~8.6 kb of plasmid DNA upstream of P_{fabHaF}, we created a control strain (HB13117) with pMUTIN4 inserted upstream of *fabHa* while maintaining wild type P_5.

Gene deletions were generated using long-flanking homology PCR (LFH-PCR) as described previously (30). To create HB13118 (*fabHb::kan*), 800 bp DNA fragments flanking *fabHb* were amplified using primers 5059 and 5060 for the upstream (24) fragment and 5061 and 5062 for the downstream (DO) fragment. Extensions of ~25 nucleotides were added to the 5' end of the UP-reverse and the DO-forward primers that were complementary (opposite strand and inverted sequence) to

the ends of the kanamycin cassette. 100-150 ng of the UP and DO fragments and 250-300 ng of the resistance cassette were used together with the specific UP-forward and DO-reverse primers in a second joining PCR and the product used to transform *B. subtilis*. To generate a *fabHa* null mutant, while retaining *fabF* under control of P_{fabHaF} (HB13127), an ~800 bp UP fragment was amplified with primers 5164 and 5174. The DO fragment (a P_{fabHaF}-*fabF* fusion) required the fusion of two PCR product encompassing the P_{fabHaF} promoter (primers 5165 and 5166) and *fabF* (primers 5167 and 5169). The UP and DO fragments were joined with an *MLS* cassette by PCR and used to transform *B. subtilis*. These extra steps were taken to insert the *MLS* resistance cassette upstream of P_{fabHaF} instead of between P_{fabHaF} and *fabF* to minimize the effect of deleting *fabHa* on *fabF* expression.

To generate HB13128 (P_{xyl}-*fabF*), a PCR-amplified fragment (primers 5168 and 5169) containing a ribosome binding site followed by *fabF* was cloned into pSWEET-*bgaB* (4) and the resulting plasmid (pTK047) was cleaved with PstI and integrated into W168 at *amyE*.

5'-RACE. The start site of the P_5 transcript was determined using 5' rapid amplification of cDNA ends (5'-RACE) (35). The W168 strain was grown to an OD_{600} of 0.4 and treated with 0.004% triton X-100 for 30 minutes at 37 ^{o}C with aeration. 2 μg of extracted RNA was reverse-transcribed to cDNA using TaqMan reverse transcription reagents (Applied Biosystems) and oligo P_5-GSP1 (4520) as a primer. The 3' end of the cDNA was tailed with poly(dC) using dCTP and terminal deoxynucleotidyl transferase (New England Biolabs). The tailed cDNAs were amplified by PCR with primers AAP (3314) and P_5-GSP2 (4521) and sequenced.

Disk diffusion assays. Disk diffusion assays were performed as described (29). Briefly, strains were grown to an OD_{600} of 0.4. A 100 µl aliquot of these cultures was mixed with 4 ml of 0.7% LB soft agar (kept at 50°C) and directly poured onto LB plates (containing 15 ml of 1.5% LB agar). The plates were dried for 20 min in a laminar airflow hood. Filter paper disks containing the chemicals to be tested were placed on the top of the agar and the plates were incubated at 37°C overnight. The distances between the edge of the inhibition zones and the edge of the filter paper disks were measured. For promoter-*lacZ* strains, 80 µg ml^{-1} Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to the agar and the plates were analyzed for the appearance of a blue ring around the edge of the zone of inhibition. The following chemicals and quantities were used in the disk diffusion assays: Triton X-100 10 µl of a 25% solution, amitriptyline 200 µg, polymyxin B 100 µg, vancomycin 100 µg, daptomycin 100 µg, D-cycloserine 1 mg, cephalosporin C 10 µg.

β-galactosidase assays. Strains carrying promoter-*lacZ* fusions were grown to an OD_{600} of 0.4 in LB. Cultures were then treated with alkali shock (24 mM NaOH) or a control (H_2O) and samples were taken 30 min after treatment. β-galactosidase assays were performed as described by Miller (32).

Western Blots. Strains expressing FLAG-tagged copies of FabHa or FabF were grown to an OD_{600} of 0.4 and treated with either 2% xylose, 24 mM NaOH, or 200 µL H_2O for 30 minutes at 37 ^oC with aeration. Cells were lysed and subjected to Western blot analysis using anti-FLAG antibodies to detect the fusions as described (39). Relative levels of each FLAG-tagged protein were compared using densitometry analysis with ImageJ.

Spot-on-lawn assays. Spot-on-lawn assays were performed as described (6). Briefly, lawn cells were grown to an OD_{600} of 0.4 in LB. A 100 µl aliquot of these cultures was mixed with 4 ml of 0.7% LB soft agar (kept at 50°C) and directly poured onto LB plates (containing 15 ml of 1.5% LB agar). Plates were dried for 20 min in a laminar flow hood, and 2 µl of the producer strain (OD_{600} of 0.6) was spotted on top of the agar. Plates were incubated at 37°C overnight (18 h) before observation.

Fluorescence Anisotropy. We analyzed fluorescence anisotropy of *B. subtilis* strains treated with 1,6-diphenyl-1,3,5-hexatriene (DPH) using a modification of described methods (40). Strains were grown to early-log phase (OD_{600} of 0.2 ± 0.01) in 5 ml LB medium. Cultures were treated with or without 2% xylose and/or 2.5 µM DPH (from a 2.5 mM stock in acetone) and incubated at 37°C with aeration for 30 minutes. For each culture, a 1 ml sample was washed once and suspended in 2 mL phosphate buffer (100 mM, pH 7.0). Fluorescence anisotropy measurements (λ_{ex}=358 nm, slit width=10 nm; λ_{em}=428 nm, slit width=15 nm) were performed with a PerkinElmer LS55 luminescence spectrometer. The correction for the fluorescence intensity of non-labeled cells was calculated according to (24).

Lipid analysis. Cells were grown in M9 minimal medium supplemented with 0.05% casamino acids, 10 µg ml^{-1} tryptophan, 0.1% glutamate, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 500 nM $MnCl_2$, 10 µM $FeCl_3$, 0.5% glucose and 2% xylose. Cells were harvested and lipids extracted as described by Bligh and Dyer (5). FA methyl esters were prepared using methanol-HCl and were identified and quantified using a Hewlett-Packard model 5890 gas chromatograph equipped with a flame ionization detector as described (11). FA compositions are expressed as weight percent. Samples

for molecular species analysis were dried and resuspended in chloroform:methanol:formic acid (50:50:1). Mass spectra were obtained using a Thermo Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific) operated in the positive ESI mode using neutral loss scanning for 141 *m/z* corresponding to the loss of the phosphoryl headgroup of phosphatidylethanolamine (25). Ion source parameters were: spray voltage 3000 V, capillary temperature 270°C, capillary offset 35 V, and tube lens offset set by infusion of the polytyrosine tuning and calibration solution (Thermo Electron, San Jose, CA) in electrospray mode. Acquisition parameters for phosphatidylethanolamine were: scan range 600-900 *m/z*, scan time 0.5 s, neutral loss mass 141.0 *m/z*, collision energy 30 V, peak width Q1 and Q3 0.7 FWHM, and Q2 CID gas 0.5 mTorr. The sample was injected into the loop using a syringe pump at a flow rate of 5 μl min^{-1} and the data were collected for 3 min. and analyzed using QuantumTune software version 1.2 (Thermo Fisher Scientific).

Statistical analysis. All experiments were performed with a minimum of three biological replicates. Unless otherwise noted, data is presented as mean ± standard error. Statistical evaluation of the data was performed by the use of unpaired Student's *t* tests. A value of $P \leq 0.05$ was considered statistically significant.

2.4 Results and discussion:

Identification of an active σ^W-dependent promoter within *fabHa*. Because ECF σ promoters are, as a class, highly conserved (19, 23), computer-based searches have been effective in identifying candidate promoters. The first efforts to identify regulons

controlled by ECF σ factors relied on promoter consensus sequence searches directed to intergenic regions (20, 21). Subsequently, the search was expanded to incorporate microarray-based methods to detect mRNAs produced by ECF σ factors *in vivo* (transcriptional profiling) and *in vitro* (ROMA; run-off transcription microarray analysis) (8-10, 14). In the σ^M regulon, several promoter sites are located within genes (14), a finding consistent with whole genome chromatin immunoprecipitation studies in other systems (43). We therefore performed a computer search of the *B. subtilis* genome to determine if other candidate ECF σ promoters were present within annotated genes. Here, we focus on one such element, arbitrarily designated P_5, within the *fabHa* gene. P_5 matches the σ^W consensus in both the -35 (TGAAAC) and -10 (CGTA) elements (19, 29) (Figure 2.1A). A 5′-RACE analysis of detergent-treated wild type cells confirmed the presence of a transcript with a 5′ end corresponding to transcription from P_5 that ROMA and microarrays failed to detect.

Several of the seven ECF σ factors in *B. subtilis* overlap in their promoter recognition properties (28, 29). To determine which ECF σ factors activate the P_5 promoter we used a strain with an ectopic P_5-*lacZ* fusion inserted at the *amyE* locus (HB13069). Using a disk diffusion assay, P_5-promoted β-galactosidase activity was observed with activators of σ^W such as polymyxin B, vancomycin, cephalosporin C, D-cycloserine, and triton X-100 (Table 2.1, Figure S2.1) (10). A *sigW* null mutant (HB13099) exhibited no induction of β-galactosidase. Moreover, a strain deleted for all ECF σ factor genes except for *sigW* behaved like wild-type. Therefore, σ^W is both necessary and sufficient for activating P_5 in response to antibiotics and detergents.

Figure 2.1: A σ^W dependent promoter (P_5) in *fabHa*.
A. Schematic map of P_5, P_{fabHaF}, FabHa, FabF, and the associated FapR binding site (black triangle). The -35 and -10 elements of P_5 are in bold. The locations of the P_5 and P_{fabHaF} transcriptional start sites (+114 and -68 respectively) are described relative to the first base of the FabHa translation initiation codon. The ‡ indicates a putative terminator. The DNA regions included in the P_{fabHaF}-P_5-*lacZ* and P_5-*lacZ* fusions are also illustrated.
B. The β-galactosidase activity of the P_5-*lacZ* (HB13069), P_5*-*lacZ* (HB13080), P_{fabHaF}-P_5-*lacZ* (HB13001), and P_{fabHaF}-P_5*-*lacZ* (HB13082) strains grown to mid-log phase in LB with and without alkaline shock. This experiment was performed in biological triplicate and repeated at least three times. Bars represent mean values with error bars indicating standard error. Student's *t* tests were performed, and a statistically significant difference (*P* value $\geq$ 0.05) between the control and alkaline shocked cells is denoted as * above the bar graph while a non-significant difference is denoted as NS.
C. Detection of FLAG-tagged FabHa and FabF by Western blot with anti-FLAG antibodies in the strains *fabHa-FLAG* (HB13054), *fabF-FLAG* (HB13056), *fabHa-FLAG* P_{xyl}*-sigW* (HB13058), and *fabF-FLAG* P_{xyl}*-sigW* (HB13060) with and without xylose treatment. This experiment was performed in biological triplicates and repeated at least three times. The numbers below each band represent the average intensity of that band (± standard error) relative to the non-xylose treated control for that strain. Using Student's t-tests, a statistically significant difference (*P* value $\leq$ 0.05) between the control and xylose treated cells was found in strains containing the P_{xyl}-sigW construct but not in strains lacking this construct.
D. Overview of FA biosynthesis. Chain initiation requires either FabHa or FabHb. FabHb has a greater ability to accept straight chain precursors than FabHa. Increased abundance of the FabF elongation enzyme can increase the chain length of the resulting FA (Choi *et al.*, 2000; (37).

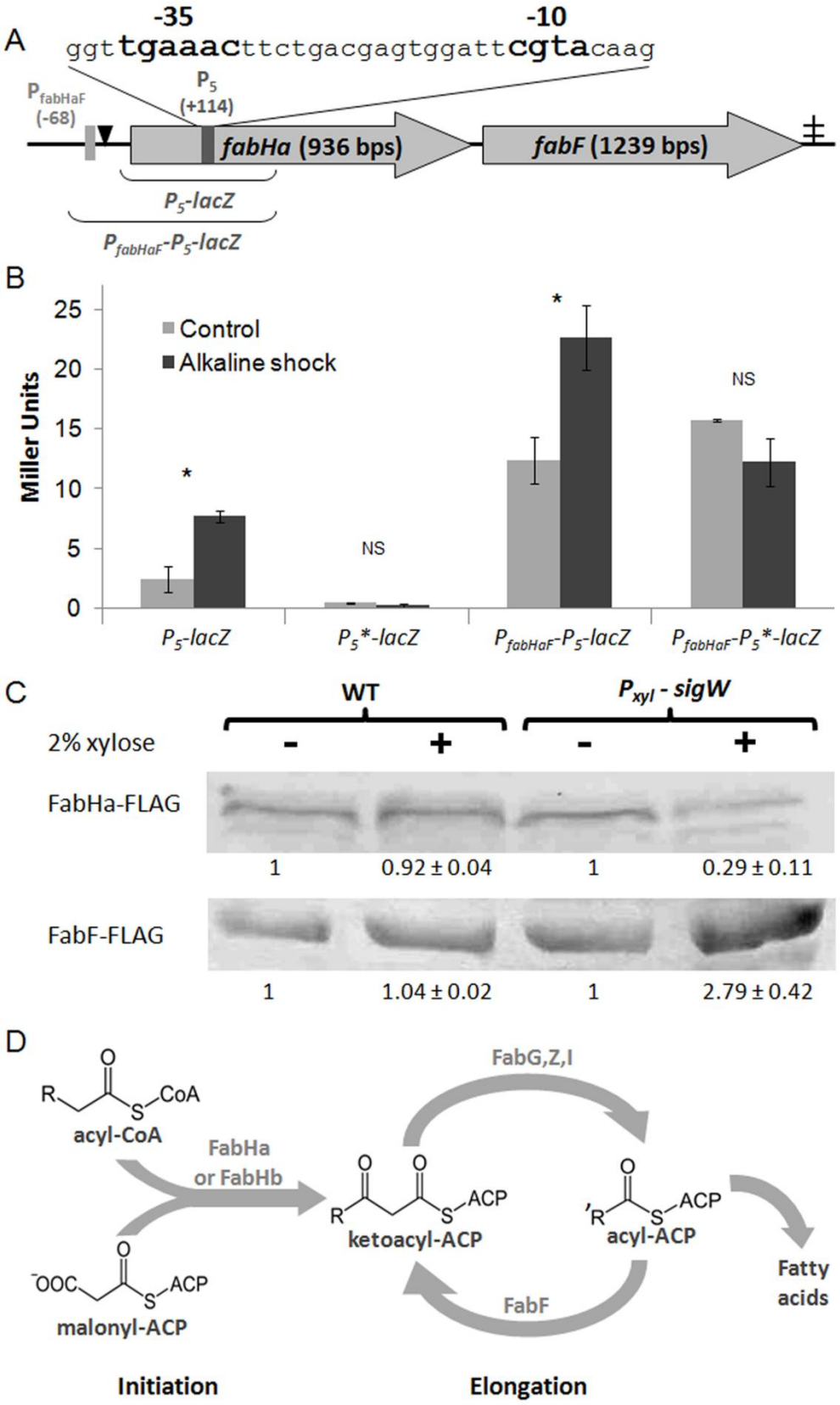

A
-35
-10
ggttgaaacttctgacgagtggattcgtacaag
P5
(+114)
PfabHaF
(-68)
fabHa (936 bps)
fabF (1239 bps)
P5-lacZ
PfabHaF-P5-lacZ
B
Miller Units
Control
Alkaline shock
*
NS
*
NS
P5-lacZ
P5*-lacZ
PfabHaF-P5-lacZ
PfabHaF-P5*-lacZ
C
WT
Pxyl - sigW
2% xylose
-
+
-
+
FabHa-FLAG
1
0.92 ± 0.04
1
0.29 ± 0.11
FabF-FLAG
1
1.04 ± 0.02
1
2.79 ± 0.42
D
acyl-CoA
FabHa
or FabHb
malonyl-ACP
ketoacyl-ACP
FabG,Z,I
acyl-ACP
FabF
Fatty
acids
Initiation
Elongation

Table 2.1: Summary of disk diffusion assays to monitor induction of *P_5-lacZ* by cell envelope antibiotics

Stress	***P_5-lacZ***	***P_5-lacZ*** $\Delta\sigma^{M, V, X, Y, ylaC, Z}$	***P_5-lacZ*** $\Delta\sigma^{W}$
Triton X-100	+++	+++	-
Vancomycin	++	++	-
Polymyxin B	+	+	-
Cephalosporin	+	+	-
D-cycloserine	+	+	-

[a] The induction ability of the listed antibiotics was measured in the reporter strains HB13069 (*P_5-lacZ*), HB13151 (P_5-lacZ *ΔsigM ΔsigY ΔsigZ ΔsigV ΔylaC ΔsigX),* and HB13099 (*P_5-lacZ sigW::MLS*) using disk diffusion assays with various stresses to induce the promoter (see Fig. S1 for examples). Blue halos were observed after overnight incubation: +++ (strong blue) > ++ (blue) > + (light blue) > - (white after 7 days, no induction). Each disk diffusion assay was performed at least three times with biological triplicates. Data shown is representative of all experiments.

σ^W regulates transcription of the *fabHa-fabF* operon. To obtain a more quantitative assessment of P_5 activity, we performed β-galactosidase assays on a strain carrying an ectopically located P_5-*lacZ* fusion after induction by alkaline shock as described (45). P_5 displayed a low basal activity that increased ~3-fold in response to alkaline shock (Figure 2.1B). To verify that this activity was due to P_5, a P_5*-*lacZ* strain was constructed in which the -10 element was changed from CGTA to CGGA (HB13082). No known σ^W promoters have a G at this position (9, 19), and as predicted this P_5*-*lacZ* strain expressed no detectable β-galactosidase activity either with or without alkaline shock.

The *fabHaF* operon expressed from the promoter P_{fabHaF} is negatively regulated by FapR, a repressor of genes involved in membrane lipid biosynthesis (37). β-galactosidase activity in strain HB13001 containing an ectopically located P_{fabHaF} -P_5-*lacZ* fusion (Figure 2.1B) inserted at *amyE* increased by ~2-fold in response to alkaline shock, whereas no induction was noted for the corresponding P_{fabHaF} -P_5*-*lacZ* fusion strain. Thus, σ^W-dependent activation of P_5 has a measurable impact even in the presence of P_{fabHaF}.

Activating σ^W increases FabF and reduces FabHa. To determine how P_5 affects FabHa and FabF protein levels, strains with ectopic copies of *fabHa* and *fabF* containing C-terminal FLAG sequences (HB13054 and HB13056, respectively) were constructed. The *fabHa-flag* and *fabF-flag* constructs were expressed under the control of both native promoters (P_{fabHaF} and P_5) to ensure that the amount of FabHa-FLAG and FabF-FLAG within the cell approximated wild type levels and was appropriately

influenced by P_5. Since P_5 is part of the σ^W regulon, it was activated using a xylose-inducible copy of *sigW*. Induction of σ^W reduced FabHa-FLAG (HB13058) levels by ~4-fold and increased FabF-FLAG (HB13060) by ~3-fold compared to uninduced control cells (Figure 2.1C). In contrast, expression was unaffected in control strains lacking the P_{xyl}-*sigW* fusion (Figure 2.1C) or containing the inactive P_5* promoter (Figure S2.2A). Analogous experiments using alkaline shock to activate σ^W resulted in similar effects, albeit of reduced magnitude: FabHa decreased by 53% while FabF increased by 25% (Figure S2.2B). Thus, P_5 activation directly downregulates FabHa and upregulates FabF protein levels.

Activation of σ^W alters membrane composition. *B. subtilis* has two initiating condensing enzymes, FabHa and FabHb, that have been biochemically characterized (Figure 2.1D): Both enzymes utilize branched-chain primers (that become branched fatty acids) but FabHb is significantly more capable of utilizing the straight chain precursor acetyl-CoA (11). The function of the two condensing enzymes *in vivo* was assessed by determining the FA composition of knockout strains. The inactivation of FabHa (HB 13127) led to a significant increase in the proportion of straight-chain FA (Table 2.2) with a concomitant increase in 31:0-carbon phosphatidylethanolamine species (Figure 2.2A & 2.2B) compared to W168. The phosphatidylglycerol molecular species profiles were the same as phosphatidylethanolamine (not shown), consistent with phosphatidic acid being a common precursor to both phospholipids. The *fabHB* deletion strain (HB13115) had a FA composition (Table 2) and molecular species composition (2.2A & 2.2C) similar to strain W168, although there was a small decrease in the proportion of straight-chain FA. These data are consistent with the

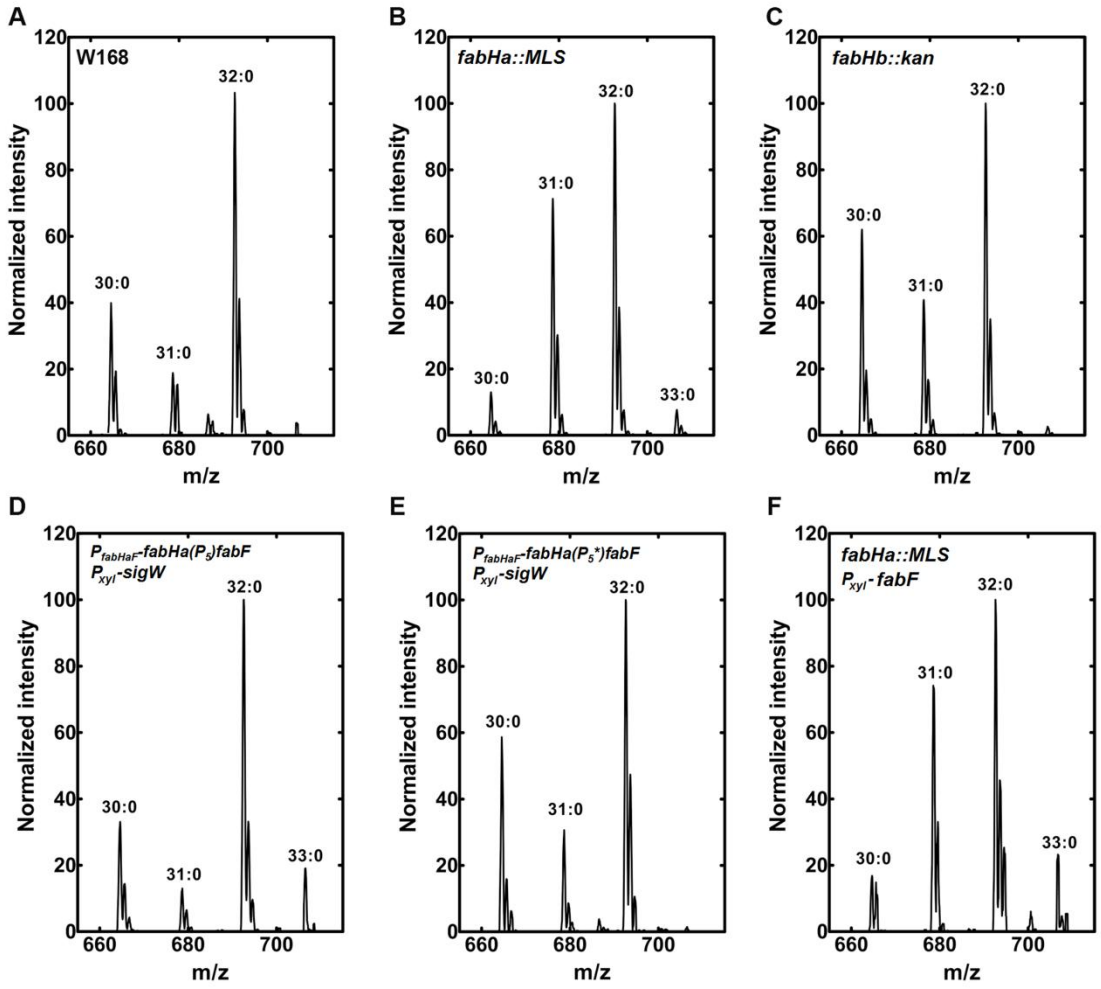


Figure 2.2: Phosphatidylethanolamine molecular species of genetically modified strains. The phosphatidylethanolamine molecular species fingerprint was obtained by ESI mass spectrometry of cells grown under inducing conditions (+ xylose). Data shown are representative of least three separate experiments performed in biological triplicates.

A. Wild-type control strain (W168)
B. *fabHa::MLS* (HB13127)
C. *fabHb::kan* (HB13115)
D. P_{fabHaF}-*fabHa*(P_5)*fabF* P_{xyl}-*sigW* (HB13121)
E. P_{fabHaF}-*fabHa*(P_5*)*fabF* P_{xyl}-*sigW* (HB13122)
F. *fabHa::MLS* P_{xyl}-*fabF* (HB13132).

Table 2.2: The effect of σ^W on membrane composition and fluidity

Strain	*W168*	*P_{fabHaF}-fabHa(P_5)-fabF P_{xyl}-sigW*	*P_{fabHaF}-fabHa(P_5*)-fabF P_{xyl}-sigW*	*fabHa::MLS*	*fabHb::kan*	*P_{xyl}-fabF*	*fabHa::MLS P_{xyl}-fabF*
% C15:0 iso [a]	9.6 ± 0.6	6.5 ± 0.8	10.7 ± 1.4	1.8 ± 0.8	15.6 ± 0.3	6.5 ± 0.9	1.9 ± 0.2
% C15:0 anteiso [a]	40.0 ± 3.1	31.8 ± 3.2	42.2 ± 2.4	30.3 ± 1.9	39.0 ± 2.8	32.4 ± 3.1	30 .0 ± 2.7
% C16:0 iso [a]	3.6 ± 0.4	2.3 ± 0.5	1.7 ± 0.5	5.2 ± 0.4	4.8 ± 0.8	3.5 ± 1.1	4.3 ± 0.4
% C16:0 [a]	8.3 ± 1.6	8.3 ± 0.5	7.5 ± 1.1	22.2 ± 1.8	6.8 ± 1.2	8.5 ± 1.6	16.8 ± 1.2
% C17:0 iso [a]	10.3 ± 2.0	9.0 ± 1.7	8.1 ± 0.7	4.8 ± 0.7	10.6 ± 2.2	11.9 ± 2.4	5.4 ± 1.0
% C17:0 anteiso [a]	26.0 ± 1.8	32.5 ± 0.8	24.8 ± 2.1	27.7 ± 0.7	20.3 ± 0.4	34.0 ± 2.0	35.4 ± 2.2
% C18:0 [a]	2.2 ± 0.6	9.6 ± 1.0	2.8 ± 0.8	7.8 ± 1.1	2.0 ± 0.7	3.3 ± 0.4	6.0 ± 0.9
% straight FAs [a]	10.5 ± 1.0	17.9 ± 1.2	10.3 ± 1.1	29.3 ± 0.7	8.8 ± 1.0	11.8 ± 1.8	22.0 ± 0.7
% iso FAs [a]	23.5 ± 2.9	17.8 ± 2.9	20.6 ± 1.6	11.4 ± 1.1	31.0 ± 2.0	21.9 ± 3.9	12.0 ± 1.3
% anteiso FAs [a]	66.0 ± 3.5	64.3 ± 3.9	67.0 ± 2.1	58.0 ± 1.5	59.3 ± 2.5	66.4 ± 4.0	65.7 ± 0.9
17:15 FA ratio [a]	0.73 ± 0.07	1.08 ± 0.09	0.62 ± 0.08	1.03 ± 0.11	0.57 ± 0.08	1.18 ± 0.12	1.32 ± 0.22
Fluorescence anisotropy analysis[b]	0.185 ± 0.005	0.217 ± 0.003	0.202 ± 0.004	ND	ND	ND	ND

[a] Data derived from FAME analysis of the following strains under xylose inducing conditions: W168, *P_{fabHaF}-fabHa(P_5)fabF P_{xyl}-sigW* (HB13121), *P_{fabHaF}-fabHa(P_5*)fabF P_{xyl}-sigW* (HB13122) *fabHa::MLS* (HB13127), *fabHb::kan* (HB13115), *P_{xyl}-fabF* (HB13128), and *fabHa::MLS P_{xyl}-fabF* (HB13132). Data is presented as the average of three trials (± standard error).

[b] Data derived from fluorescence anisotropy analysis of the wild type (W168), *P_{fabHaF}-fabHa(P_5)fabF amyE::P_{xyl}-sigW* (HB13121), and *P_{fabHaF}-fabHa(P_5*)fabF amyE::P_{xyl}-sigW* (HB13122) strains under xylose inducing conditions. Data is presented as the average of three trials (± standard error). Student's t-tests were performed, and all three values were found to be statistically different (P value $\leq$ 0.05) from each other.

biochemical properties of the two enzymes and indicate that FabHa is the principal condensing enzyme responsible for the initiation of FA synthesis in non-stressed *B. subtilis* cells. A decrease in branched-chain FA synthesis is seen following σ^W activation when P_5 is present (in strain *P_{fabHaF}-fabHa(P_5)fabF P_{xyl}-sigW;* HB13121), but not in the strain with the mutant P_5* promoter (*P_{fabHaF}-fabHa(P_5*)fabF P_{xyl}-sigW;* HB13122) consistent with the observed downregulation of FabHa expression and an increased reliance on FabHb (Table 2; Figure 2.2D & 2.2E).

Activation of σ^W (HB13121) also led to an increase in average FA chain length. As measured by the ratio of 17 to 15 carbon length FA (17:15 ratio), activation of σ^W led to an increase from 0.73 (wild-type) to 1.08. This increase was not observed (ratio of 0.62) in the strain containing the inactive P_5* promoter (HB13122, Table 2.2). An increase in FabF expression is sufficient to account for the increase in 17:15 ratio since overexpression of FabF led to a ratio of 1.18 in strain HB13128. However, an increase in FabF levels alone does not account for all effects of σ^W on FA chain length: a hallmark of σ^W activation is the appearance of the C33:0 phosphatidylethanolamine species (Figure 2.2D) that reflects a significant increase in 18 carbon FA (Table 2.2). Overexpression of FabF (HB13128) led to only a modest increase in 18 carbon FA chains which was substantially increased when combined with a *fabHa* null mutation (HB13132) (Table 2.2, Figure 2.2F). Thus, an increase in the supply of 16 carbon acyl-ACP due to the down-regulation of FabHa provides the substrate for FabF elongation to an 18 carbon acyl-ACP. We conclude that the influence of σ^W on FA synthesis results from both the down-regulation of FabHa and

the up-regulation of FabF protein levels. The net effect is an increase in both the proportion of straight-chain FA and the average FA chain length leading to an altered phospholipid molecular signature indicative of a less fluid bilayer.

The σ^W-dependent changes in membrane composition reduce membrane fluidity. To determine whether these σ^W-dependent changes in FA composition were sufficient to significantly alter membrane fluidity we monitored the fluorescence anisotropy of *B. subtilis* cells using 1,6-diphenyl-1,3,5-hexatriene (DPH). For this analysis we used isogenic strains expressing the intact *fabHa-fabF* operon containing either P_5 (HB13121) or the inactive P_5* point mutation (HB13122) following treatment with xylose to induce σ^W for 30 minutes. Induction of σ^W led to a substantially higher anisotropy (a lower degree of rotational freedom) consistent with alterations in membrane composition that reduce fluidity (Table 2.2). The control strain carrying the inactive P_5* promoter exhibited a smaller increase in anisotropy indicating that although P_5 reduces membrane fluidity, other σ^W-dependent pathways may also contribute to this reduction. The magnitude of the change in anisotropy dependent on a functional P_5 promoter was comparable to that previously reported for a shift in growth temperature from 37^{o}C to 25^{o}C (3), consistent with the hypothesis that P_5 can have a physiologically relevant impact on membrane fluidity.

σ^W contributes to detergent resistance by activating P_5. Activation of σ^W confers resistance to numerous cell envelope active antibiotics and detergents such as vancomycin and triton X-100 (29). The resistance to antibiotics and detergents in strains containing the intact *fabHa-fabF* operon with either P_5 (HB13117) or the inactive P_5* promoter (HB13118) was compared to a *sigW* null mutant (HB6208)

using disk diffusion assays. All of the detergents tested (triton X-100, N-lauryl sarcosine, DTAB, amitriptyline, and bile salts) had larger zones of inhibition in the P_5* strain than in the strain containing active P_5, although the effects were not as pronounced as in the *sigW* null mutant (Figure 2.3). Therefore, σ^W-dependent alteration of membrane fluidity via activation of P_5 contributes to detergent resistance. In contrast, activation of P_5 did not affect resistance to either the membrane-active antibiotic daptomycin or the cell wall antibiotic vancomycin (Figure S2.3).

To determine whether detergent resistance is due to downregulation of FabHa, up-regulation of FabF, or both, we monitored detergent resistance in the *fabHa::MLS* (HB13127), *fabHb::kan* (HB13115), and xylose-inducible P_{xyl}-*fabF* (HB13128) strains. Although inactivating *fabHa* had little effect on detergent susceptibility, inactivating *fabHb* increased detergent susceptibility (Figure 2.3). The increased susceptibility of the *fabHb* null strain underscores the importance of FabHb-initiated straight-chain fatty acids in detergent resistance which is driven by FabHa downregulation. The absence of a resistance phenotype in the *fabHa::MLS* strain could be attributed to the fact that deleting *fabHA* also removes P_5 which renders the strain incapable of upregulating FabF in response to stress. Finally, the FabF-overexpressing strain was more resistant to all detergents tested illustrating the contribution of chain length to resistance (Figure 2.3). We conclude that both effects of σ^W activation contribute to the detergent resistance phenotype.

σ^W contributes to resistance to antimicrobials made by other *Bacillus* spp. by activating P_5. The σ^W regulon also confers resistance to antimicrobial compounds produced by other *Bacillus* species (6). We used a spot-on-lawn assay in which an

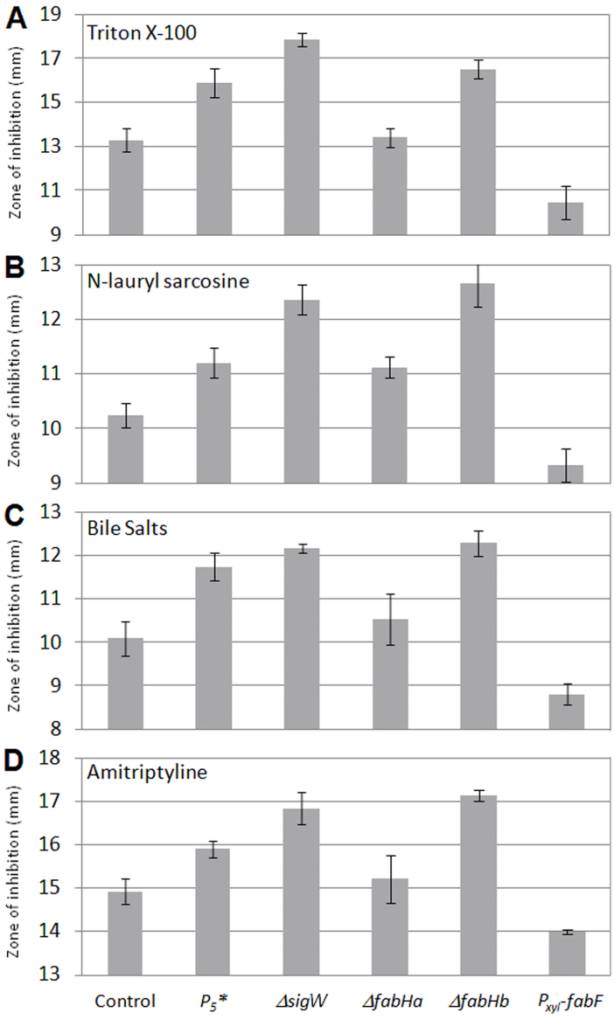


Figure 2.3: Disk diffusion assays of detergent sensitivity for the P_{fabHaF}-*fabHa*(P_5)*fabF* control (HB13117), P_{fabHaF}-*fabHa*(P_5*)*fabF* (HB13118), *sigW::spec* (HB6208), *fabHa::MLS* (HB13127), *fabHb::kan* (HB13115), and P_{xyl}-*fabF* (HB13128) strains. Each bar represents the average zone of inhibition of at least three assays performed with three independent clones of each strain. The *y* axis shows the zone of inhibition (in millimeters), expressed as total diameter minus diameter of the filter paper disk (5.5 mm). Note that the scales of individual compounds vary for clarity. Error bars represent standard error. Student's *t* tests were performed, and P_5*, *sigW::spec*, *fabHb::kan*, and P_{xyl}-*fabF* were found to be significantly different (*P* value $\leq$ 0.05) from the wild-type control for all four stresses. The P_5* and *sigW::spec* strains were also significantly different from each other under triton X-100, N-lauryl sarcosine, and amitriptyline treatment.
A. triton X-100
B. N-lauryl sarcosine
C. bile salts
D. amitriptyline

antibiotic producing strain is spotted at high cell density on a lawn of the target strain to test whether σ^W activation of P_5 affects interspecies competition. The six *Bacillus* species tested are known to produce compounds to which σ^W confers resistance (6). When the reporter (lawn) strain contained the inactive P_5* promoter there was an increased sensitivity to compound(s) produced by three strains: *B. amyloliquefaciens, B. subtilis ssp. spizizenii 2A8*^{T}, and *B. atrophaeus ESM rplV str* (Figure 2.4). No significant P_5-dependent differences in sensitivity were observed with *B. subtilis ssp. spizizenii 2A9* (Figure 2.4), *B. licheniformis,* or *B. atrophaeus NRS-213* (Figure S2.4) as the producer strains. In those strains where P_5-dependent modulation of membrane composition may be important, the P_5* strain was not as sensitive as the *sigW* null strain (Figure 2.4), consistent with the previous demonstration that other σ^W-dependent operons contribute to antimicrobial resistance (6).

2.5 Conclusion

Our study uncovers a novel ECF σ factor dependent pathway that protects against environmental insults to the cell membrane by altering FA synthesis to produce a more rigid phospholipid bilayer. A unique aspect of the system is the presence of an internal, sense-oriented σ^W promoter that reduces the expression of the gene it is within (*fabHa*) and elevates expression of the downstream gene (*fabF*). The coordinate decrease in FabHa and increase in FabF protein levels combine to produce a bilayer with a different constellation of phospholipid species that leads to decreased fluidity. This biophysical response contributes to adaptation to membrane active compounds like detergents and some, yet uncharacterized, antimicrobial compounds

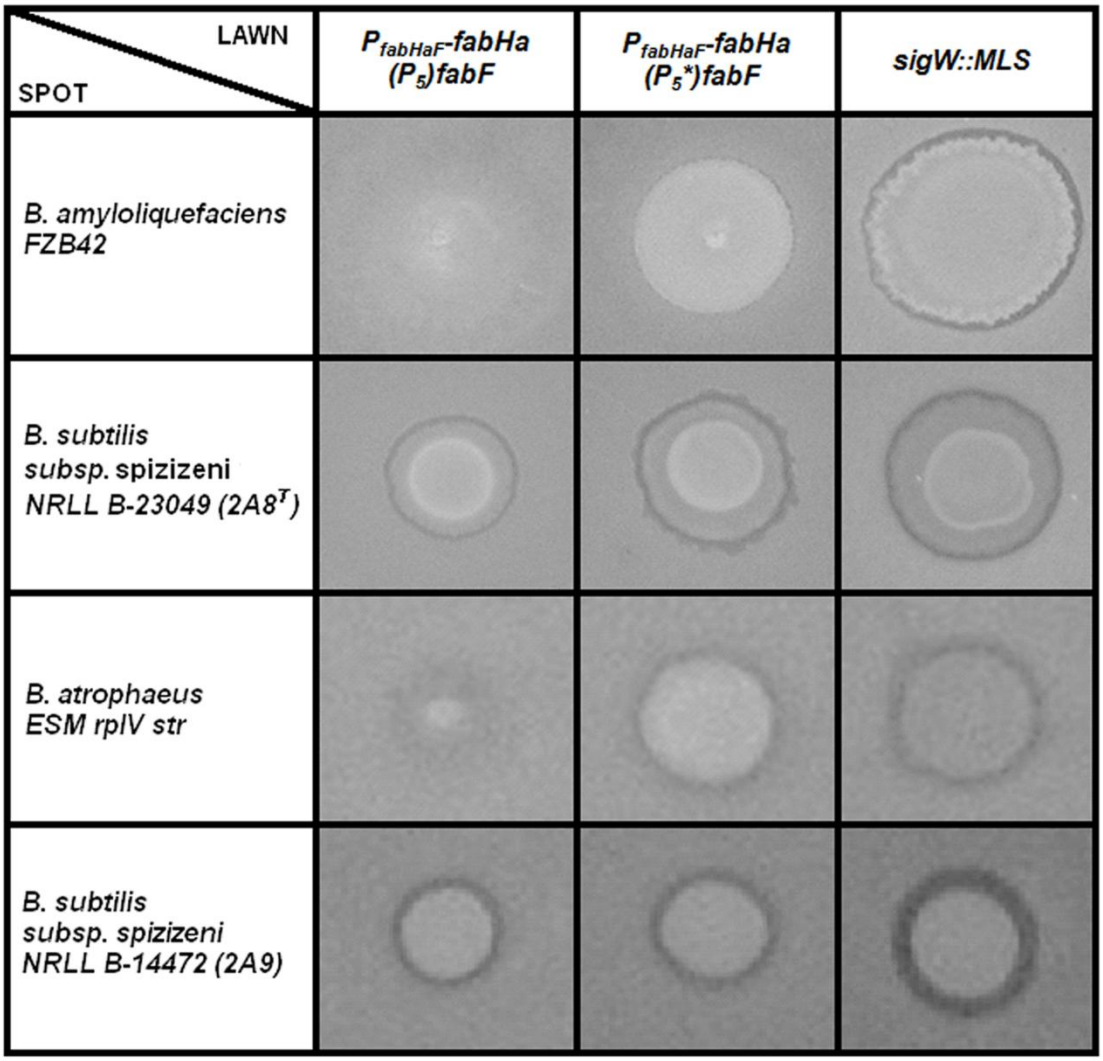


Figure 2.4: Spot on lawn assays depicting the sensitivity of the *pMUTIN-P_5* (HB13117), *pMUTIN-P_5** (HB13118), and *sigW::spec* (HB6208) lawn strains to spots of *B. amyloliquefaciens FZB42*, *B. subtilis subsp. spizizeni 2A8^T*, *B. atrophaeus ESM rplV str*, and *B. subtilis subsp. spizizenii 2A9*. The relative sensitivity of the lawn strains to each spotted strain is reflected by the size of the spot and the zone of inhibition surrounding it after 24 hours growth. A larger spot size and zone of inhibition represents increased sensitivity of the lawn strains to the metabolites produced by the spotted strains. Pictures are representative of at least three assays performed with three independent clones of each strain.

produced by other Bacilli. The alterations of membrane phospholipid composition observed upon genetic manipulations of FabHa, FabHb and FabF expression further illustrate the relative importance of each of these enzymes in modulating membrane FA composition and thereby the resistance of cells to environmental insults.

The mechanism by which changes in FabHa and FabF protein levels affect FA composition are understood based on prior enzymological analysis in this and other systems. FabH condenses an acyl-CoA with malonyl-ACP to form a 3-ketoacyl-ACP that initiates new cycles of FA elongation (44). The two *B. subtilis* FabH isozymes, FabHa and FabHb (27), catalyze the same reaction although FabHb has a higher specific activity for straight chain FA substrates than FabHa (11). Thus, downregulation of FabHa by σ^W increases the proportion of straight chain FAs by increasing the cell's reliance on FabHb (Figure 2.1D). The other enzyme affected by P_5 is FabF, the elongation condensing enzyme that adds two carbons to the growing FA chain in each round of elongation (44). As the only elongation condensing enzyme in *B. subtilis*, FabF activity plays a determining role in the final chain length of the acyl-ACP (31, 36). The acyl-ACPs have 3 possible fates: 1) conversion to acyl-PO_4 by PlsX; 2) acylation of lysophosphatidic acid by PlsC; or 3) elongation by another 2 carbons by FabF. Therefore, the σ^W-dependent upregulation of FabF alters the competitive balance between these 3 fates to increase the average FA chain length, as illustrated in strains where FabF is conditionally induced under xylose control. Together, these increases in the proportion of straight chain FA and average FA chain length reduce membrane fluidity. Sequence comparisons suggest that this regulatory mechanism is likely conserved in those *Bacillus* spp. containing two FabH paralogs,

but conservation of the P_5 promoter is not apparent in more distantly related bacteria that contain neither *fabHb* nor an obvious *sigW* ortholog (Table S1). In *Streptococcus pneumoniae* a similar regulatory effect is exerted instead by the essential YycFG TCS which downregulates *fabH*, upregulates *fabF*, and increases the average FA chain length of phospholipids in the plasma membrane (33). The physiological role for this homeoviscous adaptation could be similar to that of the P_5 pathway. Unlike the situation in *B. subtilis*, *Listeria monocytogenes* has only a single FabH which displays an altered substrate specificity at different temperatures and thereby contributes to temperature-dependent adjustments in membrane fluidity (38).

One of the best characterized mechanisms of homeoviscous adaptation is the DesRK TCS of *B. subtilis* that regulates expression of a FA desaturase to conditionally increase membrane fluidity (Aguilar & Mendoza, 2006). This system is initiated by the sensor kinase DesK which becomes activated in response to an increase in membrane thickness due to a temperature decrease (12). In contrast with DesRK, which is activated by conditions that decrease membrane fluidity, the σ^W-dependent response can be activated, either directly or indirectly, by conditions that increase membrane fluidity. The σ^W regulon mediates resistance to antimicrobial compounds by activating expression of detoxification enzymes, immunity proteins, and efflux pumps. Here, we extend this suite of mechanisms to include chemical alterations to the membrane that contribute to resistance against the action of membrane-destabilizing compounds. Since the σ^W regulon is induced by detergents and related membrane-active compounds, this system provides a novel mechanism of homeoviscous adaptation.

2.6 Acknowledgements

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2.7 Supplementary information

Table S2.1: Consensus P_5 nucleotide sequences in *B. subtilis* and related species

Strain(s)	Consensus P_5 nucleotide sequence[a]	# of *fabH* genes	# of σ^{ECF}
Bacillus subtilis	GTTGAAACTTCTGACGAGTGGATTCGTACAAGAACA	2	7
Consensus from 6 species closely related to B. subtilis[b]		2	7-10
Consensus from 4 *Bacillus ACT* species[c]		2	7-10
Consensus from 17 *Listeria* species[d]		1	1
Consensus from 17 *Staphylococcus* species[d]		1	1
Amino acid consensus from all analyzed strains		NA	NA

[a] For each sequence, the region corresponding to the P5 -35 and -10 elements is underlined.
[b] *Bacillus subtilis subsp. subtilis str. 168, Bacillus subtilis subsp. spizizenii str. W23, Bacillus licheniformis ATCC 14580, Bacillus atrophaeus 1942, Bacillus pumilus SAFR-032,* and *Bacillus amyloliquefaciens DSM7*
[c] *Bacillus anthracis str. Sterne, Bacillus thuringiensis str. Al Hakam, Bacillus cereus ATCC 14579,* and *Bacillus cereus subsp. cytotoxis NVH 391-98*
[d] Includes all 17 *listeria/Staphylococcus* genomes on the U Chicago SEED server (http://www.theseed.org)

Note: Residues W38, R42, and T43 are part of the FabH substrate binding site. W38 and T43 (last residue shown) are highly conserved with R42 showing some variability (15).

Table S2.2: Bacterial strains, plasmids, and oligos used in Chapter 2.

Strain, plasmid, or oligo	Genotype or description	Source, reference, or construction[1]
***B. subtilis* strains**		
W168	*trpC2*	Lab Stock
HB0020	*CU1065 sigW::MLS*	(7)
HB6208	*W168 sigW::spec*	(6)
HB13001	*W168 amyE::P_{fabHaF}-P_5-lacZ*	pTK001 --> W168
HB13042	*W168 amyE::P_{xyl}-sigW*	pVG003 --> W168
HB13054	*W168 thrC::P_{fabHaF}-fabHa-FLAG*	pTK013 --> W168
HB13056	*W168 thrC::P_{fabHaF}-fabHa-fabF-FLAG*	pTK015 --> W168
HB13058	*W168 thrC::P_{fabHaF}-fabHa-FLAG amyE::P_{xyl}-sigW*	HB13042 chrDNA --> HB13054
HB13060	*W168 thrC::P_{fabHaF}-fabHa-fabF-FLAG amyE::P_{xyl}-sigW*	HB13042 chrDNA --> HB13056
HB13069	*W168 amyE::P_5-lacZ*	pTK022 --> W168
HB13077	*W168 thrC::P_{fabHaF}-fabHa-FLAG sigW::kan*	HB0020 chrDNA --> HB13054
HB13078	*W168 thrC::P_{fabHaF}-fabHa-fabF-FLAG sigW::kan*	HB0020 chrDNA --> HB13056
HB13080	*W168 amyE::P_5*-lacZ*	pTK031 --> W168
HB13082	*W168 P_{fabHaF}-P_5*-lacZ*	pTK033 --> W168
HB13099	*W168 amyE::P_5-lacZ sigW::MLS*	HB0020 chrDNA --> HB13069
HB13115	*W168 fabHb::kan*	LFH-PCR --->168
HB13117	*W168 P_{fabHaF}-fabHa(P_5)fabF*	pTK043 --> W168
HB13118	*W168 P_{fabHaF}-fabHa(P_5*)fabF*	pTK044 --> W168
HB13121	*W168 P_{fabHaF}-fabHa(P_5)fabF amyE::P_{xyl}-sigW*	HB13042 chrDNA --> HB13117
HB13122	*W168 P_{fabHaF}-fabHa(P_5*)fabF amyE::P_{xyl}-sigW*	HB13042 chrDNA --> HB13118
HB13123	*W168 thrC::P_{fabHaF}-fabHa(P_5*)-FLAG*	pTK045 --> W168
HB13124	*W168 thrC::P_{fabHaF}-fabHa(P_5*)-fabF-FLAG*	pTK046 --> W168
HB13125	*W168 thrC::P_{fabHaF}-fabHa(P_5*)-FLAG amyE::P_{xyl}-sigW*	HB13042 chrDNA --> HB13123
HB13126	*W168 thrC::P_{fabHaF}-fabHa(P_5*)-fabF-FLAG amyE::P_{xyl}-sigW*	HB13042 chrDNA --> HB13124
HB13127	*W168 fabHa::MLS*	LFH-PCR --->168
HB13128	*W168 amyE::P_{xyl}-fabF*	pTK047 --> W168
HB13132	*W168 fabHa::MLS amyE::P_{xyl}-fabF*	HB13128 chrDNA --> HB13127
BSU2006	*W168 ΔsigM ΔsigY ΔsigZ ΔsigV ΔylaC ΔsigX*	(2)
HB13151	*W168 ΔsigM ΔsigY ΔsigZ ΔsigV ΔylaC ΔsigX P_5-lacZ*	HB13069 chrDNA --> BSU2006

Plasmids		
pdg1661	Vector for integration of *lacZ* fusions at *amyE*	(16)
pdg1664	Vector for integration at *thrC* locus	(16)
pMUTIN	Vector for allelic replacement	(42)
pVG003	*pSWEET-sigW (cat)*	Veronica Guariglia-Oropeza (unpublished results)
pTK001	*pDG1661-P_{fabHaF}-P_5-lacZ (cat)*	This work
pTK013	*pDG1664-P_{fabHaF}-fabHa-FLAG (MLS)*	This work
pTK015	*pDG1664-P_{fabHaF}-fabHa-fabF-FLAG (MLS)*	This work

pTK022	*pDG1661-P_5-lacZ (cat)*	This work
pTK031	*pDG1661-P_5*-lacZ (cat)*	This work
pTK033	*pDG1661-P_{fabHaF}-P_5*-lacZ (cat)*	This work
pTK043	*pMUTIN- P_{fabHaF}-P_5 (MLS)*	This work
pTK044	*pMUTIN- P_{fabHaF}-P_5* (MLS)*	This work
pTK045	*pDG1664-P_{fabHaF}-fabHa(P_5*)-FLAG (MLS)*	This work
pTK046	*pDG1664-P_{fabHaF}-fabHa(P_5*)-fabF-FLAG (MLS)*	This work
pTK047	*pSWEET-fabF (cat)*	This work

Oligos	**Name**	**Sequence**
4520	P_5 GSP1	CGTACATGAAGCCCGCACA
4521	P_5 GSP2	CCGAGTTGTTCTTGAATCATA
4549	AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
4778	FabHa UP BamHI	AGCTGGATCCCAACTGCATACGCCTCCTT
4780	FabHa Flag DO EcoRI	AGCTGAATTCTTATTATTTATCATCATCATCTTTATAA TCCGGCCGAACGACCACATCGCCGTCT
4781	FabF Flag DO EcoRI	AGCTGAATTCTTATTATTTATCATCATCATCTTTATAA TCCGGCCGGAATCCTAATGAGTTGCTGA
4883	P_5 Fwd cgGa -10 element	TCTGACGAGTGGATTCGGACAAGAACAGGAATAGAA G
4884	P_5 Rev cgGa -10 element	TCTATTCCTGTTCTTGTCCGAATCCACTCGTCAGAAG
4576	P_{fabHaF}-P_5 UP EcoRI	AGTCGAATTCCAACTGCATACGCCTCCT
4577	P_5 DO BamHI	AGTCGGATCCCGTACATGAAGCCCGCACA
4852	P_5 UP EcoRI	AGTCGAATTCAGGATAGAATTAGTACCTGATA
5059	fabHb up-fwd	CGTACAAAACAAGCAGAGACA
5060	fabHb up-rev (kan)	CCTATCACCTCAAATGGTTCGCTGTCACTCCTTATGG TCAGATTA
5061	fabHb do-fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGGATTAACCTAT ACAGGATTGCT
5062	fabHb do-rev	GACAGGAAGCAGCCAAGTAT
5164	P_{fabHaF}-up-rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCGATGTGTTTTCAA GAATGATGT
5165	P_{fabHaF}-do-fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCCAACTGCATACG CCTCCTT
5166	P_{fabHaF}-do-rev	TTAGTCATTAGGGAAGACTCCTTTATATCT
5167	fabF-do-fwd	AGATATAAAGGAGTCTTCCCTAATGACTAAAAAAAG AGTAGTTGT
5168	fabF UP RBS PacI	AGTCTTAATTAA*AGGAGG*ACACAAGATGACTAAAAA AAGA
5169	fabF DO BamHI	AGTCGGATCCTCATGTGATCGCCTCCTCT

Other Bacillus Strains	**Bacillus Genetic Stock Center (BGSC) #**
B. subtilis ssp. spizizenii NRRL B-23049	$2A8^T$
B. subtilis ssp. spizizenii NRRL B-14472	2A9
B. licheniformis ATCC14580	5A36T
B. amyloliquefaciens FZB42	NA
B. atrophaeus NRS-213	11A2T
B. atrophaeus ESM rplV str	12A1

[1] Abbreviations used: --> indicates transformation; chrDNA indicates chromosomal DNA.

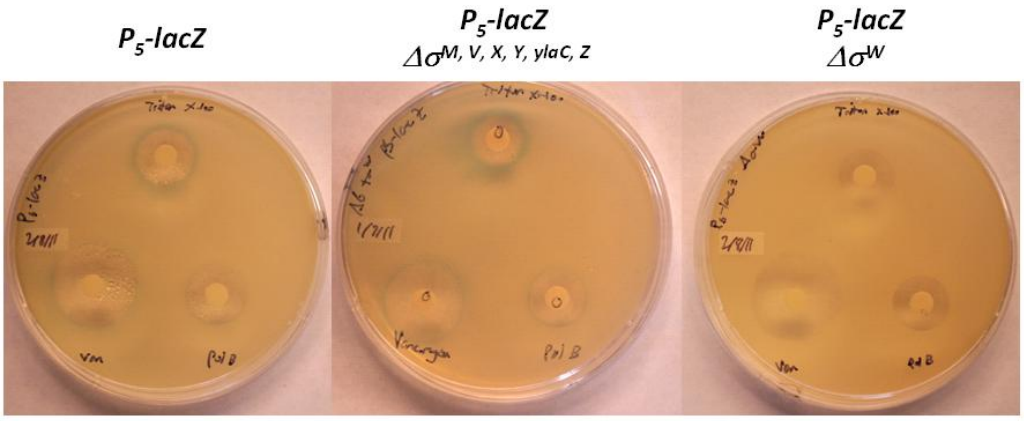


Figure S2.1: Disk diffusion assays to monitor induction of *P_5-lacZ* by cell envelope antibiotics. Representative results are shown for HB13069 (*P_5-lacZ*), HB13151 (*P_5-lacZ ΔsigM ΔsigY ΔsigZ ΔsigV ΔylaC ΔsigX),* and HB13099 (*P_5-lacZ sigW::MLS*) under triton X-100 (top disk), vancomycin (bottom left disk), and polymyxin B (bottom right disk) treatments. Pictures are representative of at least three independent experiments performed with biological triplicates.

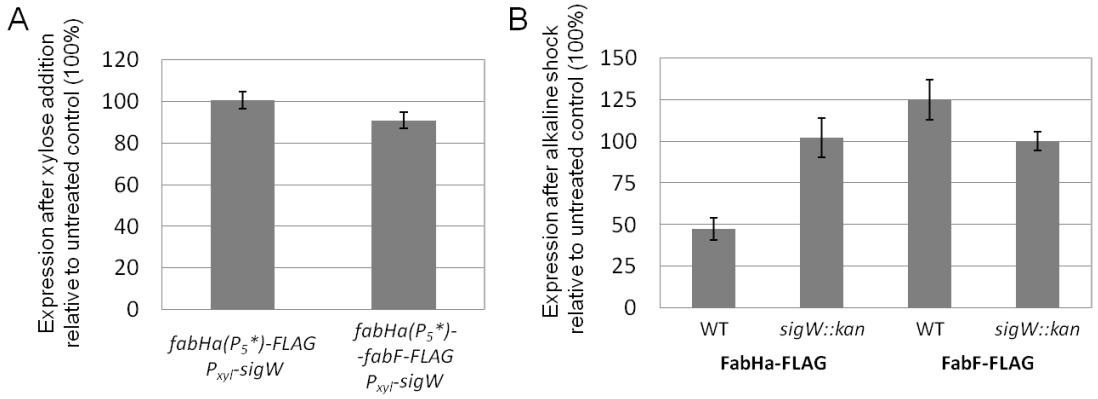


Figure S2.2: Supplementary Western blot quantification.
A. Change in expression of FabHa-FLAG in *fabHa(P_5*)-FLAG P_{xyl}-sigW* (HB13125) and of FabF-FLAG in *fabHa(P_5*)-fabF-FLAG P_{xyl}-sigW* (HB13126) upon xylose treatment. This experiment was performed in biological triplicate and repeated at least three times. Bars represent the relative levels of FabHa-FLAG or FabF-FLAG compared to an untreated control (error bars indicate standard error). Student's *t* tests showed no statistically significant difference (*P* value $\geq$ 0.05) between the xylose and non-xylose treated cells for both FabHa-FLAG and FabF-FLAG expression.
B. Change in expression of FabHa-FLAG and FabF-FLAG levels following alkaline shock in strains *fabHa-FLAG* (HB13054), *fabF-FLAG* (HB13056), *fabHa-FLAG sigW::kan* (HB13077), and *fabF-FLAG sigW::kan* (HB13078). Bars represent the relative levels of FabHa-FLAG or FabF-FLAG compared to an untreated control with error bars indicating standard error. Student's *t* tests showed a statistically significant difference (*P* value $\leq$ 0.05) between the *fabHa-FLAG* and *fabHa-FLAG sigW::kan* strains, but a non-significant difference (*P* value $\geq$ 0.05) between the *fabF-FLAG* and *fabF-FLAG sigW::kan* strains.

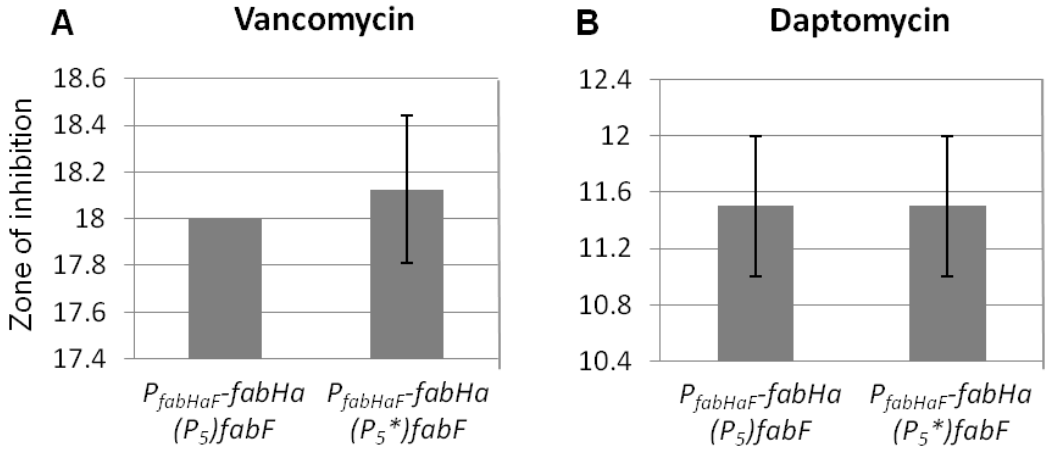


Figure S2.3: Disk diffusion assays of vancomycin (A) and daptomycin (B) sensitivity in the P_{fabHaF}-*fabHa*(P_5)*fabF* (HB13117), and P_{fabHaF}-*fabHa*(P_5*)*fabF* (HB13118) strains. Each bar represents the average zone of inhibition of at least three assays performed with three independent clones of each strain. Error bars represent standard error. Student's *t* tests were performed, and the zones of inhibition for the two strains were not significantly different (*P* value $\geq$ 0.05) under vancomycin or daptomycin treatment.

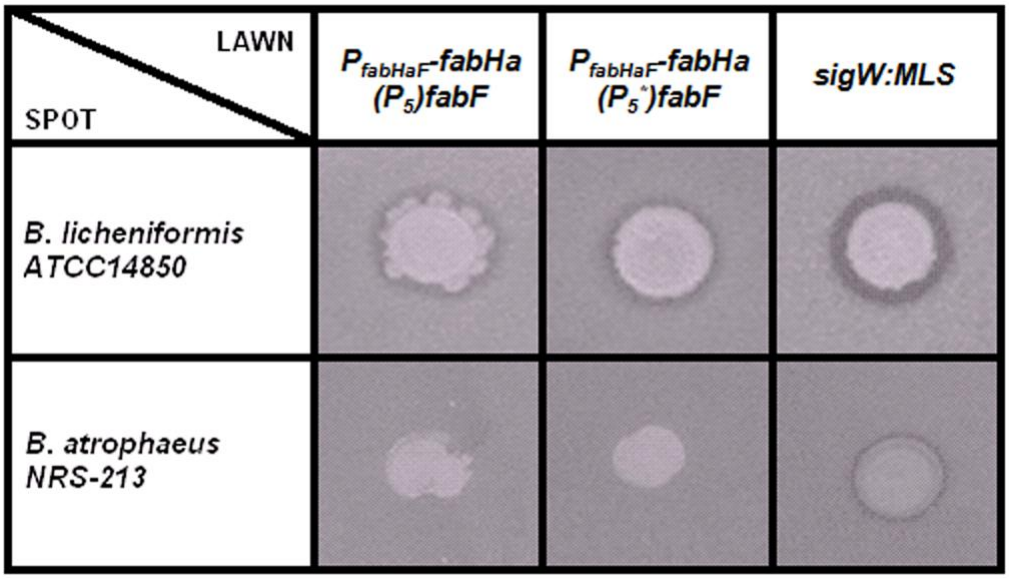


Figure S2.4: Spot on lawn assays showing the sensitivity of the P_{fabHaF}-*fabHa*(P_5)*fabF* (HB13117), P_{fabHaF}-*fabHa*(P_5*)*fabF* (HB13118), and *sigW::spec* (HB6208) lawns to *B. licheniformis ATCC14850* and *B. atrophaeus NRS-213*. Pictures are representative of at least three assays performed with three independent clones of each strain.

REFERENCES

1. **Aguilar, P. S., and D. de Mendoza.** 2006. Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. Mol Microbiol **62:**1507-1514.

2. **Asai, K., K. Ishiwata, K. Matsuzaki, and Y. Sadaie.** 2008. A viable *Bacillus subtilis* strain without functional extracytoplasmic function sigma genes. J Bacteriol **190:**2633-2636.

3. **Beranova, J., M. C. Mansilla, D. de Mendoza, D. Elhottova, and I. Konopasek.** 2010. Differences in cold adaptation of *Bacillus subtilis* under anaerobic and aerobic conditions. J Bacteriol **192:**4164-4171.

4. **Bhavsar, A. P., X. Zhao, and E. D. Brown.** 2001. Development and characterization of a xylose-dependent system for expression of cloned genes in *Bacillus subtilis*: Conditional complementation of a teichoic acid mutant. Appl Environ Microbiol **67:**403-410.

5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:**911-917.

6. **Butcher, B. G., and J. D. Helmann.** 2006. Identification of *Bacillus subtilis* σ^W-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli. Mol Microbiol **60:**765-782.

7. **Cao, M., B. A. Bernat, Z. Wang, R. N. Armstrong, and J. D. Helmann.** 2001. FosB, a cysteine-dependent fosfomycin resistance protein under the control of σ^W, an extracytoplasmic-function σ factor in *Bacillus subtilis*. J Bacteriol **183:**2380-2383.

8. **Cao, M., and J. D. Helmann.** 2004. The *Bacillus subtilis* extracytoplasmic-function σ^X factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. J Bacteriol **186:**1136-1146.

9. **Cao, M., P. A. Kobel, M. M. Morshedi, M. Fang, and W. Wu.** 2002. Defining the *Bacillus subtilis* σ^W regulon: A comparative analsis of promoter consensus search, run-off transcription/macroarrray analysis (ROMA), and transcriptional profiling approaches. J Mol Biol **315:**443-457.

10. **Cao, M., T. Wang, R. Ye, and J. D. Helmann.** 2002. Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ^W and σ^M regulons. Mol Microbiol **45:**1267-1276.

11. **Choi, K. H., R. J. Heath, and C. O. Rock.** 2000. β-ketyacyl-acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. J Bacteriol **182:**365-370.

12. **Cybulski, L. E., M. Martin, M. C. Mansilla, A. Fernandez, and D. de Mendoza.** 2010. Membrane thickness cue for cold sensing in a bacterium. Curr Biol **20:**1539-1544.

13. **Denich, T. J., L. A. Beaudette, H. Lee, and J. T. Trevors.** 2003. Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Meth **52:**149-182.

14. **Eiamphungporn, W., and J. D. Helmann.** 2008. The *Bacillus subtilis* σ^M regulon and its contribution to cell envelope stress responses. Mol Microbiol **67:**830-848.

15. **Gajiwala, K. S., S. Margosiak, J. Lu, J. Cortez, Y. Su, Z. Nie, and K. Appelt.** 2009. Crystal structures of bacterial FabH suggest a molecular basis for the substrate specificity of the enzyme. FEBS Lett **583:**2939-2946.

16. **Guerout-Fleury, A.-M., N. Frandsen, and P. Stragier.** 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57-61.

17. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for Bacillus. John Wiley & Sons Ltd, Chichester, United Kingdom.

18. **Hashimoto, M., H. Takahashi, Y. Hara, H. Hara, K. Asai, Y. Sadie, and K. Matsumoto.** 2009. Induction of extracytoplasmic function sigma factors in *Bacillus subtilis* cells with membranes of reduced phosphatidylglycerol content. Genes Genet Syst **84:**191-198.

19. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol **46:**47-110.

20. **Huang, X., A. Gaballa, M. Cao, and J. D. Helmann.** 1999. Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function σ factor, σ^W. Mol Microbiol **31:**361-371.

21. **Huang, X., and J. D. Helmann.** 1998. Identification of target promoters for the *Bacillus subtilis* sigma X factor using a consensus-directed search. J Mol Biol **279:**165-173.

22. **Jordan, S., M. I. Hutchings, and T. Mascher.** 2008. Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol Rev **32:**107-146.

23. **Koo, B. M., V. A. Rhodius, G. Nonaka, P. L. deHaseth, and C. A. Gross.** 2009. Reduced capacity of alternative sigmas to melt promoters ensures stringent promoter recognition. Genes Dev **23:**2426-36.

24. **Kuhry, J.-G., G. Duportail, C. Bronner, and G. Laustriat.** 1985. Plasma membrane fluidity measurements on whole living cells by fluorescence anisotropy of trimethylammoniumdiphenylhexatriene. Biochim Biophys Acta **845:**60-67.

25. **Leonardi, R., M. W. Frank, P. D. Jackson, C. O. Rock, and S. Jackowski.** 2009. Elimination of the CDP-ethanolamine pathway disrupts hepatic lipid homeostasis. J Biol Chem **284:**27077-89.

26. **Los, D. A., and N. Murata.** 2004. Membrane fluidity and its roles in the perception of environmental signals. Biochim Biophys Acta **1666:**142-157.

27. **Lu, Y. J., Y. M. Zhang, and C. O. Rock.** 2004. Product diversity and regulation of type II fatty acid synthases. Cell Biol **82:**145-155.

28. **Luo, Y., K. Asai, Y. Sadaie, and J. D. Helmann.** 2010. Transcriptomic and phenotypic characterization of a *Bacillus subtilis* strain without extractyoplasmic function σ factors. J Bacteriol **192:**5736-5745.

29. **Mascher, T., A. B. Hachmann, and J. D. Helmann.** 2007. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. J Bacteriol **189:**6919-6927.

30. **Mascher, T., N. G. Margulis, T. Wang, R. W. Ye, and J. D. Helmann.** 2003. Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. Mol Microbiol **50:**1591-1604.

31. **Mendoza, D. d., P. Aguilar, and G. Schujman.** 2001. Biosynthesis and function of membrane lipids, p. 43-55. *In* J. A. Hoch, A. L. Sonenshein, and R. Losick (ed.), *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells. American Society for Microbiology, Washington D.C.

32. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

33. **Mohedano, M. L., K. Overweg, A. d. l. Fuente, M. Reuter, S. Altabe, F. Mulholland, D. de Mendoza, P. Lopez, and J. M. Wells.** 2005. Evidence that the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of fatty acid biosynthesis genes and alters membrane composition J. Bacteriol **187:**2357-2367.

34. **Rivera-Milla, E., C. A. O. Stuermer, and E. Málaga-Trillo.** 2006. Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain. Cell Mol Life Sci **63:**343-357.

35. **Schramm, G., I. Bruchhaus, and T. Roeder.** 2000. A simple and reliable 5'-RACE approach. Nuc Acids Res **28:**e96.

36. **Schujman, G. E., S. Altabe, and D. de Mendoza.** 2008. A malonyl-CoA-dependent switch in the bacterial response to a dysfunction of lipid metabolism. Mol Microbiol **68:**987-996.

37. **Schujman, G. E., and D. de Mendoza.** 2008. Regulation of type II fatty acid synthase in Gram-positive bacteria. Curr Opin Microbiol **11:**148-152.

38. **Singh, A. K., Y.-M. Zhang, K. Zhu, C. Subramanian, Z. Li, R. K. Jayaswal, C. Gatto, C. O. Rock, and B. J. Wilkinson.** 2009. FabH selectivity for anteiso branched-chain fatty acid precursors in low-temperature adaptation in *Listeria monocytogenes*. FEMS Microbiol Lett **301:**188-192.

39. **Soonsanga, S., J.-W. Lee, and J. D. Helmann.** 2008. Conversion of *Bacillus subtilis* OhrR from a 1-Cys to a 2-Cys peroxide sensor. J Bacteriol **190:**5738-5745.

40. **Svobodová, J., and P. Svoboda.** 1988. Cytoplasmic membrane fluidity measurements on intact living cells of *Bacillus subtilis* by fluorescence anisotropy of 1,6-diphenyl 1,3,5-hexatriene. Folia Microbiol (Praha) **33:**1-9.

41. **Tamehiro, N., Y. Okamoto-Hosoya, S. Okamoto, M. Ubukata, M. Hamada, H. Naganawa, and K. Ochi.** 2002. Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrob Agents Ch **46:**315-320.

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

43. **Wade, J. T., D. C. Roa, D. C. Grainger, D. Hurd, S. J. W. Busby, K. Struhl, and E. Nudler.** 2006. Extensive functional overlap between σ factors in *Escherichia coli*. Nat Struct Mol Biol **13:**806-814.

44. **White, S. W., J. Zheng, Y.-M. Zhang, and C. O. Rock.** 2005. The structural biology of type II fatty acid biosynthesis. Annu Rev Microbiol **74:**791-831.

45. **Wiegert, T., G. Homuth, S. Versteeg, and W. Schumann.** 2001. Alkaline shock induces the *Bacillus subtilis* σ^W regulon. Mol Microbiol **41:**59-71.

46. **Zhang, Y. M., and C. O. Rock.** 2008. Membrane lipid homesostasis in bacteria. Nat Rev Microbiol **6:**222-233.

CHAPTER 3

CONTRIBUTIONS OF THE σ^W, σ^M, AND σ^X REGULONS TO THE LANTIBIOTIC RESISTOME OF *BACILLUS SUBTILIS*[1]

3.1 Summary

In *Bacillus subtilis*, the extracytoplasmic function (ECF) σ factors σ^M, σ^W, and σ^X all contribute to resistance against lantibiotics. Nisin, a model lantibiotic, has a dual mode of action: it inhibits cell wall synthesis by binding lipid II, and this complex also forms pores in the cytoplasmic membrane. These activities can be separated in a nisin hinge-region variant (N20P M21P) that binds lipid II, but no longer permeabilizes membranes. The major contribution of σ^M to nisin resistance is expression of *ltaSa*, encoding a stress-activated lipoteichoic acid synthase, and σ^X functions primarily by activation of the *dlt* operon controlling D-alanylation of teichoic acids. Together, σ^M and σ^X regulate cell envelope structure to decrease access of nisin to its lipid II target. In contrast, σ^W is principally involved in protection against membrane permeabilization as it provides little protection against the nisin hinge region variant. σ^W contributes to nisin resistance by regulation of a signal peptide peptidase (SppA), phage shock proteins (PspA and YvlC, a PspC homolog), and tellurite resistance related proteins (YceGHI). These defensive mechanisms are also effective against other lantibiotics such as mersacidin, gallidermin, and subtilin and comprise an important subset of the intrinsic antibiotic resistome of *B. subtilis*.

[1]The results of this study are pending publication in Molecular Microbiology. Authors: Kingston AW, Liao X, and Helmann JD. X.L. contributed to some disk diffusion

assays and β-galactosidase assays. All other experiments were performed by A.W.K. A.W.K and J.D.H wrote the manuscript.

3.2 Introduction

As a normal resident of the soil and rhizosphere, *B. subtilis* encounters a diverse array of antimicrobial compounds produced by competing microbes, including other *Bacilli*. Typically, gene clusters involved in antibiotic production encode specific immunity functions that protect the producer organism against self-imposed toxicity. However, non-producers also display significant intrinsic antibiotic resistance, imparted by genes collectively known as the antibiotic resistome (89). In many cases, key components of the resistome are induced when cells encounter antibiotics and, as explored here, this induction may be mediated by extracytoplasmic function (ECF) σ factors.

Antimicrobial compounds often target the cell envelope by inhibiting the synthesis or impairing the function of the peptidoglycan cell wall (74) and the phospholipid membrane (35). Upon encountering cell envelope active antibiotics, *B. subtilis* activates the expression of one or more cell envelope stress responses (CESRs), often including genes that protect against antibiotic-mediated killing (40). Here, we focus specifically on the responses to a subset of bacterially produced peptide antibiotics (bacteriocins) known as lantibiotics (14) and the corresponding lantibiotic resistance determinants.

Lantibiotics (class I bacteriocins) are antimicrobial peptides containing lanthionine, thioether bridged amino acids that introduce intramolecular rings within the peptide. They play critical roles in interspecies competition (64) and share

similarities with cationic antimicrobial peptides (CAMPs) produced by the immune systems of humans and higher organisms (63, 79) (72). In addition, lantibiotics show promise for clinical applications because they are effective against a wide spectrum of Gram positive bacteria including known pathogens (7) (16) (20) (50). The most widely used and extensively studied lantibiotic is nisin, a 34 amino acid cationic antimicrobial peptide produced by *Lactococcus lactis*. This bacteriocin is active against a broad range of Gram-positive bacteria and has been used as a food preservative for almost 60 years (14, 17, 20). Partly because of its ubiquity, nisin serves as a model for studying lantibiotics (5).

Nisin employs a potent mechanism of action that is shared by many lantibiotics. Nisin binds with high affinity to lipid II (30), a membrane bound cell wall precursor essential for peptidoglycan synthesis. Once bound, nisin permeabilizes the membrane through lipid-II-dependent pore formation. Pore formation is made possible by a flexible hinge region in the middle of the peptide that allows the C-terminus to insert into the membrane while the N-terminus remains bound to lipid II (5). Thus, nisin has a dual mechanism and acts both by inhibiting cell wall synthesis and disrupting cell membrane integrity. Other lantibiotics that share this dual mechanism of action (such as subtilin, ericin, gallidermin, epidermin and entianin) are usually type A(I) lantibiotics (linear peptides) whereas type B lantibiotics (globular peptides), such as mersacidin and cinnamycin typically bind lipid II, but do not insert into membranes (37).

In the presence of nisin, *B. subtilis* activates multiple CESR pathways including those controlled by ECF σ factors (collectively, σ^{ECF}) and two component

systems (9, 65). *B. subtilis* encodes seven ECF σ factors, among which four (σ^M, σ^W, σ^X, and σ^V) are known to be activated by, and confer resistance against, cell envelope active compounds (18, 23, 31). These four ECF σ factors are activated by overlapping sets of cell envelope stresses and subsequently up-regulate distinct, but overlapping, sets of genes that confer resistance (53).

In this study, we demonstrate that σ^M, σ^W, and σ^X contribute to intrinsic nisin resistance through their collective activation of six operons: *pspA*, *yvlC*, *sppA*, *ltaSa*, *dltABCD*, and *yceGHI*. The σ^M and σ^X controlled operons contribute broadly to lantibiotic resistance including compounds that bind lipid II, but do not permeabilize the membrane. In contrast, σ^W regulated genes function primarily to protect against the membrane-permeabilization activity of lantibiotics. These same resistance determinants protect against related lantibiotics, such as gallidermin and mersacidin, and against competing *Bacillus* strains that produce lantibiotics and other antimicrobial compounds.

3.3 Materials and methods

Strains, plasmids, and growth conditions. All *B. subtilis* strains, plasmids, and oligonucleotides (oligos) used in this study are listed Table S7. Bacteria were grown in liquid Luria-Bertani (LB), penassay broth (PAB), Belitsky minimal (MM) (78) or Mueller Hinton (MH) medium at 37°C with vigorous shaking or on solid LB or MH medium containing 1.5% Bacto agar (Difco) with appropriate selection. Plasmids were amplified in *Escherichia coli* DH5α before transformation of *B. subtilis* strains.

Ampicillin (amp; 100 μg ml^{-1}), kanamycin (kan; 30 μg ml^{-1}), chloramphenicol (cat; 34 μg ml^{-1}) were used to select *E. coli* transformants. For *B. subtilis*, antibiotics used for selection were: spectinomycin (spec; 100 μg ml^{-1}), kanamycin (kan; 15 μg ml^{-1}), chloramphenicol (cat; 10 μg ml^{-1}), tetracycline (20 μg ml^{-1}), neomycin (neo; 10 μg ml^{-1}), and macrolide-lincosoamide-streptogramin B (MLS; contains 1 μg ml^{-1} erythromycin and 25 μg ml^{-1} lincomycin).

Genetic techniques. Chromosomal and plasmid DNA transformations were performed as described previously (29). Unless otherwise stated, all PCR products were generated using WT168 chromosomal DNA as a template and all strains were verified by sequence analysis (Cornell University Life Sciences Core Laboratories Center). Gene deletions were generated using long-flanking homology PCR (LFH-PCR) as described previously (54).

Disk diffusion assays. Disk diffusion assays were performed as described (53). Briefly, strains were grown to an OD_{600} of 0.4. A 100 μl aliquot of these cultures was mixed with 4 ml of 0.7% MH soft agar (kept at 50°C) and directly poured onto MH plates (containing 15 ml of 1.5% MH agar). The plates were dried for 20 min in a laminar airflow hood. Filter paper disks containing the chemicals to be tested were placed on the top of the agar and the plates were incubated at 37°C overnight. The distances between the edge of the inhibition zones and the edge of the filter paper disks were measured. For IPTG treated cells, the indicated concentration of IPTG was added only to the soft agar except for 1 mM IPTG treatments which contained 1 mM IPTG in the MH plates as well. For promoter-*lacZ* strains, 80 μg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to the agar and the plates were

analyzed for the appearance of a blue ring around the edge of the zone of inhibition. Unless otherwise noted, the following chemicals and quantities were used in the disk diffusion assays: Triton X-100 10 µl of a 10% solution, sodium dodecyl sulfate 500 µg, bile salts 1 mg, colistin 100 µg, protamine 1 mg, Clofazimine 1 mg, poly-L-lysine 5 µg, ethylenediaminetetraacetic acid 365 µg, polymyxin B 50 µg, novobiocin 50 µg, vancomycin 50 µg, daptomycin 100 µg, D-cycloserine 500 µg, mersacidin 10 µg, gallidermin 5 µg, nisin 20 µg. For Nisin assays, a 2.5 mg ml^{-1} nisin stock solution was prepared by dissolving a 2.5% nisin mixture balanced with sodium chloride and denatured milk solids (Sigma-Aldrich Co. St. Louis, MO USA) in 0.02 M HCl. For daptomycin and mersacidin assays, the media was supplemented with 1.25 mM $CaCl_2$.

Broth Dilution Assays

We determined the minimal inhibitory concentration (MIC) and the 50% inhibition concentration (IC_{50}) of *B. subtilis* strains to nisin using a variation of the broth dilution assay described previously (48). Briefly, strains were grown to an OD_{600} of 0.4 in MH media. For MIC determination, strains were diluted 1:200 in MH broth, and 200 µl aliquots of the diluted cultures were dispensed in a Bioscreen 100-well microtitre plate. Each strain was grown in nisin concentrations ranging from 0.0625 to 10 µg ml^{-1} with concentrations near the MIC of a specific strain increasing by increments of 10% or less. For (IC_{50}) determination, 200 µl aliquots of the undiluted culture were dispensed into the wells of a Bioscreen 100-well microtitre plate. Each strain was grown in nisin concentrations ranging from 0.25 to 2.5 µg ml^{-1} with concentrations increasing by increments of 25% or less. Growth was measured spectrophotometrically (OD_{600}) every 15 min for 24 h using a Bioscreen C incubator

(Growth Curves USA, Piscataway, NJ) at 37 °C with continuous shaking. MIC was defined as the lowest concentrated that prevented growth ($OD_{600} < 0.2$) at the 10 h time point and IC_{50} was defined as the lowest concentration that reduced cell density to $OD_{600} < 0.2$ (a 50% reduction).

β-galactosidase assays. Strains carrying promoter-*lacZ* fusions were grown to an OD_{600} of 0.4 in LB. Cultures were then treated with the indicated concentrations of nisin or control (H_2O) and samples were taken 60 min after treatment. β-galactosidase assays were performed as described by Miller (57).

Western Blots. LTA detection in *B. subtilis* was adapted from Wörmann *et al*. (88). Wild-type and ltaSa::spec strains were grown to an OD_{600} of 0.6 in PAB media and treated with or without 0.5 μg ml^{-1} nisin for 60 minutes at 37 ^{o}C with aeration. Bacteria from 4.5 mL culture was pelleted by centrifugation and resuspended in 150 μL 2x protein sample buffer per mL culture of $OD_{600} = 3$. Samples were boiled for 45 min, centrifuged for 5 min, and a volume containing 100 ug of protein from each supernatant was analyzed on a 15% SDS PAGE gel. Blocking was performed with 3% BSA. LTA detection was accomplished with an overnight incubation in 1:250 Gram positive bacteria LTA monoclonal antibody (Thermo Fisher Scientific Inc.; Rockford, IL USA) followed by a 5 hour incubation in 1:2000 anti-mouse IgG alkaline phosphatase (Sigma-Aldrich Co. St. Louis, MO USA). Relative levels of each LTA were compared using densitometry analysis with ImageJ.

Spot-on-lawn assays. Spot-on-lawn assays were performed as described (8). Briefly, lawn cells were grown to an OD_{600} of 0.4 in LB. A 100 μL aliquot of these cultures was mixed with 4 mL of 0.7% LB soft agar (kept at 50°C) and directly poured onto

LB plates (containing 15 ml of 1.5% LB agar). Plates were dried for 20 min in a laminar flow hood, and 3 µl of the producer strain (OD_{600} of 0.6) was spotted on top of the agar. Plates were incubated at 37°C overnight (18 h) before observation.

Nisin production and purification. Production and purification of nisin and the N20PM21P nisin variant was adapted from (25) with some modifications. HE13090 (DE3 pRSFDuet-1-His6nisAB pACYCDuet-1-NisC) and HE13091 (DE3 pRSFDuet-1-nisAB-N20PM21P pACYCDuet-1-NisC) were each grown to an OD_{600} of 0.6 in 1 L terrific broth media containing 30 µg ml^{-1} kan and 34 µg ml^{-1} cat at 37 ^{o}C. The media was then supplemented with 0.5 mM IPTG and grown at 18 ^{o}C for 18 hours with continuous shaking. Each culture was pelleted, resuspended in 25 mL LEW buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), and incubated at 4 ^{o}C for 30 minutes with 1 mg ml^{-1} lysozyme. The samples were then lysed by sonication and subsequently centrifuged for 30 minutes at 5000 g. The resulting supernatants were each incubated with 0.75 g PrepEase his-tagged high yield purification resin (Affymetrix) for 30 minutes. The resins were loaded onto columns and washed with 50 mL LEW buffer containing 10 mM imidazole. Prenisin was eluted from each resin with three 4 mL fractions of LEW buffer containing 250 mM imidazole. The eluents were concentrated to 0.5 mL and desalted with Amicon Ultra-4 centrifugal filtration units, 3K MWCO (Millipore). Concentrated prenisin was treated with 2.5 µg trypsin (Sigma) in 100 mM tris at pH 8.0 and incubated for 18 hours @ 37 ^{o}C to generate mature nisin. At each step, the presence of prenisin or mature nisin was confirmed with a coomassie-stained tris-tricine gel. For disk diffusion assays, 10 µL of mature nisin or mature (N20PM21P)-nisin was used.

Statistical analysis. All experiments were performed with a minimum of three biological replicates. Unless otherwise noted, data is presented as mean ± standard error. Statistical evaluation of the data was performed by the use of unpaired Student's *t* tests. A value of $P \leq 0.05$ was considered statistically significant.

3.4 Results and discussion:

Three ECF σ factors (σ^M, σ^W, and σ^X) contribute to intrinsic nisin resistance. We previously reported that a *B. subtilis* NCIB3610 strain lacking σ^M, σ^W, and σ^X (the ΔMXW strain) displayed a significant increase in sensitivity to cell envelope active compounds and, in the case of nisin, the effects of these three σ factors were additive (53). In subsequent studies, we analyzed a *B. subtilis* W168 in which all seven genes encoding ECF σ factors were replaced by unmarked deletions (Δ7) (3). For most tested compounds, the Δ7 strain was not significantly more sensitive than an isogenic ΔMXW strain, although lantibiotics were not tested in that study (47).

Here, we have investigated nisin sensitivity in the *B. subtilis* 168 strain background and, consistent with previous results (53), we find that σ^M, σ^W, and σ^X contribute additively to nisin resistance (Figure 3.1A). Removal of the four remaining ECF σ factor genes, in the Δ7 strain, does not further increase nisin sensitivity. We also quantified the contribution of ECF σ factors to nisin resistance with a broth

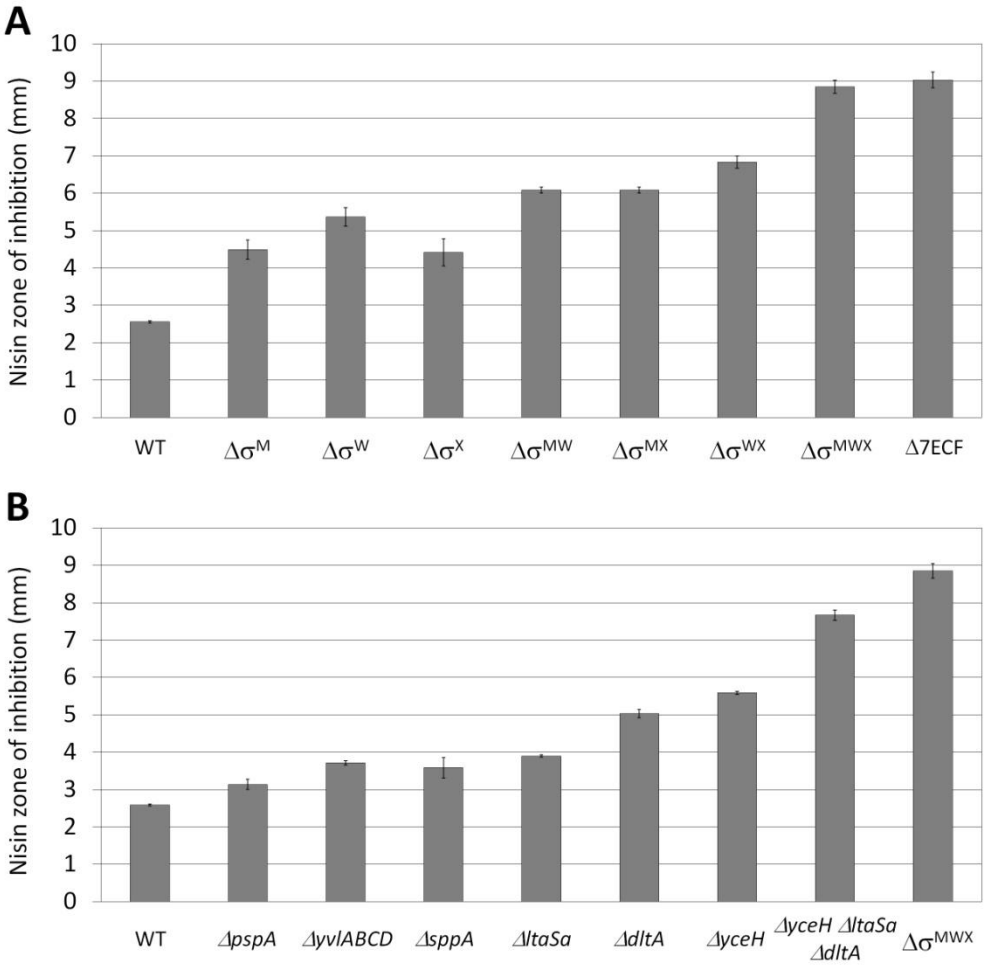


Figure 3.1: Disk diffusion assays that led to the identification of extracytoplasmic function sigma factor regulated nisin resistance genes. For all graphs representing disk diffusion assays, the *y-axis* shows the zone of inhibition (in millimeters), expressed as total diameter minus diameter of the filter paper disk (5.5 mm). Each bar represents the average zone of inhibition of at least three assays performed with three independent clones of each strain with error bars representing standard error. Statistically significant differences in zone of inhibition were determined with a Student's t-test (P-value 0.05). A. Nisin sensitivity for the WT (168), $\Delta\sigma^{M}$ (HB10216), $\Delta\sigma^{W}$ (HB10102), $\Delta\sigma^{X}$ (HB10103), $\Delta\sigma^{MW}$ (HB13218), $\Delta\sigma^{MX}$ (HB13217), $\Delta\sigma^{WX}$ (HB13219), $\Delta\sigma^{MWX}$ (HB10107), and Δ7ECF (BSU2007) strains. All mutant strains had significantly larger zones of inhibition than the WT strain, and the $\Delta\sigma^{MWX}$ zone of inhibition was significantly larger than that of the single or double ECF σ deletion strains. B. Nisin sensitivity for the WT (168), Δ*pspA* (HB13243), Δ *yvlABCD* (HB13242), Δ*sppA* (HB13251), Δ*ltaSa* (HB13210), Δ*dltA* (HB12084), Δy*ceH* (HB13281), *ΔltaSa ΔdltA ΔyceH* (HB13287), and $\Delta\sigma^{MWX}$ (HB10107) strains. All single gene deletion strains exhibited significantly larger zones of inhibition than the WT strains. The zone of inhibition of the *ΔltaSa ΔdltA ΔyceH* strain was significantly lower than that of the $\Delta\sigma^{MWX}$ strain.

dilution assay. The ΔMXW mutant had a 7-fold lower MIC than that of wild-type (0.45 ± 0.04 μg ml^{-1} vs. 3.25 ± 0.11 μg ml^{-1}).

This σ^{ECF}-mediated nisin resistance is augmented by the LiaRS two component system (TCS). In *B. subtilis*, nisin strongly activates LiaRS which upregulates the nisin resistance determinants LiaIH (28, 87). Our disk diffusion assays confirmed that *liaIH* null strain was significantly more sensitive to nisin than WT cells, and when *liaIH* was deleted from the ΔMXW strain, the nisin MIC further decreased to over 11 times its original value (0.29 ± 0.03 μg ml^{-1}).

Identification of σ^{ECF}-dependent operons that confer nisin resistance. Presumably, the ΔMXW strain is more susceptible to nisin because it is unable to activate the expression of specific, σ^{ECF}-dependent resistance genes. Previous studies have defined the regulons activated by each of these ECF σ factors (9, 10, 18). Each regulon contains ~30-60 target genes, including several that can be activated by more than one ECF σ factor. As a result of this regulon overlap, the total number of genes activated by σ^M, σ^W, and σ^X is ~80 (9, 10, 18). Within these regulons, the only operon known to confer nisin resistance is *dltABCD* which encodes a pathway (Dlt) for D-alanylation of teichoic acids (9, 62). However, a *ΔdltA* strain, previously shown to inactivate Dlt function (62), is not as sensitive to nisin as the ΔMXW strain (Figure 3.1), indicating that there are additional σ^{ECF}-activated genes that confer nisin resistance.

To identify σ^{ECF}-dependent operons that contribute to nisin resistance, we screened a library of strains containing mutations in operons activated by σ^M, σ^W, and/or σ^X, with a focus on genes that are associated with the cell envelope. Although

many of the mutant strains did not display increased nisin sensitivity (Table S1), there were generally small but measurable effects for the singly mutant strains lacking *pspA*, *yvlABCD*, *sppA*, *ltaSa*, or *yceH* (Figure 3.1B). Among these mutants, the strongest phenotypes were observed upon inactivation of *ltaSa*, *yceH*, or *dltA* (Figure 3.1B), and a triple mutant was almost as sensitive to nisin as the ΔMXW strain. These results indicate that *ltaSa*, *yceH*, and the *dlt* operon are each major contributors to σ^{ECF}-dependent nisin resistance in *B. subtilis*.

σ^{ECF} regulation of nisin resistance operons. The σ^{M}, σ^{W}, and σ^{X} factors are all moderately active in growing cells, depending on growth medium and growth phase (47, 53, 92). However, their activities can be induced in response to a variety of cell envelope stress conditions. To explore the contributions of each σ^{ECF} factor to both the basal and induced expression of these operons, we fused the promoter regions for each resistance determinant (P_{yvlA}, P_{sppA}, P_{pspA}, P_{dltA}, P_{ltaSa}, and P_{yceC}) to the *lacZ* reporter gene and integrated the resulting promoter-*lacZ* fusions into both WT and various $\Delta\sigma^{ECF}$ backgrounds. Initial studies were done using disk diffusion assays which provide a gradient of antibiotic concentrations and thereby allow a clear qualitative visualization of promoter induction. Strong induction was noted in response to both vancomycin and triton X-100, consistent with prior studies (10, 18, 65). More modest induction was seen in response to nisin (Table 3.1, and Figure S3.1). In the Δ*sigW* background, the P_{pspA}-*lacZ*, P_{sppA}-*lacZ*, and P_{yvlA}-*lacZ* strains had no detectable activity, consistent with the assignment of these promoters to the σ^{W} regulon (10). The activity of the P_{yceC}-*lacZ* fusion was substantially reduced in *ΔsigW*, but was only

completely abolished in the ΔMXW background. P_{yceC} is therefore primarily driven by σ^W but can be partially activated by σ^M and/or σ^X. A similar analysis with P_{dltA} showed that this promoter is primarily activated by σ^X and weakly activated by σ^M, which is in agreement with previous results (9). The P_{ltaSa}-*lacZ* fusion displayed diminished activity in the *ΔsigM* background, but still retained a small level of basal activity even in the ΔMXW strain. Thus, *ltaSa* can be activated by σ^M, but is also controlled by other transcriptional factors.

These induction results were corroborated with β-galactosidase assays on cells grown in liquid culture (Figure 3.2). Nisin concentrations ranged from low levels that did not affect growth (25 and 125 ng ml^{-1}), moderate levels that inhibited growth (250 and 375 ng ml^{-1}), to high levels (500 and 625 ng ml^{-1}) that induce cell lysis. The σ^W-dependent promoters P_{pspA}, P_{sppA}, $P_{yvlA,}$ and P_{yceH} were only upregulated by high nisin concentrations with a maximal induction of ~2 fold above their basal activity. $P_{dltA,}$ which is primarily activated by σ^X, was significantly upregulated by moderate nisin stress and was induced ~3.5 fold by high nisin levels. The σ^M-activated P_{ltaSa} had significant basal activity and was activated ~1.75 times by nisin. These assays demonstrate that the nisin resistance genes are expressed even in the absence of nisin stress, and this basal activity is due largely or entirely to the basal activity of σ^{ECF} factors in growing cells (Table 3.1). Addtionally, the promoters regulating these genes can by upregulated by nisin stress, but their induction is modest compared to P_{liaI} which can be induced over 400 by fold nisin (55).

Table 3.1: Detailed summary of the inducing activity of the nisin resisting σ^{ECF} promoters in WT and $\Delta\sigma^{ECF}$ backgrounds.[a]

Strain	Stress	WT	$\Delta\sigma^{W}$
P_{pspA}-lacZ	Triton X-100	++	-
	Vancomycin	++	-
	Nisin	+	-
P_{yvlA}-lacZ	Triton X-100	+++	-
	Vancomycin	+++	-
	Nisin	+	-
P_{sppA}-lacZ	Triton X-100	+++	-
	Vancomycin	+++	-
	Nisin	+	-

Strain	Stress	WT	$\Delta\sigma^{M}$	$\Delta\sigma^{W}$	$\Delta\sigma^{X}$	$\Delta\sigma^{MW}$	$\Delta\sigma^{MX}$	$\Delta\sigma^{WX}$	$\Delta\sigma^{MWX}$
P_{ltaSa}-lacZ	Triton X-100	++++	+	++++	++++	+	+	++++	+
	Vancomycin	++++	+	++++	++++	+	+	++++	+
	Nisin	++	+	++	++	+	+	++	+
P_{dltA}-lacZ	Triton X-100	++++	++++	NA	+	NA	-	NA	NA
	Vancomycin	++++	++++	NA	+	NA	-	NA	NA
	Nisin	++	++	NA	+	NA	-	NA	NA
P_{yceC}-lacZ	Triton X-100	++++	+++	+	++++	+	++	+	-
	Vancomycin	++++	+++	+	++++	+	++	+	-
	Nisin	++	+	+	++	+	+	+	-

[a.] The induction ability of *P_{pspA}-lacZ, P_{yvlA}-lacZ, P_{pspA}-lacZ, P_{ltaSa}-lacZ, P_{dltA}-lacZ*, and *P_{yceC}-lacZ* gene fusions in WT and $\Delta\sigma^{ECF}$ backgrounds to triton X-100, vancomycin, and nisin as measured by disk diffusion assays. The reported activity of each strain represents intensity of the blue halos induced by each stress after overnight incubation: ++++ (dramatic blue) > +++ (strong blue) > ++ (blue) > + (light blue) > -(white after 3 days, no induction). A sample image of a disk diffusion assay can be found in Figure S3.2.

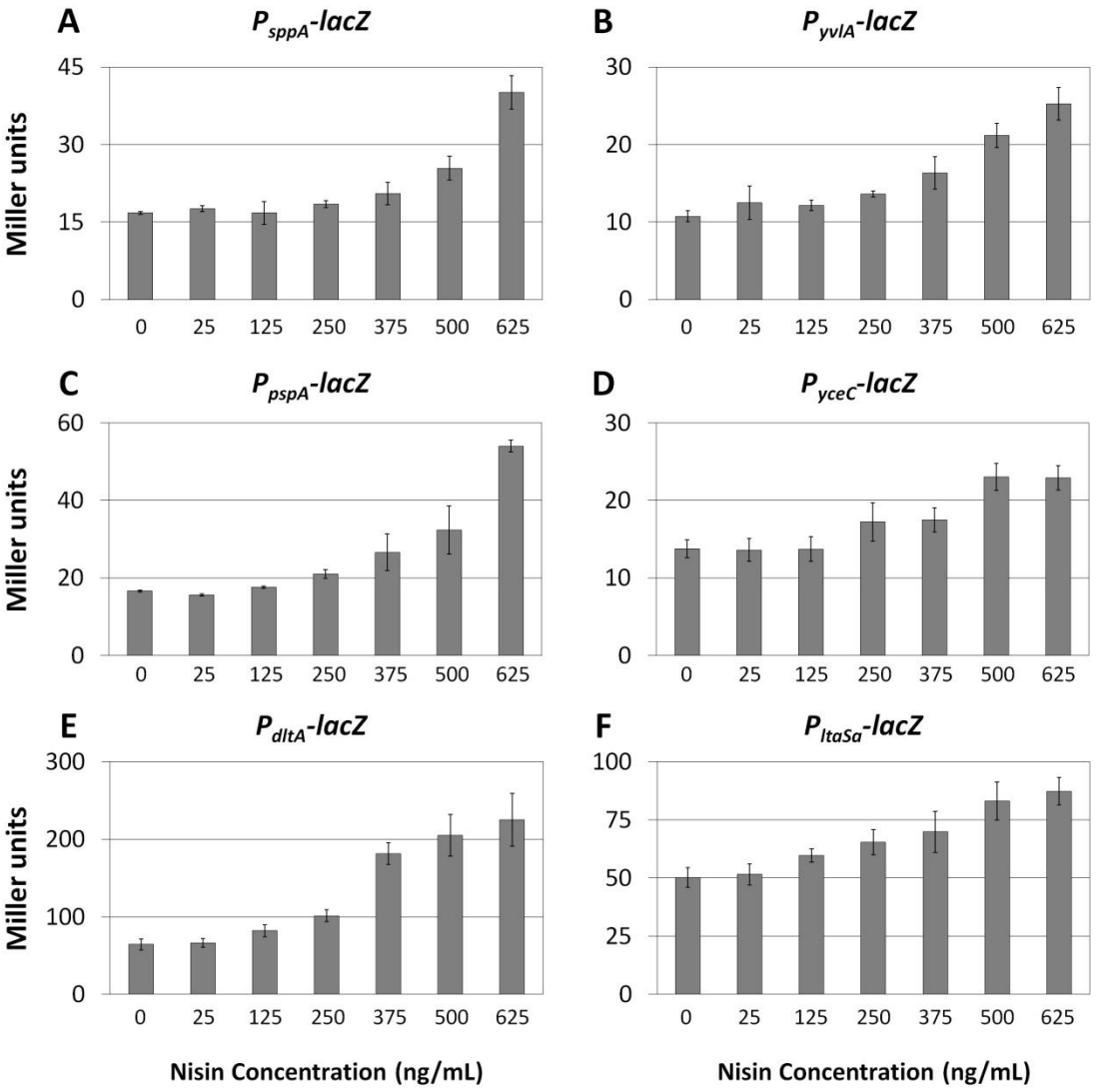


Figure 3.2: Nisin-dependent induction of the σ^{ECF} promoters regulating nisin resistance genes. β-galactosidase activity of the A. P_{sppA}-*lacZ* (HB13308), B. P_{yvlA}-*lacZ* (HB13310), C. P_{pspA}-*lacZ* (HB13306), D. P_{yceC}-*lacZ* (HB13298), E. P_{dltA}-*lacZ* (HB12060), and F. P_{ltaSa}-*lacZ* (HB13225) strains grown to mid-log phase and treated with varying concentrations of nisin for 1 hour. This experiment was performed with at least three biological replicates. Bars represent mean values with error bars indicating standard error. Error bars represent standard error.

σ^{ECF} factors confer nisin resistance by the activation of five operons *(dltABCDE*, *sppA*, *yceCDEFGHI*, *ltaSa*, and *yvlABCD*). We used genetic epistasis to determine if these σ^{ECF}-regulated operons are sufficient to account for the roles of these three σ^{ECF} factors in nisin resistance. Indeed, the unique contribution of σ^{M} is mediated largely, if not entirely, by *ltaSa*: Inactivation of *sigM* in the *ltaSa* mutant does not further increase nisin sensitivity (Figure 3.3A). Similarly, the *dlt* operon is sufficient to account for the unique role of σ^{X}: Inactivation of *sigX* in a *dlt* mutant strain does not further increase nisin sensitivity (Figure 3.3B). In contrast, σ^{W} contributes to nisin resistance by activation of several resistance determinants. Among the σ^{W}-regulated loci, *pspA* had the smallest effect (Figure 3.1B). We therefore focused our attention on a triple mutant in the remaining three (largely) σ^{W}-dependent loci (Δ*yvlABCD* Δ*sppA* Δ*yceH*). This mutant is at least as sensitive as a *sigW* mutant and, importantly, does not exhibit an increase in nisin sensitivity upon deletion of *sigW* (Figure 3.3C). Collectively, these results suggest that the nisin sensitivity of the ΔMXW mutant strain is due, in large part, to decreased transcription of these five operons encoding a total of 18 genes.

Mechanisms of nisin resistance: Modification of the cell envelope. The *dlt* operon and *ltaSa* represent two distinct σ^{ECF} activated nisin resistance mechanisms that affect teichoic acids (TAs). TAs are long chains of glycopolymers that can either be anchored to the cell membrane (lipoteichoic acid or LTA) or covalently attached to peptidoglycan (wall-teichoic acid or WTA) (59). LTA and WTA are synthesized by distinct pathways, but share many of the same functions (85). The cell can modulate

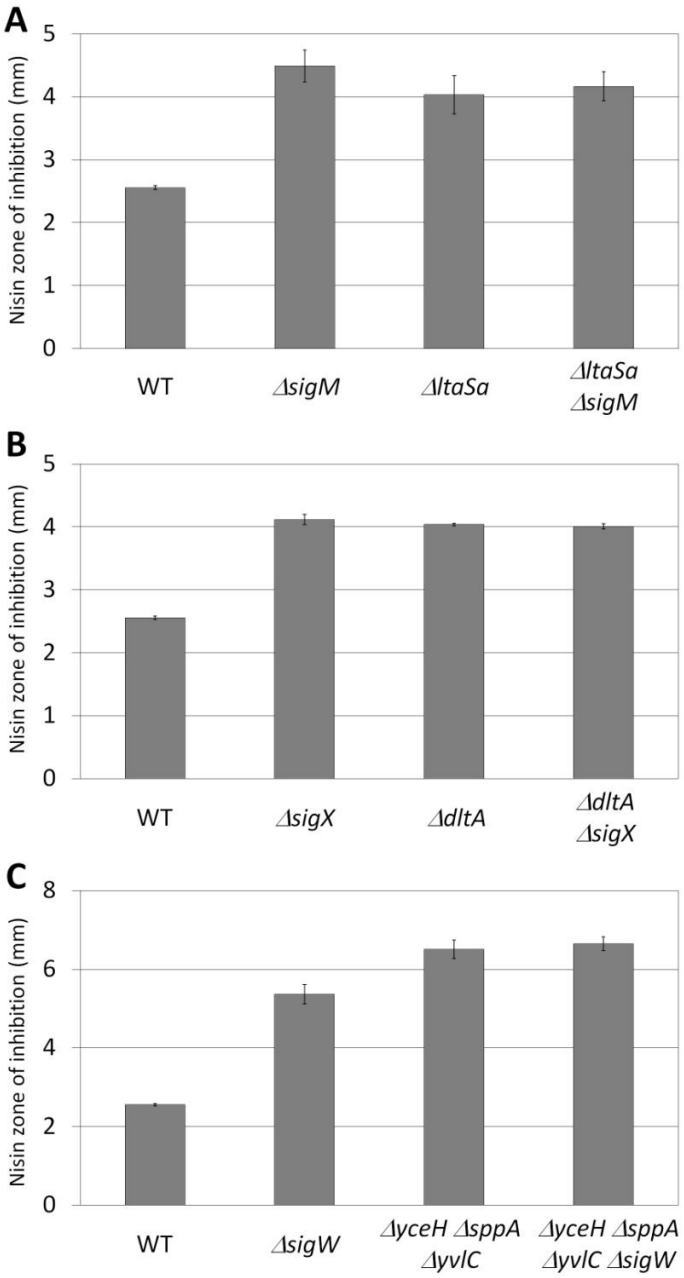


Figure 3.3: Nisin disk diffusion assays to confirm that the identified nisin resistance genes account for the entirety of σ^{ECF} mediated nisin resistance. Each graph compares nisin sensitivity between A. ΔsigM (HB10216), *ΔltaSa* (HB13210) and *ΔltaSa ΔsigM* (HB13215); B. ΔsigX (HB10103), *ΔdltA* (HB12084) and *ΔdltA ΔsigX* (HB13337); and C. ΔsigW (HB10102), *ΔyceH ΔsppA ΔyvlC* (HB13289) and *ΔyceH ΔsppA ΔyvlC ΔsigW* (HB13290). For all comparisons, no significant difference in nisin sensitivity was found when the gene encoding an ECF σ factor was deleted from a strain lacking the nisin resistance gene(s) activated by that σ factor. Error bars represent standard error

the protective effects of the cell envelope by altering the chemical composition of LTA and WTA. To investigate the roles of teichoic acids in nisin resistance, we analyzed the effects of deletion mutations affecting LTA synthesis, WTA synthesis, or D-alanylation of teichoic acids.

LTA protects cells against nisin. LTA is synthesized on a glycolipid (Glc_2-diacylglycerol) anchor by transfer of glycerophosphate (GroP) head groups from the membrane lipid phosphatidylglycerol. In *B. subtilis,* the LTA primase, YvgJ, or one of three distinct lipoteichoic acid synthases (LtaS, LtaSa, or YqgS) are responsible for creating the initial GroP-Glc_2-DAG product that is then elongated by one or more LTA synthases (88). LtaS is the primary synthase responsible for the bulk of LTA synthesis, LtaSa (formerly YfnI) is a σ^M-regulated and stress-induced LTA synthase, and YqgS may play a specific role in sporulation (73). The chemical differences in the LTA produced by these three synthases, and the implications for CAMP resistance, are not fully understood.

Because of their polyanionic nature, LTA and WTA adsorb cations from the environment, including cationic antimicrobial peptides (CAMPs) such as nisin. Binding of cations to LTA and WTA may inhibit their transit through the cell wall, and thereby increase resistance. Indeed, *ltaS* mutants display an increased sensitivity to Mn^{2+} (73). To dissect the contribution of LTA to nisin resistance, we measured the nisin susceptibility of strains lacking one, two, or all three of the LTA synthases (Figure 3.4A). Deleting *ltaS* greatly increased nisin susceptibility, consistent with its role as the primary LTA synthase. As noted (Figure 3.1B & 3.4A) an *ltaSa* mutant also displays a small but significant increase in nisin susceptibility. Both LtaS and

LtaSa contribute to nisin resistance and their effect is additive, as evidenced by the increased sensitivity of the *ltaS ltaSa* double mutant strain. Inactivation of *yqgS* had no effect on nisin susceptibility (Table S2), presumably because it was not active under the growth conditions tested. The high level of nisin sensitivity exhibited by the *ltaS ltaSa* double mutant can be complemented fully by ectopic expression of LtaS (Figure 3.4B). Curiously, induction of LtaSa increased nisin resistance in wild-type cells (Figure S3.7A), but failed to complement the *ltaS ltaSa* mutant strain and actually increased sensitivity in this background (Figure 3.4B). The reason for this latter effect could be due to the synthesis of LTAs that are so long that they impair the cell.

LtaS and LtaSa have been previously shown to produce distinct LTA polymers as visualized by immunoblotting: The LTA synthesized by the σ^M-regulated LtaSa is substantially longer than that produced by LtaS or YqgS (88). We have confirmed this general effect in our background. An *ltaS* mutant has greatly decreased staining in the region corresponding to LTA, and induction of *ltaSa* leads to an increase in the average molecular weight of LTA as visualized by immunoblotting (Figure S3.2). Moreover, our results indicate that treatment of wild-type cells, but not the *ltaSa* mutant, with nisin leads to an apparent increase in average LTA chain length. (Figure S3.2). These results are reminiscent of a previous study which found that a nisin resistant *Streptococcus bovis* strain produces longer and denser LTAs than the corresponding WT strain (51). Collectively, these data are consistent with the notion that LTA plays a role in the general defense mechanism of *B. subtilis* against nisin, presumably by limiting access of this cationic antimicrobial to its target in the cell membrane.

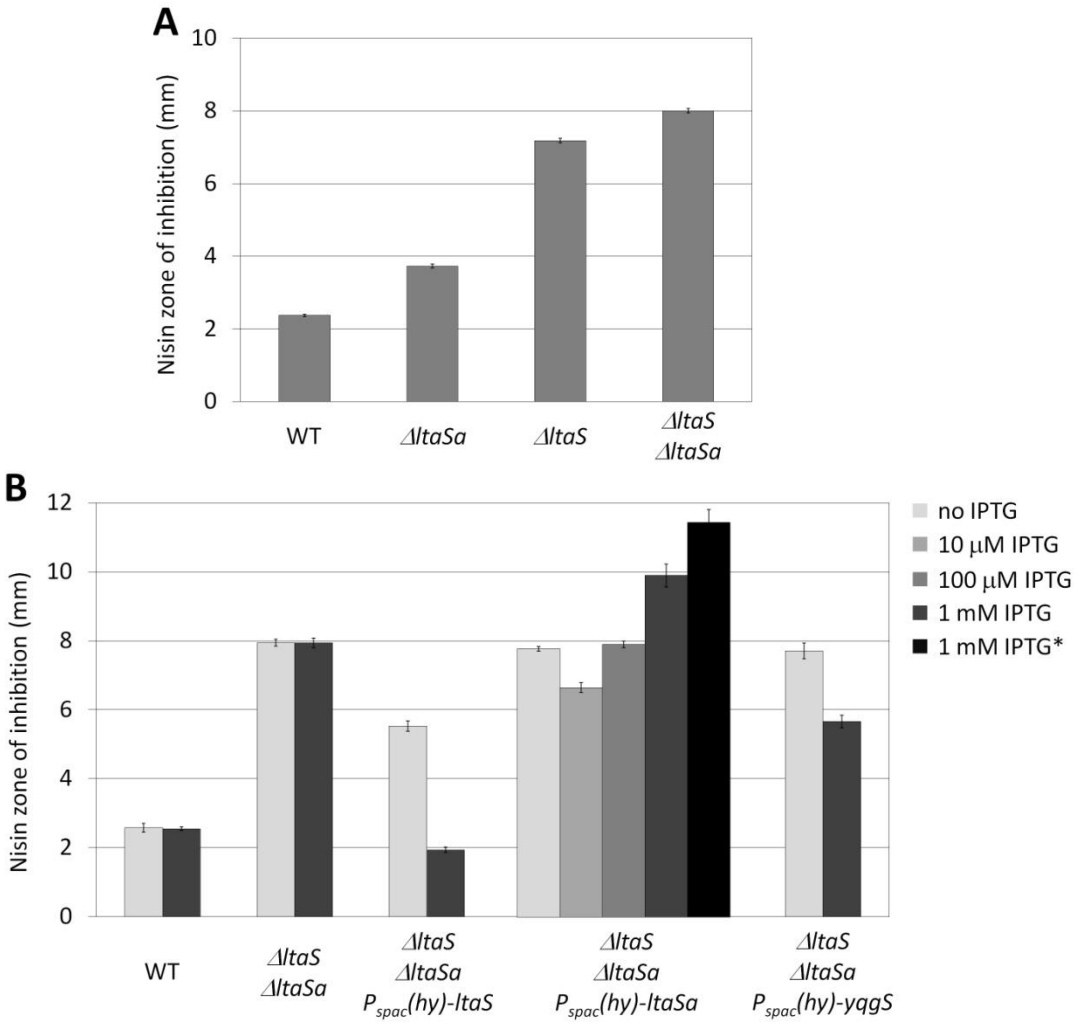


Figure 3.4: LTA synthesis influences nisin resistance. A. Nisin disk diffusion assays for WT (168), *ΔltaSa* (HB13210), *ΔltaS* (HB13255), Δ*ltaS* Δ*ltaSa* (HB13258). Significant increases in sensitivity are observed upon deletion of *ltaSa* and *ltaS*. B. Nisin disk diffusion assays for WT (168), *ΔltaS ΔltaSa* (HB13258), *ΔltaS ΔltaSa* P_{spac}*(hy)-ltaS* (HB13264), *ΔltaS ΔltaSa* P_{spac}*(hy)-ltaSa* (HB13261), and *ΔltaS ΔltaSa* P_{spac}*(hy)-yqgS* (HB13265) strains on media with varying concentrations of IPTG. * indicates prior growth in IPTG for one hour before nisin treatment. All strains containing a IPTG induicible LTA synthase exhibited a significant change in zone of inhibition due to IPTG treatment with the exception of the 100 mM treatment for *ΔltaS ΔltaSa* P_{spac}*(hy)-ltaSa*. Error bars represent standard error.

WTA protects cells against nisin. Like LTA, WTA is a major constituent of the anionic cell envelope, but it is unknown whether WTA also contributes to lantibiotic resistance. We note that a strain unable to synthesize WTA (due to a *tagO* deletion) was extremely susceptible to nisin (Table S3). This suggests that, like LTA, WTA may contribute to an anionic barrier that impedes the permeation of cations through the cell wall to targets in the membrane. WTA may not be a permeability barrier for all CAMPs, however. It is also possible that, by facilitating accumulation of CAMPs from the medium, WTA and other anionic polymers could sensitize cells to their antimicrobial action. Indeed, in *S. aureus* depletion of WTA has been shown to confer resistance to mammalian phospholipase A_2 and β-defensin 3 (Koprivnjak *et al.,* 2008).

As noted above for LTA synthesis, the final steps of WTA synthesis can be catalyzed by any of several functionally overlapping enzymes. Specifically, three LytR-Cps2A-Psr (LCP) family proteins (TagTUV) were recently identified as encoding enzymes that attach the WTA polymer to peptidoglycan (41). Interestingly, two of the three *B. subtilis* genes that encode WTA attaching enzymes are regulated by σ^{ECF} factors: *tagT* (formerly, *ywtF*) and *tagU* (formerly, *lytR*) are partially regulated by σ^{M} and σ^{X}, respectively (18) (34). This led to the hypothesis that one mechanism by which σ^{ECF} factors might contribute to nisin resistance is by modulating the extent or the nature of the attachment between WTA and peptidoglycan. However, deleting these genes did not affect nisin resistance (Table S3). Thus, these enzymes are either complemented by TagV, or have only a minor role in mediating resistance to nisin. We conclude that WTA itself is an intrinsic resistance determinant for nisin, but the

role (if any) of σ^{ECF}-mediated modulation of WTA synthesis and attachment is not yet clear.

D-alanylation of LTA and WTA contributes to nisin resistance. The *dlt* operon encodes proteins that esterify D-alanine residues to the glycerol backbone of WTA and LTA (62). The D-alanyl esters introduce positively charged free amino groups that partially compensate for the negative charge of the phosphodiester linkages, thereby reducing the net negative charge of the TAs.

The contribution of teichoic acid D-alanylation to nisin resistance had been recognized in multiple Gram-positive bacteria including *B. subtilis* (9), *Clostridium difficile* (56), *Lactococcus lactis* (45), *Staphylococcus aureus* (63), and *Streptococcus pneumoniae* (44). Originally, it was proposed that the anionic TAs attracted CAMPs and thus sensitized the cells to their action, and the Dlt system reduced this electrostatic attraction. The finding that LTA and WTA both serve to decrease, rather than increase, nisin sensitivity challenges this interpretation. Recently, an alternative model was proposed that suggests that D-alanylation allows LTA to form a more compact and less permeable barrier that serves to physically restrict the access of CAMPs to the membrane (71). Thus, the key role of the Dlt pathway may be to decrease CAMP permeation by altering the structure of TAs, rather than by reducing the extent of CAMP binding.

We sought to investigate whether the contribution of the Dlt pathway to nisin resistance was dependent upon LTA, WTA, or both. DltABCD has only been shown to directly attach D-alanine to LTA (83), and one model suggests that a separate enzyme, encoded outside the *dlt* operon, transfers the D-alanyl residues from LTA to

WTA (26, 69). A recent study supporting this model has shown that a strain lacking LTAs exhibits significantly less D-alanylated WTA in *Staphylococcus aureus* (68). If this were the case in *B. subtilis*, the *dlt* operon would be primarily dependent upon LTAs to resist nisin. We noted that a *dltA* mutation only slightly increased nisin sensitivity in an Δ*ltaS ltaSa* double mutant background that synthesizes little LTA (Table S3.4). This agrees with the hypothesis that LTA is needed to efficiently transfer D-alanine to WTA, but also indicates that WTA can still be D-alanylated in the absence of LTA.

Mechanisms of nisin resistance: σ^W activates multiple resistance pathways. The above results indicate that σ^X and σ^M play a major role in defending the cell against nisin by activation of an alternative LTA synthase (LtaSa) and by increasing the D-alanylation of teichoic acids, which collectively serve to decrease permeation of nisin through the envelope to the membrane. In contrast, σ^W regulated resistance genes have a variety of functions that are linked to membrane homeostasis. These are encoded by the *sppA, pspA, yvlABCD,* and *yceCDEFGHI* operons.

The SppA signal peptide peptidase protects against nisin. The σ^W activated nisin resistance gene *sppA* encodes a signal peptide peptidase, SppA. Signal peptide peptidases are ubiquitous enzymes present in eukaryotic, bacterial, and archaeal cells (42). Their proposed function is to cleave signal peptides left behind in the membrane after they have been cleaved by signal peptidases. However, the regulation of *sppA* by the antibiotic-inducible σ^W presents an alternative hypothesis: SppA may serve to bind

and possibly cleave peptide antibiotics that insert into the membrane and thereby provide protection. This hypothesis is supported by the fact that lantibiotics and Gram positive signal peptides share similar structures (both are small peptides with a hydrophobic core and cationic residues (4)) and thus may have similar affinities for SppA. Additionally, a recent report has shown that *B. subtilis* SppA is capable of digesting folded proteins other than signal peptides *in vivo* (58).

Other examples of bacterial proteases that degrade antimicrobial peptides have been described (66), including the digestion of LL-37 by the metalloprotease aureolysin from *S. aureus* (77) and the cleavage of nisin by the nisin resistance protein NSR in some *Lactococcus lactis* strains (82). Moreover, an SppA homolog is encoded within the enterocin A gene cluster in *Enterococcus faecium* (60), suggesting a possible role in immunity.

In addition to SppA, *B. subtilis* contains two other signal peptide peptidase homologs, TepA, and YqeZ (6, 31). To determine whether these SppA paralogs also influence nisin resistance, the nisin susceptibility of single, double, and triple mutants lacking *sppA*, *tepA*, and/or *yqeZ* was analyzed with disk diffusion assays. Deleting *tepA* and/or *yqeZ* had no effect on nisin susceptibility, even in an *sppA* mutant background (Table S3.5). Since only *sppA* deletion increased sensitivity to nisin, we conclude that SppA is the only signal peptide peptidase in *B. subtilis* that contributes to nisin resistance.

PspA, YvlC, and LiaH are phage shock proteins that confer nisin resistance. Two additional σ^W-regulated resistance genes encode homologs of the phage shock protein (Psp) system originally described in Gram negative bacteria (36) (39). In *E. coli,* the

key phage shock proteins are PspA, PspB, PspC, and PspF (15). The Psp response is inactive under non-stressed conditions due to PspA binding and inhibiting the transcriptional activator PspF. When activated by membrane stress, PspB and/or PspC sequester PspA to the inner membrane (90) leaving PspF free to activate the expression of all phage shock proteins (36). Membrane disruption is ameliorated by accumulated PspA at the cell membrane, but other phage shock proteins contribute to membrane stress resistance as well (39).

B. subtilis encodes two PspA homologs, PspA and LiaH, and one PspC homolog, YvlC. Both PspA and YvlC are expressed from σ^W-dependent promoters, whereas LiaH is activated by the antibiotic-sensing LiaRS TCS (55). LiaR activates transcription of an autoregulatory promoter driving expression of the *liaIHGFSR* operon. However, we find that nisin resistance only requires *liaH* (Figure S3.7). Purified LiaH has been previously shown to form oligomeric rings similar to those formed by active *E. coli* PspA (87). In addition, both PspA and LiaH have been implicated in resistance to daptomycin (27) (Figure S3.6), which, like nisin, damages cells by depolarizing the membrane. These observations suggest that the Psp homologs of *B. subtilis* may function similarly to their *E. coli* orthologs: YvlC may function to recruit PspA and LiaH to the membrane where they prevent or repair membrane damage.

Here, we show that all three *B. subtilis* Psp proteins contribute to nisin resistance. We first showed that within the *yvlABCD* operon, *yvlC* is necessary and sufficient for nisin resistance (Fig S3 & supplemental text). We then analyzed mutants lacking *pspA*, *liaIH*, and/or *yvlABCD* with nisin disk diffusion assays (Figure 3.5). The

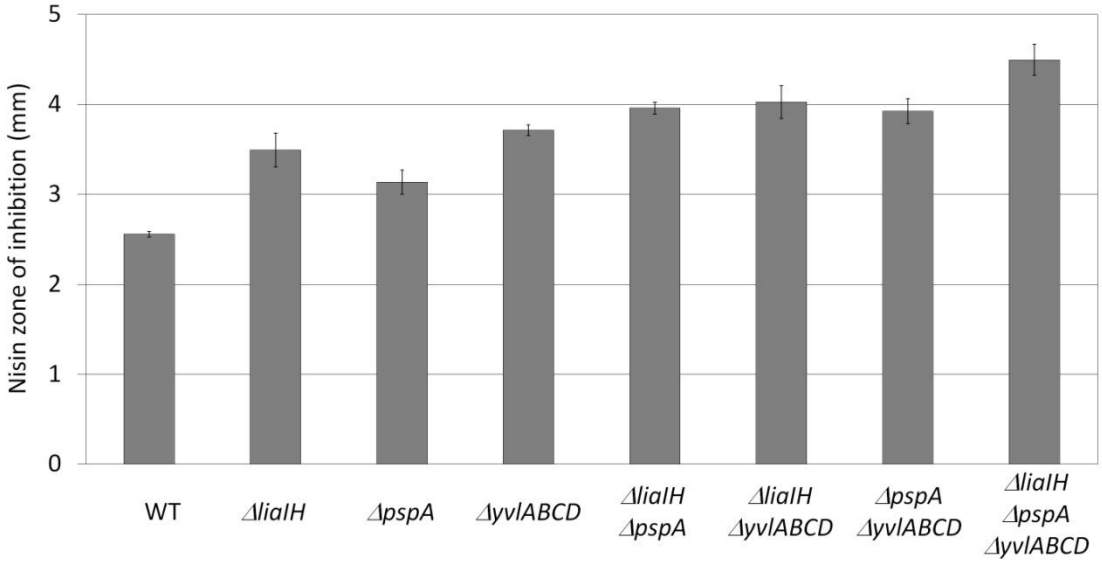


Figure 3.5: Nisin resistance among *psp* deletion strains. Nisin disk diffusion assays for WT (168), Δ*liaH* (HB13245), Δ*pspA* (HB13243), Δ*yvlABCD* (HB13242), Δ*liaIH* Δ*pspA* (HB13247), Δ*liaIH* Δ*yvlABCD* (HB13246), Δ*pspA* Δ*yvlABCD* (HB13244), and Δ*liaIH* Δ*pspA* Δ*yvlABCD* (HB13248) strains. The Δ*liaIH* strain has a higher zone of inhibiton than the WT strain, the Δ*liaH* Δ*pspA* strain has a higher zone of inhibition than the Δ*liaH or* Δ*pspA* strains*,* and the triple mutant has a higher zone of inhibition than the Δ*liaH* Δ*yvlABCD* or Δ*pspA* Δ*yvlABCD* strains. These differences are modest yet statistically significant. Error bars represent standard error.

pspA liaIH double mutant was significantly more sensitive than either of the single gene deletions. Deleting *pspA* or *liaIH* from a *yvlABCD* background did not significantly increase sensitivity, but the triple mutant (*pspA liaIH yvlABCD*) was significantly more sensitive than the *yvlABCD* null strain. Thus, *pspA*, *yvlC*, and *liaH* all independently contribute to nisin resistance, but the PspA homologs may partially complement each other. These findings are consistent with a previous report that *liaIH* contributes to nisin resistance (28).

Our genetic studies indicate the the PspA and PspC pathways are at least partially independent and contribute additively to nisin resistance. This is consistent with reports that *E. coli* PspA is capable of repairing proton leakage of damaged membranes independent of PspC (43), and that PspC can function independently of PspA in *Yersinia enterocolitica* (32). Previous studies have also identified roles for the Psp system in nisin resistance in Gram positive bacteria. Mutations in *Listeria monocytogenes* leading to increased expression of a *yvlC* homolog (*lmo2485*) or a *liaS* homolog (*lmo1021*) decreased nisin sensitivity, while interrupting the operon containing the *yvlC* homolog or the *liaS* homolog increased sensitivity (22). In addition, Psp proteins are activated by Lipid II interacting antibiotics in *Lactococcus lactis* (52) and *Streptococcus pneumonia (19), and by membrane perturbing agents in Streptomyces lividans (84). Although the functions of Psp proteins may be broadly conserved, the regulatory pathways that activate expression in response to cell envelope stress appear quite diverse involving, depending on the bacterium, one or more two-component systems, ECF σ factor(s), or RpoN-dependent regulation.*

The YceGHI proteins contribute to nisin resistance. Of all the σ^{ECF}-activated nisin

resistance loci identified in this paper, a *yceH*::pMUTIN insertion mutation had the single largest effect (Figure 3.1B). Since *yceH* is the penultimate gene in the *yceCDEFGHI* operon, we first set out to determine which gene(s) from this operon were important for nisin resistance. Expression of *yceH* from an IPTG inducible promoter was able to restore nisin resistance to a *ΔyceH* strain, but not to a *ΔyceCDEFGHI* strain, indicating that an additional component within this operon is necessary for *yceH*-mediated nisin resistance (Figure S3.4A). Our studies to date indicate that YceGHI are likely sufficient for wild-type levels of nisin resistance (Figure S3.4 & accompanying text).

The roles of the YceGHI proteins are not entirely clear. YceI, also known as NiaP, is a nicotinate transporter (70) (38), YceH is classified as a tellurite resistance protein (TelA) homolog (2), and YceG is largely uncharacterized. YceH shares significant homology with *Listeria monocytogenes* TelA, which also contributes to innate nisin and gallidermin resistance (13). The *B. subtilis* genome encodes a second TelA homolog, YaaN, which is also activated by a σ^{ECF}-dependent promoter (2). However, deleting *yaaN* from either WT cells or a *yceH* mutant background did not affect nisin sensitivity (Table S6). A recent study of TelA domain architecture concluded that TelA proteins likely function as part of a membrane associated sensory complex (2). Additionally, TelA homologs likely interact with YceG because the genes encoding these proteins often co-occur.

Finally, we looked at the nicotinate transporter YceI. Nicotinate is primarily used by *B. subtilis* as a substrate for NAD synthesis. One hypothesis is that YceG and/or YceH are NAD-dependent enzymes and YceI increases the amount of NAD

available to these proteins. This would explain why *yceI* contributes to, but is not essential for, this nisin resistance mechanism. To test this hypothesis, we performed a nisin disk diffusion assay on WT and *yceCDEFGHI* cells grown on minimal media supplemented with or without 4 μM nicotinate (Figure S3.5). The addition of nicotinate resulted in a small increase in nisin resistance in the WT cells, but had no effect on the Δ*yceCDEFGHI* or Δ*yceI* strain, suggesting that the *yceI*-dependent import of nicotinate into the cell benefits this nisin resistance mechanism.

Mechanistic inferences from analysis of a nisin hinge region mutant. To better understand the origins of nisin resistance, we assessed the susceptibility of gene knockout strains to the N20PM21P nisin variant hinge-region variant (*N20PM21P-nisin)*. This variant can still bind lipid II and inhibit cell wall synthesis, but no longer forms membrane pores (21).

Disk diffusion assays using (*N20PM21P*)-*nisin, purified as described* (25, 76), revealed that *ltaSa, dltA,* and *yceH* still functioned as resistance determinants (Figure 3.6A). This is consistent with the roles of LtaSa and the Dlt pathway in reducing the permeation of nisin through the cell envelope. In contrast, comparison of wild-type and singly mutant strains failed to show any role for *pspA, yvlABCD, liaH*, or *sppA* in resistance against the (*N20PM21P*)-*nisin. This supports the notion that the* Psp proteins (PspA, YvlC, and LiaH) and SppA only defend against the membrane-perturbing and pore forming activities of nisin.

Mechanistic inferences from comparison of nisin and other antibiotics. Nisin, gallidermin and mersacidin are all lipid II-binding lantibiotics that inhibit cell wall synthesis. As noted above, nisin binds lipid II and this complex nucleates pore

formation and membrane permeabilization. Gallidermin binds lipid II and inserts its N-terminal region into the membrane, possibly leading to some membrane perturbation, but the N-terminal tail does not readily form pores (86). Mersacidin binds lipid II but does not integrate into the membrane.

In support of the mechanistic inferences described above, susceptibility to gallidermin and mersacidin was only increased in the *ltaSa, dltA*, an*d yceH* knockout strains, which mimic the results seen with the non-pore forming N20PM21P nisin variant (Figure 3.6B, 3.6C). Conversely, the *pspA*, *yvlC*, and *liaH* genes do not provide resistance against those compounds (N20PM21P nisin, mersacidin, and gallidermin) which cannot form pores in the membrane (Figure 3.6). However, the *sppA* strain was more sensitive to gallidermin, even though it was not more sensitive to mersacidin or (*N20PM21P*)-*nisin*. Since the major difference between gallidermin and mersacidin/(*N20PM21P*)-*nisin* is that gallidermin can insert into the membrane, it is likely that SppA only resists lantibiotics that integrate into the membrane. SppA may be targeting gallidermin directly (perhaps by degradation) once it enters the membrane where the active site of the SppA protease resides (58).

To determine if the effects of these nisin resistance genes were specific for lantibiotics, we also tested the sensitivity of various mutant strains to other antibiotics including polymyxin B, vancomycin, bacitracin, triton X-100, daptomycin, D-cycloserine, novobiocin, and ampicillin. These stresses were chosen since, in each case, an increased susceptibility was noted in the MXW triple mutant (53). Daptomycin, polymyxin B, novobiocin, and triton X-100 disrupt different aspects of

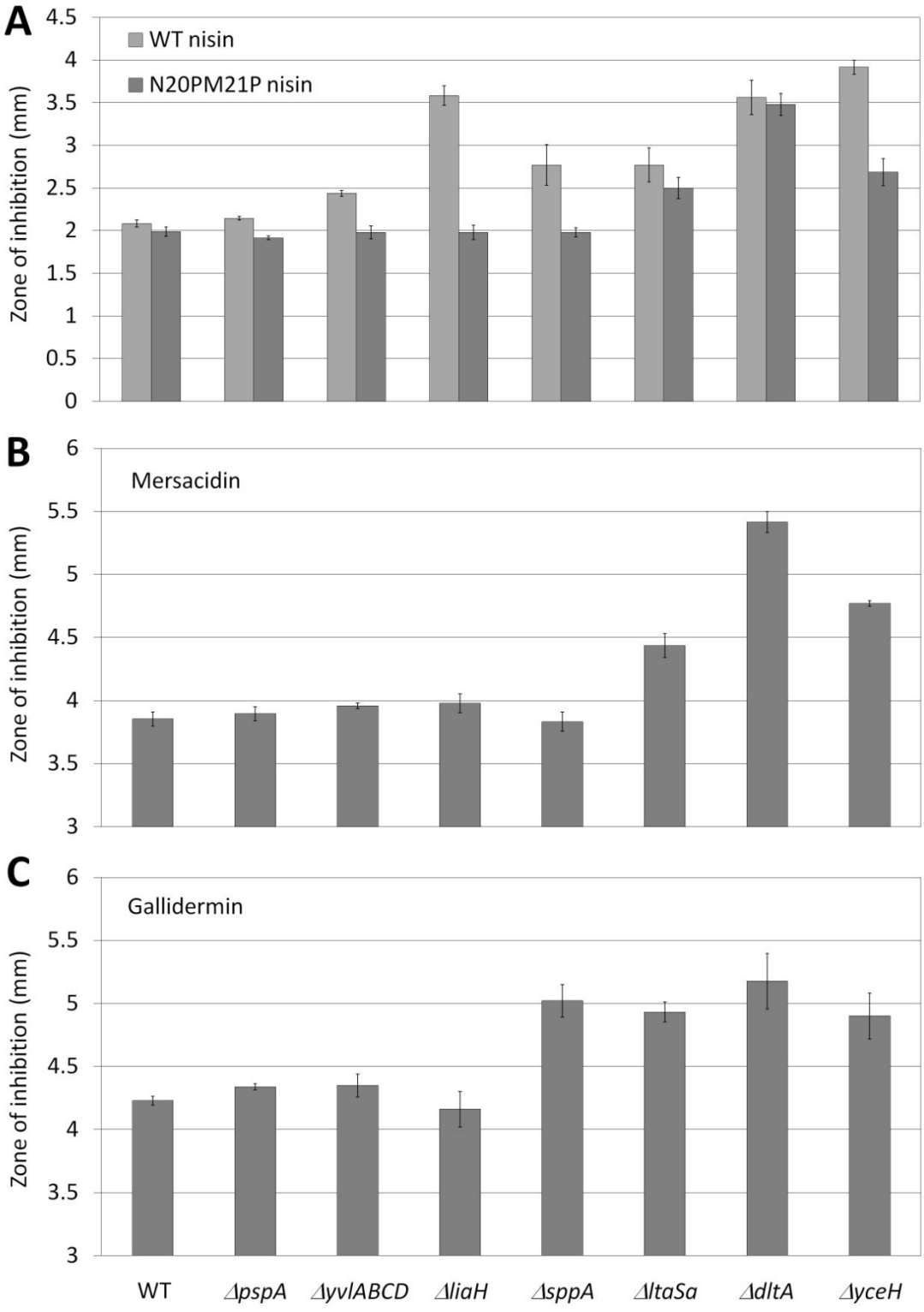

A
WT nisin
N20PM21P nisin
Zone of inhibition (mm)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
B
Mersacidin
Zone of inhibition (mm)
6
5.5
5
4.5
4
3.5
3
C
Gallidermin
Zone of inhibition (mm)
6
5.5
5
4.5
4
3.5
3
WT
ΔpspA
ΔyvlABCD
ΔliaH
ΔsppA
ΔltaSa
ΔdltA
ΔyceH

Figure 3.6: Comparison of A. WT- and N20PM21P-Nisin, B. mersacidin, and C. gallidermin sensitivity for the WT (168), *ΔpspA* (HB13243), *ΔyvlABCD* (HB13242), Δ*liaH* (HB13245), *ΔsppA* (HB13251), *ΔltaSa* (HB13210), *ΔdltA* (HB12084), and *ΔyceH* (HB13281) strains. The *ΔltaSa*, *ΔdltA*, and *ΔyceH* strains exhibit significantly larger zones of inhibition than WT cells to these stresses. Additionally, *ΔsppA* exhibits significantly increased sensitivity to gallidermin.

the cell membrane, while vancomycin, ampicillin, D-cycloserine, and bacitracin inhibit various steps of cell wall synthesis. Like nisin, vancomycin binds to lipid II. However, these nisin resistance gene knockout strains were not more sensitive to the stresses tested, with the exception of slight increases in sensitivity to daptomycin for the *pspA* mutant, to novobiocin for *yceH*, and to polymyxin B and novobiocin for *dltA* (Figure S3.6). Thus, the resistance mechanisms described here seemed to have evolved to help resist the actions of bacteriocins such as nisin and related lantibiotics.

Creating a nisin resistant *B. subtilis* strain. Our identification of σ^{ECF}-activated genes that contribute to nisin resistance provided us with an opportunity to engineer a nisin resistant *B. subtilis* strain. Such a strain could be useful in developing a cost-effective method of producing nisin or other lantibiotics for industrial, clinical, or research purposes (28). Previous efforts to create such a strain relied on the introduction of nisin immunity genes from *Lactococcus lactis* or overexpression of a single resistance determinant, *liaIH* (28, 80).

To artificially increase nisin resistance, we fused each resistance gene or operon to the IPTG inducible promoter $P_{spac(hy)}$ and assessed nisin sensitivity for these strains with and without 1 mM IPTG using disk diffusion assays (Figure S3.7A). Induction slightly increased nisin resistance in the $P_{spac(hy)}$-*yvlC*, $P_{spac(hy)}$-*liaH*, and $P_{spac(hy)}$-*ltaSa* strains, but did not affect sensitivity in the $P_{spac(hy)}$-*pspA* and $P_{spac(hy)}$-*dltABCDE* strains. In contrast with our hypothesis, induction increased nisin sensitivity in the P_{spac}(hy)-*sppA* and P_{spac}(hy)-*yceGHI* strains. These results highlight the fact that high level overexpression of resistance determinants (as obtained with the strong P_{spac} promoter) is not necessarily beneficial compared to the normal expression

level and, in some cases, can be detrimental.

Based these results, we decided to create a *B. subtilis* strain that could moderately upregulate all ECF σ-dependent nisin resistance genes. We first deleted the genes encoding the anti-σ factors to σ^W and σ^X, *rsiW* and *rsiX* respectively, thereby creating a strain in which σ^W and σ^X constitutively upregulate their target genes (including *pspA*, *yvlC*, *sppA*, *yceGHI*, and *dltABCD*). To increase expression of LtaSa, we included a P_{spac}*(hy)-ltaSa* fusion in the *rsiW rsiX* strain. A disk diffusion assay confirmed that this P_{spac}*(hy)-ltaSa rsiW rsiX* strain was significantly more nisin-resistant than WT cells on MH media, and this resistance was increased by the addition of 1 mM IPTG (Figure S3.7B).

To quantify the increase in nisin resistance achieved by these mutations, we determined the minimal inhibitory concentration (MIC) of this strain with a broth dilution assay. The MIC of the P_{spac}*(hy)-ltaSa rsiW rsiX* to nisin in MH media + 1 mM IPTG was 1.3x higher than wildtype (4.16 ± 0.21 μg ml^{-1} vs. 3.25 ± 0.11 μg ml^{-1}). This modest increase may be limited by the use of very low density cultures in the MIC assay (OD_{600} = 0.002). As an alternative, we determined the concentration of nisin required to kill 50% of cells at OD_{600} = 0.4 (50% inhibition concentration). In this assay, we observed a ~3.8 fold increase for the P_{spac}*(hy)-ltaSa rsiW rsiX* over the WT strain in MH media + 1 mM IPTG (1.72 ± 0.16 μg ml^{-1} vs. 0.45 ± 0.02 μg ml^{-1}).

Nisin resistance genes defend against competing microbes. Since the nisin resistance genes identified in this study presumably evolved through interspecies competition, we reasoned that they might also play a role in protection against lantibiotics, peptide antibiotics, and other antimicrobial compounds made by other

Bacillus spp. We therefore analyzed the sensitivity of strains lacking one or more nisin resistance genes to other *Bacilli* using spot on lawn assays in which an antibiotic producing strain is spotted at high density on lawns of *B. subtilis*. We tested six *Bacillus* strains known to produce compounds to which *B. subtilis* σ^{ECF} factors confer resistance (8) (49) (75) (Table 3.2, Figure 3.7 and Figure S3.8A). In general, the lawns lacking individual nisin resistance genes showed little differences in sensitivity to the *Bacilli* tested, but progressively deleting these potential resistance genes led to increased sensitivity. For example, the *dltA ltaSa* double mutant was more sensitive to *B. atrophaeus ESM,* and *B. atrophaeus NRS-213* than either single mutant. In addition, while the *pspA*, *yvlABCD*, *yceH*, *liaIH* and *sppA* strains displayed little to no sensitivity to *B. subtilis* ssp. *spizizenii W23* and *B. subtilis* ssp. *spizizenii ATCC 6633,* sensitivity was evident in double mutant strains *(pspA yvlABCD*, *sppA yvlABCD*, and *sppA yceH*), and even more apparent in triple mutant strains (*sppA yceH yvlABCD* and *pspA yvlABCD liaIH*). A quadruple mutant *(dltA ltaSa yceH yvlABCD*) exhibited still higher sensitivity to *B. atrophaeus ESM* and *B. amyloliquefaciens* (Table 3.2). Thus, we conclude that each of these nisin resistance loci protects *B. subtilis* W168 from at least one of these *Bacillus* species, and therefore likely contributes to fitness in the complex microbial community of the soil.

Many of the antimicrobial compounds produced by *Bacillus* species have been identified (1). We can therefore make inferences regarding the likely active compound in these intermicrobial competition studies. For example, *B. subtilis* subsp. *spizizenii ATCC 6633* and *2A9* generated the largest growth inhibition phenotypes in *B. subtilis*

Table 3.2: The contribution of *liaIH*, *pspA*, *yvlABCD*, *sppA*, *ltaSa*, *dltA*, and *yceH* to resistance against other *Bacilli*[a]

	liaIH	*pspA*	*yvlABCD*	*sppA*	*ltaSa*	*dltA*	*yceH*
B. licheniformis ATCC 14580	-	-	-	-	-	-	-
B. atrophaeus NRS-213	-	-	-	-	+	+++	-
B. atrophaeus ESM rplV str	+	+	+	+	++	+++	+
B. amyloliquefaciens FZB42	-	-	+	-	+	+	+
B. spizizenii W23	+	++	+++	+++	+	+	++
B. spizizenii ATCC 6633	+++	++	+++	+++	+	+	+++

[a]. The contribution of each gene or operon to resistance against a specific strain is determined by the increase in zone of inhibition and spot size of the competing microbe due to gene deletion in the *B. subtilis* lawn strain: Representative images of all spot on lawn assays can be found in Figure S6.
- : Gene deletion has no visible effect on the spot.
+: Gene deletion increases spot size, but not the zone of inhibition. The effect is only visible in strains lacking multiple nisin resistance genes.
++: Gene deletion increases spot size and zone of inhibition, but the effect is visible only in strains lacking multiple nisin resistance genes.
+++: Gene deletion increases spot size and zone of inhibition in a WT background.

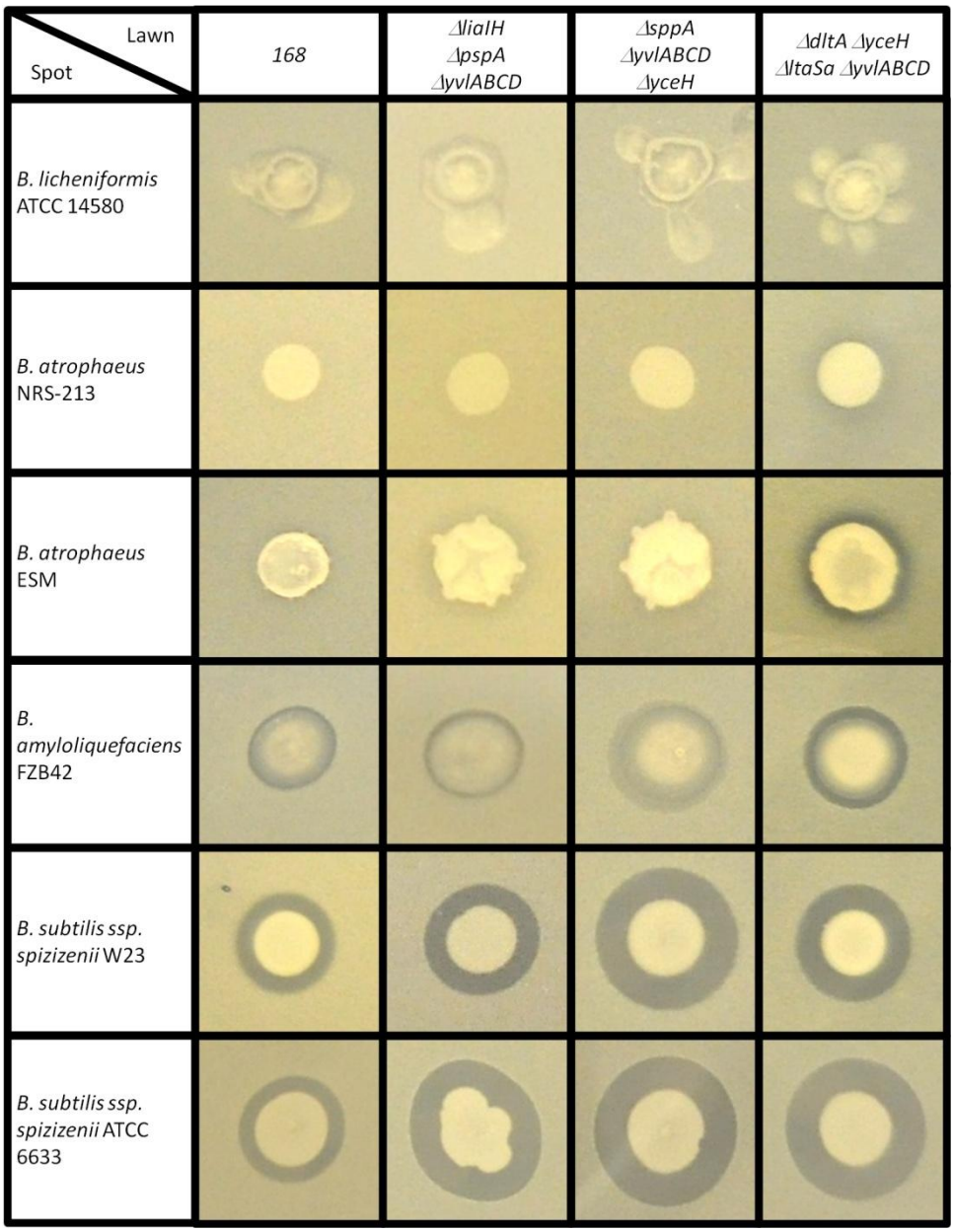


Figure 3.7: Spot on lawn assays depicting the sensitivity of the WT (168), Δ*liaIH* Δ*pspA* Δ*yvlABCD* (HB13248) Δ*sppA* Δ*yceH* Δ*yvlABCD* (HB13289), and *ΔyceH ΔdltA ΔltaSa ΔyvlABCD* (HB13335) lawn strains to spots of *B. licheniformis* ATCC 14580*, B. atrophaeus* NRS-213*, B. atrophaeus* ESM*, B. amyloliquefaciens* FZB42*, B. subtilis ssp. spizizenii* W23*,* and *B. subtilis ssp. spizizenii* ATCC 6633. The relative sensitivity of the lawn strains to each spotted strain is reflected by the size of the spot and the zone of inhibition surrounding it after 18 h growth at 37 ^{o}C. A larger spot size and zone of inhibition represents increased sensitivity of the lawn strains to the metabolites produced by the spotted strains. Pictures are representative of at least three assays performed with three independent clones of each strain.

strains lacking nisin resistance genes. Although these *B. subtilis* subsp. *spizizenii* strains are closely related to *B. subtilis* W168, one key difference is that their genomes contain a subtilin biosynthesis gene cluster (91). Subtilin is a type A lantibiotic with a size, structure, and mechanism of action that is similar to nisin (61). Indeed, the antimicrobial activity of unpurified supernatant from a *ATCC 6633* culture is considered to derive primarily from subtilin when assayed against other *B. subtilis* strains (79). Since all of the *B. subtilis* W168 nisin resistance genes protect the cell from *B. subtilis* subsp. *spizizenii,* it is likely that these genes are specifically providing protection against subtilin.

B. amyloliquefaciens FZB42 synthesizes numerous lipopeptides and polyketides, the dipeptide antibiotic bacilysin, and a recently discovered cationic thiazole/oxazole-modified microcin called plantazolicin (12). We compared the sensitivity of wild-type *B. subtilis* and the quadruple mutant *(dltA ltaSa yceH yvlABCD*) to various *B. amyloliquefaciens* mutants, including an *spf* null strain that only produces bacilysin and plantazolicin (Figure S3.8B). Since *B. subtilis* is immune to bacilysin, these nisin resistance genes are probably defending against plantazolicin. This conclusion is supported by the fact that a *B. subtilis sigW* mutant is known to be more sensitive to plantazolicin than a WT strain (75).

Collectively, these results demonstrate that although the resistance loci described here were defined with respect to their ability to confer resistance to nisin (a natural product of *Lactococcus* spp.), their role in the environment is broadly related to protection against lantibiotics and other antimicrobial peptides made by common co-habitating soil microorganisms including other *Bacillus* spp. Indeed, our results, and

those of others (86), indicate that nisin itself is a weak inducer of the σ^{ECF} stress responses and these same resistance genes are induced more strongly by other compounds including the lantibiotics mersacidin and gallidermin.

3.5 Conclusion

We have here explored ECF σ factor-mediated resistance mechanisms that protect against the model lantibiotic nisin. Intrinsic nisin resistance is primarily mediated by the *pspA*, *yvlC*, *sppA*, *dltABCD*, *ltaSa*, and *yceGHI* genes (Table 3.3). The σ^{W}-regulated *pspA* and *yvlC* genes encode phage shock protein homologs postulated to enhance membrane stability and thereby provide resistance specifically against the membrane-perturbing and pore forming activity of nisin. This resistance mechanism overlaps with that provided by a second PspA homolog, LiaH, which is activated by the lantibiotic inducible LiaRS TCS. The σ^{W}-regulated signal peptide peptidase (SppA) may function by degrading those lantibiotics that integrate into the membrane. The mechanism by which YceG and its co-expressed proteins protects against nisin is unclear, but a homolog (TelA) functions in nisin resistance in *L. monocytogenes* (13). σ^{X} activates expression of the *dlt* operon, encoding proteins that attach D-alanine residues to teichoic acids, while the σ^{M}-activated *ltaSA* encodes an alternative lipoteichoic acid synthase. Activation of these genes alters teichoic acids in the cell envelope and is postulated to reduce permeation of nisin through the envelope to access its binding partner in the membrane, lipid II. Collectively, these operons play a role in the intermicrobial competition amongst closely related *Bacillus* strains, and their regulation further highlights the role of ECF σ factors in antimicrobial resistance.

3.6 Acknowledgments:

Special thanks to the Wilfred van der Donk lab for providing the plasmids to produce nisin variants and to the Hans-Georg Sahl lab for supplying mersacidin and gallidermin.

Table 3.3: Summary of the *B. subtilis* nisin resistance genes identified in this paper

Gene or operon	Regulation[a]	Function	Resistance Contribution[b]	Resistance Target[c]	Hypothesized Resistance Mechanism
yceGHI	$\sigma^{W(MX)}$	YceG - Unknown	+++	Inhibits nisin-lipid II binding	Unknown, but nicotinate dependent
		YceH - Tellurium resistance homolog			
		YceI - Nicotinate transporter			
dltABCD	$\sigma^{X(M)}$	D-alanylation of teichoic acids	+++		Increases net charge of TAs
ltaSa	σ^{M}	Lipoteichoic acid synthase	++		Increases length and quantity of LTAs
sppA	σ^{W}	Signal peptide peptidase	++	Resists nisin's pore forming activity	Nisin degradation
yvlC		Phage shock protein C homolog	++		Enhances membrane integrity
pspA		Phage shock protein A	+		
liaH	LiaRS	Phage shock protein A homolog	+		

[a.] Identifies which ECF σ factor(s) or TCS activate each nisin resistance gene based on disk diffusion and β-galactosidase assays in Table 3.1, Fig 2, and Figure S2.
[b.] Indicates degree to which nisin sensitiviy increases upon deletion of the gene or operon based on the disk diffusion assays in Figure 1.
[c.] Infers whether the nisin resistance genes contribute to resistance against nisin's pore forming activity or its lipid II binding activity based on sensitivity assays in Figure 6.

3.7 Supplementary information

Table S3.1: ECF σ activated genes that don't influence nisin resistance.[a]

Strain	Zone of Inhibition (mm)
WT	2.6 ± 0.1
ΔydbST	2.8 ± 0.3
ΔyjoB	2.8 ± 0.1
ΔyknWXYZ	2.8 ± 0.3
ΔyfhLM	3.1 ± 0.5
ΔythPQ	2.9 ± 0.5
ΔyuaFGI	2.9 ± 0.2
ΔpssA	2.7 ± 0.1
ΔxpaCyaaN	2.7 ± 0.1

[a.] Data from nisin disk diffusion assays for WT (CU1065), *ΔydbST* (HB6196), *ΔyjoB* (HB6158), *ΔyknWXYZ* (HB6127), *ΔyfhLM* (HB6123), *ΔythPQ* (HB6132), *ΔyuaFGI* (HB6156), *ΔpssA* (HB), *ΔtagU* (HB10193), and *ΔtagT* (HB13357) strains. The zone of inhibition was not significantly different from WT cells for all mutant strains tested.

Table S3.2: The alternative LTA synthase gene *yqgS* does not contribute to nisin resistance.[a]

Mutations	Zone of Inhibition (mm)	
	WT	*ΔyqgS*
WT	2.4 ± 0.1	2.3 ± 0.1
ΔltaSA	3.7 ± 0.1	3.7 ± 0.1
ΔltaS	7.2 ± 0.1	7.2 ± 0.2
ΔltaS ΔltaSA	8.0 ± 0.1	8.0 ± 0.1

[a.] Nisin disk diffusion assays for WT (168), *Δyqgs* (HB13256), *ΔltaSa* (HB13210), *Δyqgs ΔltaSa* (HB13257), *ΔltaS* (HB13255), Δ*ltaS* Δ*yqgs* (HB13259) Δ*ltaS* Δ*ltaSa* (HB13258), and Δ*ltaS* Δ*ltaSa* Δ*yqgS* (HB13260). Significant increases in sensitivity are observed upon deletion of *ltaSa* and *ltaS*, but not upon deletion of *yqgS*.

Table S3.3: WTA synthesis genes and nisin resistance.[a]

Strain	Zone of Inhibition (mm)
WT	2.8 ± 0.1
ΔtagT	2.6 ± 0.2
ΔtagU	2.8 ± 0.3
ΔtagT ΔtagU	2.6 ± 0.1
ΔtagO	25.8 ± 2.3

[a.] Data from nisin disk diffusion assays for WT (168), *ΔtagT* (HB13357), *ΔtagU* (HB10193), *ΔtagT ΔtagU* (HB13425), and *ΔtagO* (HB13386) strains. The *ΔtagO* strain is significantly more sensitive to nisin than WT cells, but the *ΔtagU*, *ΔtagT*, and *ΔtagT ΔtagU* strains are not.

Table S3.4: *dltA* does not require LTA synthesis genes to confer nisin resistance.[a]

Mutations	Zone of Inhibition (mm)	
	WT	*ΔdltA*
WT	2.7 ± 0.1	5.0 ± 0.1
ΔltaSA	3.9 ± 0.1	5.6 ± 0.1
ΔltaS	6.4 ± 0.1	7.2 ± 0.1
ΔltaS ΔltaSA	7.3 ± 0.1	8.0 ± 0.1

[a.] Data from nisin disk diffusion assays for WT (168), *ΔltaSa* (HB13210), *ΔdltA* (HB12084), *ΔltaSa ΔdltA* (HB13216), *ΔltaS* (HB13255), *ΔltaS ΔdltA* (HB13317), *ΔltaS ΔltaSa* (HB13258), and *ΔltaS ΔltaSa ΔdltA* (HB13318) . Deleting *dltA* from strains lacking *ltaS* and/or *ltaSa* significantly increases nisin sensitivity.

Table S3.5: The SppA homologs TepA and YqeZ don't contribute to nisin resistance.[a]

Mutations	Zone of Inhibition (mm)	
	WT	*ΔsppA*
WT	3.1 ± 0.1	3.9 ± 0.1
ΔtepA	3.2 ± 0.1	3.9 ± 0.1
ΔyqeZ	3.0 ± 0.1	3.7 ± 0.2
ΔtepA ΔyqeZ	3.1 ± 0.1	3.9 ± 0.1

[a.] Data from nisin disk diffusion assays for WT (168), Δ*sppA* (HB13251), Δ*tepA* (HB13313), Δ*yqeZyqfAB* (HB13566), Δ*sppA* Δ*tepA* (HB13314), Δ*sppA* Δ*yqeZyqfAB* (HB13312), Δ*tepA* Δ*yqeZyqfAB* (HB13315), and Δ*sppA* Δ*tepA* Δ*yqeZyqfAB* (HB13316) strains. Among the strains, the deletion of *sppA* caused the zone of inhibition to significantly increase, but the deletion of *tepA* and *yqeZ* had no significant effect on nisin zone of inhibition.

Table S3.6: The YceH homolog YaaN does not contribute to nisin resistance.[a]

Mutations	Zone of Inhibition (mm)	
	WT	*ΔyceH*
WT	2.6 ± 0.1	5.6 ± 0.1
ΔxpaCyaaN	2.7 ± 0.1	5.6 ± 0.2

[a.] Data from nisin disk diffusion assays for WT (168), Δ*yaaN* (HB13280), Δ*yceH* (HB13281), and Δ*yaaN* Δ*yceH* (HB13282) strains. Among the strains tested, the deletion of *yaaN* did not significantly change nisin sensitivity.

Table S3.7: Bacterial strains, plasmids, and oligos used in Chapter 3.[a]

Strain	Genotype or description	Source, reference, or construction
***E. coli* strains**		
DE3	BL21 (DE3)	Lab Stock
HE13085	DE3 *pRSFDuet-1-His6nisAB*	DE3 --> pRSFDuet-1nisAB
HE13086	DE3 *pRSFDuet-1-nisAB-N20PM21P*	DE3 --> pRSFDuet-1nisAB-N20PM21P
HE13090	DE3 *pRSFDuet-1-His6nisAB pACYCDuet-1-NisC*	HE13085 --> pACYCDuet-1nisC
HE13091	DE3 *pRSFDuet-1-nisAB-N20PM21P pACYCDuet-1-NisC*	HE13086 --> pACYCDuet-1nisC
***B. subtilis* strains**		
168	*trpC2*	Lab Stock
BSU2007	168 Δ*sigM* Δ*sigY* Δ*sigZ* Δ*sigV* Δ*ylaC* Δ*sigX* Δ*sigW* (Δ7ECF)	(3)
CU1065	*trpC2 attspβ*	Lab Stock
HB0010	CU1065 *risW::kan*	(11)
HB0031	CU1065 *sigM::kan*	(11)
HB0047	CU1065 *rsiX::spec*	(48)
HB0077	ZB307A *SPβ[P_{pspA}-cat-lacZ] (neo, MLS)*	(10)
HB0100	CU1065 *yceCDEF::spc*	(8)
HB0101	CU1065 *yceH-pMUTIN (MLS)*	M. Cao, unpublished
HB0348	CU1065 *sppA::cat*	(8)
HB0919	CU1065 *pspA::cat*	(8)
HB0935	CU1065 *liaIH::tet*	(54)
HB5131	CU1065 *sigM::kan sigW::MLS sigX::spec liaIH::tet*	(27)
HB5361	CU1065 *pssA::spec*	(9)
HB6123	CU1065 *yfhLM::kan*	(8)
HB6127	CU1065 *yknWXYZ::kan*	(8)
HB6132	CU1065 *ythPQ::kan*	(8)
HB6153	CU1065 *yvlABCD::kan*	(8)
HB6156	CU1065 *yuaFGI::kan*	(8)
HB6158	CU1065 *yjoB::kan*	(8)
HB6168	CU1065 *xpaCyaaN::kan*	(8)
HB6196	CU1065 *ydbST::kan*	(8)
HB6208	168 *sigW::spec*	(8)
HB7007	CU1065 *sigX::spec*	(33)
HB8013	ZB307A *SPβ[P_{sppA}-cat-lacZ] (neo, MLS)*	(10)
HB8060	ZB307A *SPβ[P_{yvlA}-cat-lacZ] (neo, MLS)*	(10)
HB10016	168 *sigM::tet*	(49)
HB10102	168 *sigW::MLS*	(49)
HB10103	168 *sigX::kan*	(49)

HB10107	168 *sigM::tet, sigX::kan sigW::mls*	(49)
HB10193	168 *lytR::mls*	(48)
HB10216	168 *sigM::kan*	(49)
HB10352	168 *yybT::MLS*	(48)
HB12060	168 *SPβ[P_{dltA}-cat-lacZ] (neo, MLS)*	V. Guariglia-Oropeza unpublished
HB12084	168 *dltA::MLS*	V. Guariglia-Oropeza unpublished
HB12088	168 *sigX::spec*	HB7007 chrDNA --> 168
HB13108	168 *rsiW::kan*	HB0010 chrDNA --> 168
HB13159	168 *yuaFGI::kan*	(46)
HB13185	168 *sigM::kan*	HB0031 chrDNA --> 168
HB13210	168 *yfnI::spec*	LFH-PCR --> 168
HB13215	168 *sigM::kan yfnI::spec*	HB13210 chrDNA --> HB13185
HB13216	168 *yfnI::spec dltA::MLS*	HB12084 chrDNA --> HB13211
HB13217	168 *sigM::kan sigX::spec*	HB12088 chrDNA --> HB13185
HB13218	168 *sigW::MLS sigM::kan*	HB0031 chrDNA --> HB10102
HB13218	168 *sigW::MLS sigM::kan*	HB0031 chrDNA --> HB10102
HB13219	168 *sigW::MLS sigX::spec*	HB13088 chrDNA --> HB10102
HB13219	168 *sigW::MLS sigX::spec*	HB12088 chrDNA --> HB10102
HB13222	168 *amyE::yfnI (cat)*	pTK075 --> 168
HB13223	168 *yfnI::spec amyE::yfnI (cat)*	HB13222 chrDNA --> HB13210
HB13225	168 *amyE::P_{yfnI}-lacZ (cat)*	pTK077 --> 168
HB13241	168 *amyE::P_{yfnI}(hy)-lacZ (cat) sigM::kan*	HB0031 chrDNA --> HB13225
HB13242	168 *yvlABCD::kan*	HB6153 chrDNA --> 168
HB13243	168 *pspA::cat*	HB0919 chrDNA --> 168
HB13244	168 *yvlABCD::kan pspA::cat*	HB0919 chrDNA --> HB13242
HB13245	168 *liaIH::tet*	HB0935 chrDNA --> 168
HB13246	168 *yvlABCD::kan liaIH::tet*	HB0935 chrDNA --> HB13242
HB13247	168 *pspA::cat liaIH::tet*	HB0935 chrDNA --> HB13243
HB13248	168 *yvlABCD::kan pspA::cat liaIH::tet*	HB0935 chrDNA --> HB13244
HB13249	168 *amyE::P_{spac}(hy)-yfnI (cat)*	pTK078 --> 168
HB13250	168 *yfnI::spec amyE::P_{spac}(hy)-yfnI*	HB13249 chrDNA --> HB13210
HB13251	168 *sppA::cat*	HB0348 chrDNA --> 168
HB13253	168 *yvlABCD::kan sppA::cat*	HB0348 chrDNA --> HB13242
HB13255	168 *ltaS::MLS*	LFH-PCR --> 168
HB13256	168 *yqgS::cat*	LFH-PCR --> 168
HB13257	168 *yfnI::spec yqgS::cat*	HB13256 chrDNA --> HB13210
HB13258	168 *yfnI::spec ltaS::MLS*	HB13255 chrDNA --> HB13210
HB13259	168 *yqgS::cat ltaS::MLS*	HB13255 chrDNA --> HB13256
HB13260	168 *yfnI::spec yqgS::cat ltaS::MLS*	HB13255 chrDNA --> HB13259
HB13261	168 *yfnI::spec ltaS::MLS amyE::P_{spac}(hy)-yfnI (cat)*	HB13249 chrDNA --> HB13258
HB13262	168 *amyE::P_{spac}(hy)-ltaS (cat)*	pTK079 --> 168
HB13263	168 *amyE::P_{spac}(hy)-yqgS (cat)*	pTK080 --> 168

HB13264	168 *yfnI::spec ltaS::MLS amyE::P_{spac}(hy)-ltaS (cat)*	HB13262 chrDNA --> HB13258
HB13265	168 *yfnI::spec ltaS::MLS amyE::P_{spac}(hy)-yqgS (cat)*	HB13263 chrDNA --> HB13258
HB13266	168 *yvlBCD::kan*	LFH-PCR --> 168
HB13267	168 *yvlCD::kan*	LFH-PCR --> 168
HB13268	168 *yvlD::kan*	LFH-PCR --> 168
HB13269	168 *amyE::Pspac(hy)-yvlC (cat)*	pTK081 --> 168
HB13270	168 *yvlABCD::kan amyE::P_{spac}(hy)-yvlC (cat)*	HB13266 chrDNA --> HB13243
HB13274	*168 dltA::cat*	ECE76 --> HB12084
HB13280	168 *xpaCyaaN::kan*	HB6168 chrDNA --> 168
HB13281	168 *yceH-pMUTIN (MLS)*	HB0101 chrDNA --> 168
HB13282	168 *xpaCyaaN::kan yceH-pMUTIN (MLS)*	HB0101 chrDNA --> HB13280
HB13283	168 *yybT::cat*	ECE76 --> HB10352
HB13284	168 *yybT::cat ltaS::MLS*	HB13255 chrDNA --> HB13283
HB13285	168 *yceH-pMUTIN (MLS) dltA::cat*	HB13274 chrDNA --> HB13281
HB13286	168 *yceH-pMUTIN (MLS) yfnI::spec*	HB13210 chrDNA --> HB13281
HB13287	168 *yceH-pMUTIN (MLS) dltA::cat yfnI::spec*	HB13274 chrDNA --> HB132815
HB13288	168 *yceH-pMUTIN (MLS) sigW::spec*	HB6208 chrDNA --> HB13281
HB13289	168 *sppA::cat yvlABCD::kan yceH-pMUTIN (MLS)*	HB0101 chrDNA --> HB13253
HB13290	168 *sppA::cat yvlABCD::kan sigW::spec yceH-pMUTIN (MLS)*	HB0101 chrDNA --> HB13278
HB13291	168 *ltaS::MLS yfnI::spec yybT::cat*	HB13283 chrDNA --> HB13258
HB13292	168 *sigX::kan 168 amyE::P_{yfnI}-lacZ (cat)*	HB13255 chrDNA --> HB12088
HB13293	168 *sigW::MLS 168 amyE::P_{yfnI}-lacZ (cat)*	HB13255 chrDNA --> HB12087
HB13294	168 *sigM::kan 168 amyE::P_{yfnI}-lacZ (cat)*	HB13255 chrDNA --> HB13218
HB13295	168 *sigM::kan sigX::spec 168 amyE::P_{yfnI}-lacZ (cat)*	HB13255 chrDNA --> HB13217
HB13296	168 *sigW::MLS sigX::spec 168 amyE::P_{yfnI}-lacZ (cat)*	HB13255 chrDNA --> HB13219
HB13297	168 *sigM::tet sigW::MLS sigX::kan 168 amyE::P_{yfnI}-lacZ (cat)*	HB13255 chrDNA --> HB12090
HB13298	168 *amyE::P_{yceC}-lacZ (cat)*	pTK082 --> 168
HB13299	168 *sigM::kan amyE::P_{yceC}-lacZ (cat)*	HB13298 chrDNA --> HB13185
HB13300	168 *sigX::kan amyE::P_{yceC}-lacZ (cat)*	HB13298 chrDNA --> HB12088
HB13301	168 *sigW::MLS amyE::P_{yceC}-lacZ (cat)*	HB13298 chrDNA --> HB12087
HB13302	168 *sigM::kan sigW::MLS amyE::P_{yceC}-lacZ (cat)*	HB13298 chrDNA --> HB13218
HB13303	168 *sigM::kan sigx::spec amyE::P_{yceC}-lacZ (cat)*	HB13298 chrDNA --> HB13217
HB13304	168 *sigW::MLS sigX::spec 168 amyE::P_{yceC}-lacZ (cat)*	HB13298 chrDNA --> HB13219
HB13305	168 *sigM::tet sigW::MLS sigX::kan 168 amyE::P_{yceC}-lacZ (cat)*	HB13298 chrDNA --> HB12090
HB13306	168 *SPβ[P_{pspA}-cat-lacZ] (neo, MLS)*	HB0077 spβ--> 168
HB13307	168 *sigW::spec SPβ[P_{pspA}-cat-lacZ] (neo, MLS)*	HB0077 spβ --> HB13206
HB13308	168 *SPβ[P_{sppA}-cat-lacZ] (neo, MLS)*	HB8013 spβ --> 168
HB13309	168 *sigW::spec SPβ[P_{sppA}-cat-lacZ] (neo, MLS)*	HB8013 spβ --> HB13206
HB13310	168 *SPβ[P_{yvlA}-cat-lacZ] (neo, MLS)*	HB8060 spβ --> 168
HB13311	168 *sigW::spec SPβ[P_{yvlA}-cat-lacZ] (neo, MLS)*	HB8060 spβ --> HB13206
HB13312	168 *yqeZyqfAB::kan sppA::cat*	HB0348 chrDNA --> HB13231

HB13313	168 *tepA::MLS*	LFH-PCR --> 168
HB13314	168 *sppA::cat tepA::MLS*	HB13314 chrDNA --> HB13251
HB13315	168 *yqeZyqfAB::kan tepA::MLS*	HB13314 chrDNA --> HB13231
HB13316	168 *yqeZyqfAB::kan sppA::cat tepA::MLS*	HB13314 chrDNA --> HB13312
HB13317	168 *dltA::cat ltaS::MLS*	HB13255 chrDNA --> HB13274
HB13318	168 *ltaS::MLS yfnI::spec dltA::cat*	HB13274 chrDNA --> HB13258
HB13322	168 *amyE::Pspac(hy)-yceH(cat)*	pTK084 --> 168
HB13323	168 *yceCDEFGHI::MLS*	LFH-PCR --> 168
HB13324	168 *yceH-pPL82 yceC-I::MLS*	HB13323 chrDNA --> HB13322
HB13325	168 *yceH-pMUTIN (MLS) yceH-pPL82*	HB13322 chrDNA --> HB13281
HB13326	168 *amyE::Pspac(hy)-dltABCDE (cat)*	pTK090 -->168
HB13327	168 *amyE::Pspac(hy)-liaH (cat)*	pTK091 -->168
HB13328	168 *amyE::Pspac(hy)-pspA (cat)*	pTK092 -->169
HB13329	168 *amyE::Pspac(hy)-sppA (cat)*	pTK093 -->169
HB13330	168 *amyE::Pspac(hy)-yceG (cat)*	pTK094 -->170
HB13331	168 *amyE::Pspac(hy)-yceI (cat)*	pTK095 -->170
HB13332	168 *amyE::Pspac(hy)-yceGH (cat)*	pTK096 -->171
HB13333	168 *amyE::Pspac(hy)-yceHI (cat)*	pTK097 -->171
HB13334	168 *amyE::Pspac(hy)-yceGHI (cat)*	pTK098 -->172
HB13335	168 *yceH-pMUTIN (MLS) dltA::cat yfnI::spec yvlABCD::kan*	HB13242 chrDNA --> HB13287
HB13337	168 *dltA::MLS sigX::spec*	HB12088 chrDNA --> HB12084
HB13338	168 *amyE::Pspac(hy)-yceG (cat) yceCDEFGHI::MLS*	HB13323 chrDNA -->HB13330
HB13339	168 *amyE::Pspac(hy)-yceGH (cat) yceCDEFGHI::MLS*	HB13323 chrDNA -->HB13331
HB13340	168 *amyE::Pspac(hy)-yceHI (cat) yceCDEFGHI::MLS*	HB13323 chrDNA -->HB13332
HB13341	168 *amyE::Pspac(hy)-yceI (cat) yceCDEFGHI::MLS*	HB13323 chrDNA -->HB13333
HB13342	168 *SPβ[PdltA-cat-lacZ] (neo, MLS) sigX::spec*	HB12088 chrDNA --> HB12060
HB13343	168 *SPβ[PdltA-cat-lacZ] (neo, MLS) sigM::tet*	HB10016 chrDNA --> HB12060
HB13344	168 *SPβ[PdltA-cat-lacZ] (neo, MLS) sigX::spec sigM::tet*	HB10016 chrDNA --> HB13342
HB13347	168 *amyE::Pspac(hy)-yceGHI (cat) yceCDEFGHI::MLS*	HB13323 chrDNA -->HB13334
HB13352	168 *yceH-pMUTIN (MLS) sppA::cat*	HB0348 chrDNA --> HB13281
HB13357	168 *tagT::cat*	LFH-PCR --> 168
HB13384	168 *yceCDEFG::MLS*	LFH-PCR --> 168
HB13385	168 *yceI::MLS*	LFH-PCR --> 168
HB13386	168 *TagO::kan*	LFH-PCR --> 168
HB13392	168 *rsiX::spec*	HB0047 chrDNA --> 168
HB13393	168 *rsiW::kan rsiX::spec*	HB0047 chrDNA --> HB13108
HB13394	168 *rsiW::kan amyE::Pspac(hy)-yfnI (cat)*	HB13249 chrDNA --> HB13108
HB13395	168 *rsiW::kan amyE::Pspac(hy)-yfnI (cat) rsiX::spec*	HB0047 chrDNA --> HB13394
HB13425	168 *tagU::MLS tagT::cat*	HB13357 chrDNA --> HB10193
HB13566	168 *yqeZyqfAB::kan*	(46)
ZB307A	*trpC2 SPβc2Δ2::Tn917::pSK10Δ6*	Lab Stock

Other Bacillus Strains	BGSC #
B. subtilis ssp. spizizenii NRRL B-23049T	2A8T
B. subtilis ssp. spizizenii W23 (NRRL B-14472)	2A9
B. subtilis ssp. spizizenii ATCC 6633 (NRRL B-765)	2A13
B. licheniformis ATCC 14580	5A36T
B. amyloliquefaciens FZB42	NA
B. atrophaeus NRS-213	11A2T
B. atrophaeus ESM rplV str	12A1
B. amyloliquefacien bmyA::MLS	AK1
B. amyloliquefaciens fen::cat	AK2
B. amyloliquefaciens pks2KS1::cat pks3KS1::MLS	CH12
B. amyloliquefaciens sfp::MLS	CH3

Plasmids	Genotype or description	Source
pdg1661	Vector for integration of *lacZ* fusions at *amyE*	(24)
pdg1662	Vector for integration at *amyE* locus	(24)
pPL82	Vector for IPTG-inducible control of gene expression with integration at the *amyE* locus	(67)
ECE76	Vector for integration of a cat resistance cassette into a MLS resistance cassette	(81)
pTK075	pDG1662-*yfnI* (cat)	This work
pTK077	pDG1661-P_{yfnI} (cat)	This work
pTK078	pPL82-*yfnI* (cat)	This work
pTK079	pPL82-*ltaS* (cat)	This work
pTK080	pPL82-*yqgS* (cat)	This work
pTK081	pPL82-*yvlC* (cat)	This work
pTK082	pDG1661-P_{yceC} (cat)	This work
pTK090	pPL82-*dltABCDE* (cat)	This work
pTK091	pPL82-*liaH* (cat)	This work
pTK092	pPL82-*pspA* (cat)	This work
pTK093	pPL82-*sppA* (cat)	This work
pTK094	pPL82-*yceG* (cat)	This work
pTK095	pPL82-*yceI* (cat)	This work
pTK096	pPL82-*yceGH* (cat)	This work
pTK097	pPL82-*yceHI* (cat)	This work
pTK098	pPL82-*yceGHI* (cat)	This work
pRSFDuet-1nisAB	Vector for IPTG-inducible control of *nisAB*	(76)
pRSFDuet-1nisAB-N20PM21P	Vector for IPTG-inducible control of *nisAB* with mutations in nisA to produce N20PM21P nisin	(25)
pACYCDuet-1nisC	Vector for IPTG-inducible control of *nisC*	(76)

Oligos	Name	Sequence
5535	yfnI up-fwd	GATGAGCTTGAGCGGTTTGA
5536	yfnI up-rev (spec)	CGTTACGTTATTAGCGAGCCAGTCTTCATTCATATTTACCTGCTCTT
5537	yfnI do-fwd (spec)	CAATAAACCCTTGCCCTCGCTACGAAGACTTGCTGAGGTTCCATA
5538	yfnI do-rev	CCATGCCATCCTCAACTGT
5559	yfnI up EcoRI	AGTCGAATTCGTATGTACGCTAAGGATGTCT
5560	yfnI do BamHI	AGTCGGATCCCCGCAGAGAATGACCGCTT
5670	ltaS up-fwd	AATAGTCGTTCCATCCCATCA
5671	ltaS up-rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCGACGACCGCAATTAAGAAGAA
5672	ltaS do-fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCCCTTCTGATTATGATTACACAA
5673	ltaS do-rev	TTAATTCCGCTTGGCCTTCA
5674	yqgS up-fwd	CGGCAGGAGTGGTTTATGAA
5675	yqgS up-rev (cat)	CTTGATAATAAGGGTAACTATTGCCATCAGTCTATTCCCAACATTCT
5676	yqgS do-fwd (cat)	GGGTAACTAGCCTCGCCGGTCCACGGATCAGGCATCATAATCACAA
5677	yqgS do-rev	GTGCTGTCGGTTTTCGGTAT
5678	yfnI up XmaI	TCAGCCCGGGCAGGTAAATATGAATGAAGAACT
5679	yfnI do XbaI	AGTCTCTAGAGTAATGATATGAGAGAAAGCCA
5687	yvlB up-fwd	GCTGCTTACTTACACTCATTA
5688	yvlB up-rev (kan)	CTATCACCTCAAATGGTTCGCTGCTTGCTTCATTACGATCTCCT
5689	yvlCD up-fwd	GACACGCATTTCTTCAGCAT
5690	yvlC up-rev (kan)	CTATCACCTCAAATGGTTCGCTGCTTATTCATTCGATCACCTCT
5691	yvlD up-rev (kan)	CCTATCACCTCAAATGGTTCGCTGACTAAAATGCTGACTGCCCA
5696	ltaS up XmaI	TCAGCCCGGGCGCTCGAACTGGATCGGAA
5697	ltaS do XbaI	AGTCTCTAGATGAGGAATTGAGGGCTGCT
5698	yqgS up XmaI	TCAGCCCGGGTGAGCGTGCTGCATAGGAG
5699	yqgS do XbaI	AGTCTCTAGATCACCGTAATAAACGCCATCA
5700	yvlC up XmaI	TCAGCCCGGGATCAAACTAAAGTACTCACAGA
5701	yvlC do XbaI	AGTCTCTAGAGCTGCTGATATGGATGGAGT
5792	P_{vceC} up EcoRI	AGTCGAATTCACCGAATGCTGGAGAGTTGT
5793	P_{vceC} do BamHI	AGTCGGATCCCTCACAAGGAAAATAAGCCGTA
5816	tepA up-fwd	GAATGGCAAACAAAGCTCACA
5817	tepA up-rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCGGACGCTCTTCTTCTGTGTT
5818	tepA do-fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCGATGAAGCAAGGAAAGAAGAA
5819	tepA do-rev	TTCTTGCAGCGAGCGTCCT
5871	yceC up-fwd	ACCAAGACCGAGCACCCAT
5872	yceC up-rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCAGCCGAGACCGACCATCAA
5873	yceI do-fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCCTTTGCTCGTCGGAACCCT
5874	yceI do-rev	TCGGTCATTCTGCTTGCCTA
5875	yceH up XmaI	TCAGCCCGGGATGGTGTTTCAGCAGGACTA
5876	yceH do XbaI	AGTCTCTAGATATAGGCTGTTGTTTTCCCATT
5893	sppA up XmaI	TCAGCCCGGGTGAATCGTATAATGAGAGAGTT
5894	sppA do XbaI	AGTCTCTAGACAGGAAAGCCCAGAAACGAA

5895	pspA up XmaI	TCAGCCCGGGGTTACCGAATTAGCTTTAGGA
5896	pspA do XbaI	AGTCTCTAGAATTACCGTTCCCTCTCACATT
5897	liaH up XmaI	TCAGCCCGGGTTTGATGACGAATGGGAAGAAT
5898	liaH do XbaI	AGTCTCTAGAGGTTTCATCCTTCTCATTCATT
5899	yceG up XmaI	TCAGCCCGGGCAAGGAGGGAGGTTCAATGT
5900	yceG do XbaI	AGTCTCTAGACGCTTCACTTCTGGTTCCT
5901	yceI up XmaI	TCAGCCCGGGTGTGTAAAGACAGGTGTAAACT
5902	yceI do XbaI	AGTCTCTAGAAGCATAGGAGCGGACATTCA
5903	dltA up XmaI	TCAGCCCGGGAAACCCGCTGTCAAGTGGA
5904	dltE do XbaI	AGTCTCTAGATGGTCAATCTCCCTGCTGTT
5932	tagT up-fwd	CAAGAGCAATGACGGCACA
5933	tagT up-rev (cat)	CTTGATAATAAGGGTAACTATTGCCCGGCAACAACTTTCACCCAT
5934	tagT do-fwd (cat)	GGGTAACTAGCCTCGCCGGTCCACGCTGCCGGAGTCTATTATTTCA
5935	tagT do-rev	TTCAGTTCTTCCTTCGCTTGT
5952	yceI up-fwd	AGATGAGATGACGAAGACGAA
5953	yceI up-rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCACGCTGGGATATAGGCTGTT
5954	yceG do-fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCTCAGCAGGACTACAAAGAGC
5955	yceG do-rev	GACGGATTTGAGGTGCGGT

[a] Abbreviations used: --> indicates transformation; chrDNA indicates chromosomal DNA

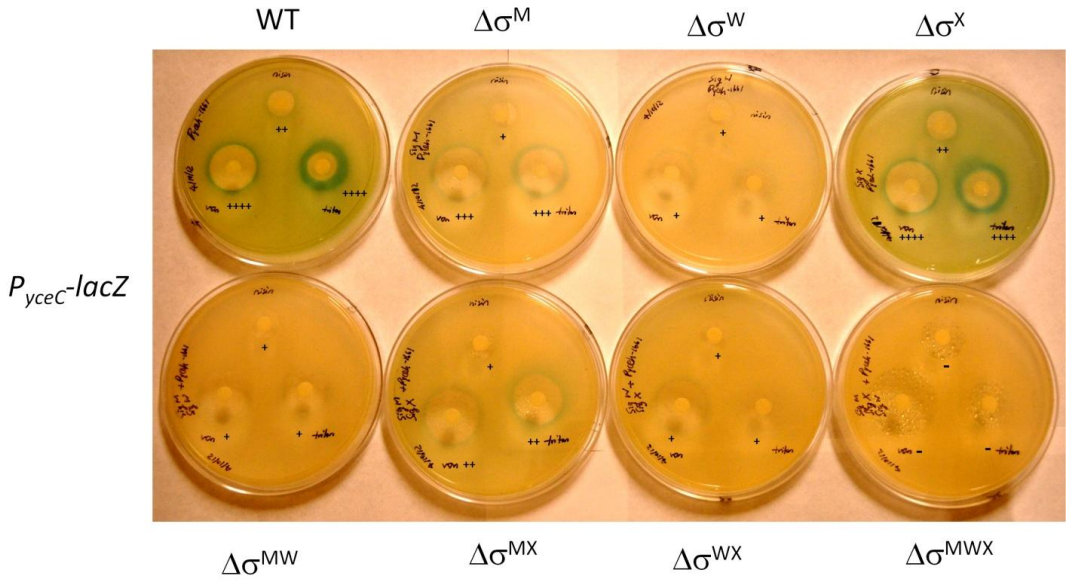


Figure S3.1: Sample image of the P_{yceC}-*lacZ* gene fusion being induced by triton X-100 (triton), vancomycin (van), and nisin in WT and $\Delta\sigma^{ECF}$ backgrounds. + and – symbols represent the blue intensity and hence, promoter activity as indicated in Table 3.1.

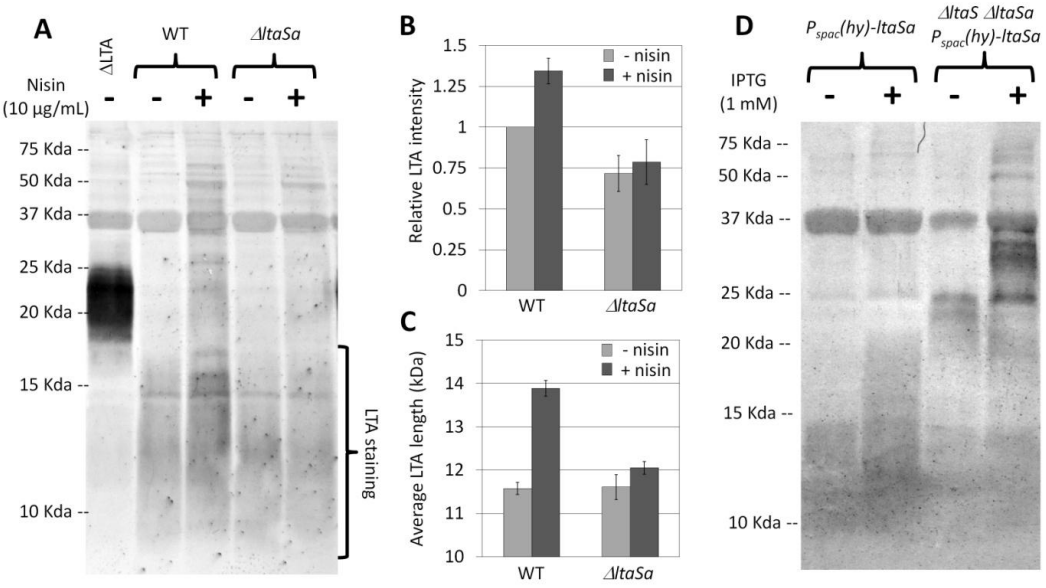


Figure S3.2: Nisin influences LTAs in *B. subtilis* in a *ltaSa*-dependent manner. A. Detection of lipoteichoic acid by Western blot with anti-LTA antibodies in the WT (168), *ΔltaSa* (HB13210), and ΔLTA (Δ*ltaS* Δ*ltaSa* Δ*yqgS* - HB13260) strains with and without nisin treatment (0.5 µg ml^{-1}). This experiment was repeated three times with independent biological replicates and the image shown is representative of typical results. The staining between 10-18 Kda, which is not present in the ΔLTA strain, represents teichoic acid. B & C. The relative intensities of LTA staining (B) and the average LTA length (C) as measured in apparent kDa (based on protein standards; note that this does not correspond to actual LTA mass) for the strains analyzed in panel A as determined by densitometry analysis. D. Detection of lipoteichoic acid by Western blot with anti-LTA antibodies in the P_{spac}*(hy)-ltaSa* (HB13249) and *ΔltaS ΔltaSa* P_{spac}*(hy)-ltaSa* (HB13261) strains with and without 1 mM IPTG treatment.

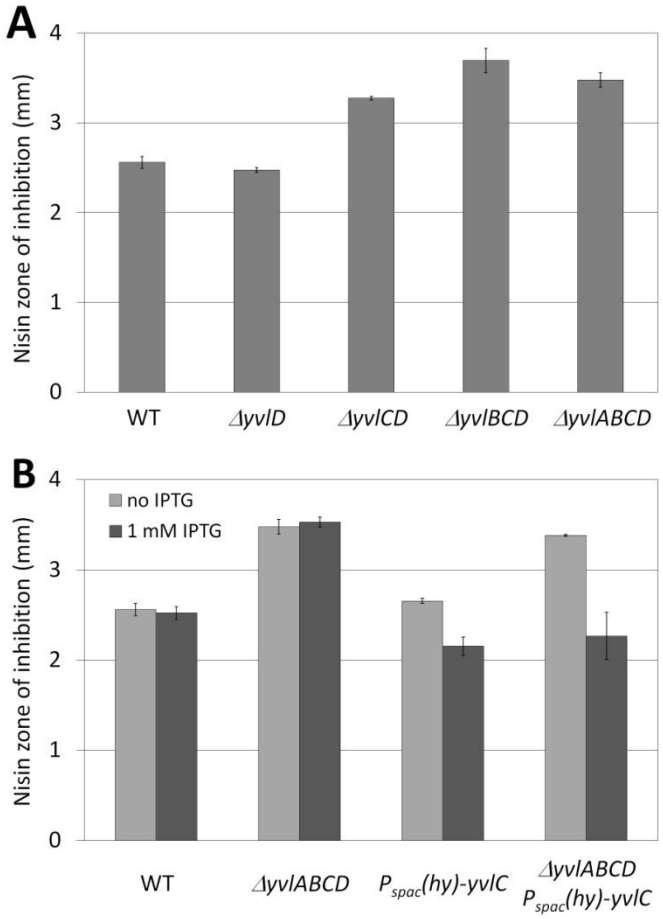


Figure S3.3: Within the *yvlABCD* operon, the *yvlC* gene is sufficient to confer nisin resistance. A. Nisin disk diffusion assays for WT (168), Δ*yvlD* (HB13268), Δ*yvlCD* (HB13267), Δ*yvlBCD* (HB13266), and Δ*yvlABCD* (HB13242) strains. The WT and Δ*yvlD* strains exhibit significantly lower zones of inhibition than the Δ*yvlCD* (HB13267), Δ*yvlBCD* (HB13266), and Δ*yvlABCD* strains. B. Nisin disk diffusion assays for WT (168), Δ*yvlABCD* (HB13242), P_{spac}*(hy)-yvlC* (HB13269), and Δ*yvlABCD* P_{spac}*(hy)-yvlC* (HB13270) strains on MH media with and without 1 mM IPTG. The zones of inhibition for the Δ*yvlABCD* P_{spac}*(hy)-yvlC* strain with and without IPTG are significantly different from each other.

To determine which gene(s) within the *yvlABCD* operon contribute to nisin resistance, we assessed the nisin sensitivity of strains lacking one or more genes (Panel A). Nisin sensitivity was increased in those strains lacking *yvlC*, and expression of *yvlC* from an IPTG-inducible promoter was sufficient to complement a *yvlABCD* deletion strain (Panel B). We conclude that *yvlC* is necessary and sufficient for nisin resistance.

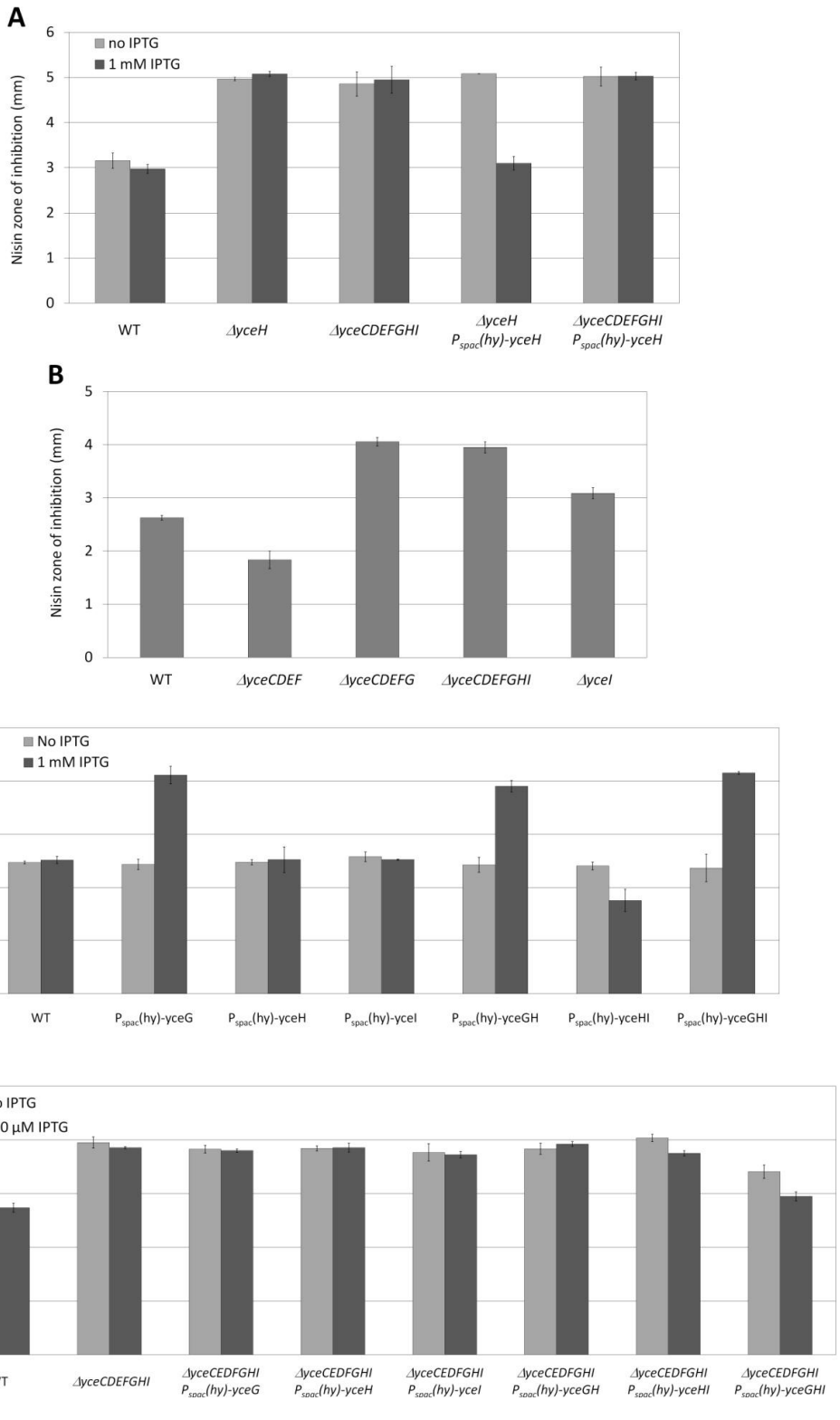

A
no IPTG
1 mM IPTG
Nisin zone of inhibition (mm)
WT
ΔyceH
ΔyceCDEFGHI
ΔyceH Pspac(hy)-yceH
ΔyceCDEFGHI Pspac(hy)-yceH
B
Nisin zone of inhibition (mm)
WT
ΔyceCDEF
ΔyceCDEFG
ΔyceCDEFGHI
ΔyceI
C
No IPTG
1 mM IPTG
Nisin zone of inhibition (mm)
WT
Pspac(hy)-yceG
Pspac(hy)-yceH
Pspac(hy)-yceI
Pspac(hy)-yceGH
Pspac(hy)-yceHI
Pspac(hy)-yceGHI
D
No IPTG
100 μM IPTG
Nisin zone of inhibition (mm)
WT
ΔyceCDEFGHI
ΔyceCEDFGHI Pspac(hy)-yceG
ΔyceCEDFGHI Pspac(hy)-yceH
ΔyceCEDFGHI Pspac(hy)-yceI
ΔyceCEDFGHI Pspac(hy)-yceGH
ΔyceCEDFGHI Pspac(hy)-yceHI
ΔyceCEDFGHI Pspac(hy)-yceGHI

Figure S3.4: *yceGHI* contributes to intrinsic nisin resistance A. Within the *yceCDEFGHI* operon, a gene other than *yceH* contrbutes to nisin resistance. Nisin disk diffusion assays of WT (168), Δ*yceH* (HB13281), *ΔyceCDEFGHI* (HB13323), *ΔyceH* P_{spac}*(hy)-yceH* (HB13325), and *ΔyceCDEFGHI* P_{spac}*(hy)-yceH* (HB13324) strains treated with and without 1 mM IPTG. The IPTG treatment restores nisin susceptibility to WT levels only in the *ΔyceH* P_{spac}*(hy)-yceH* strain. B. YceG is necessary to this nisin resistance mechanism while YceI contributes to it. Nisin disk diffusion assays of WT (168), *ΔyceCDEF* (HB0100), *ΔyceCDEFG* (HB13384), *ΔyceCDEFGHI* (HB13323), and *ΔyceI* (HB13385), strains. The *ΔyceCDEF* strain is not more susceptible to nisin than the WT strain while the Δ*yceCDEFG* strain is more susceptible. The *ΔyceI* strain is significantly more susceptible than the WT strain, but is significantly less susceptible than the *ΔyceCDEFGHI* strain. C. Nisin disk diffusion assays for WT (168), P_{spac}*(hy)-yceG* (HB13330), P_{spac}*(hy)-yceH* (HB13322), P_{spac}*(hy)-yceI* (HB13331), P_{spac}*(hy)-yceGH* (HB13332), P_{spac}*(hy)-yceHI* (HB13333), and P_{spac}*(hy)-yceGHI* (HB13334) strains with and without 1 mM IPTG. IPTG treatment significantly increased sensitivity in all the strains containing an IPTG induicible copy of *yceG* and reduced sensitivity in the P_{spac}*(hy)-yceHI* strain. D. *yceGHI* are all essential to this nisin resistance mechanism. Nisin disk diffusion assays of WT (168), *ΔyceCDEFGHI* (HB13323), Δ*yceCDEFGHI* P_{spac}*(hy)-yceG* (HB13338), Δ*yceCDEFGHI* P_{spac}*(hy)-yceH* (HB13324), *ΔyceCDEFGHI* P_{spac}*(hy)-yceI* (HB13341), *ΔyceCDEFGHI* P_{spac}*(hy)-yceGH* (HB13339), *ΔyceCDEFGHI* P_{spac}*(hy)-yceHI* (HB13340), and *ΔyceCDEFGHI* P_{spac}*(hy)-yceGHI* (HB13347) strains treated with and without 100 uM IPTG. Among all strains and treatments tested, only the *ΔyceCDEFGHI* P_{spac}*(hy)-yceGHI* strain treated with 100 uM IPTG exhibited no significant difference in nisin sensitivity compared to WT cells.

Figure S4 describes our analysis to determine which genes within the *yceCDEFGHI* operon contribute to nisin resistance. Since we originally detected a nisin sensitive phenotype for a *yceH*::pMUTIN insertion strain, we could assume that least *yceH* is implicated in resistance. Expression of *yceH* from an IPTG inducible promoter was able to restore nisin sensitivity in a *ΔyceH* strain, but not in a *ΔyceCDEFGHI* strain, indicating that an additional component within this operon is necessary for *yceH*-mediated nisin resistance (Panel A). Deleting *yceCDEF* did not increase nisin sensitivity beyond WT levels, but deleting *yceCDEFG* or *yceI* did increase nisin sensitivity which focused our attention on *yceGHI* (Panel B). We introduced the IPTG inducible P_{spac}*(hy)-yceG*, *-yceH, -yceI, -yceGH*, *-yceHI*, or *-yceGHI* gene fusions in a WT background and assessed these strains for nisin sensitivity (Panel C). Full induction (1 mM IPTG) of y*ceHI* increased nisin resistance, but all of the strains containing an IPTG inducible *yceG* displayed increased nisin sensitivity. Thus, even though YceG is essential to this nisin resistance mechanism, it can be detrimental to the cell in excessive amounts. We therefore tested moderate induction (100 μM IPTG) of these gene fusions in a *yceCDEFGHI* operon deletion strain (Panel D). Only the induction of *yceGHI* restored nisin resistance to wild-type levels suggesting that YceG, YceH, and YceI all contribute to nisin resistance.

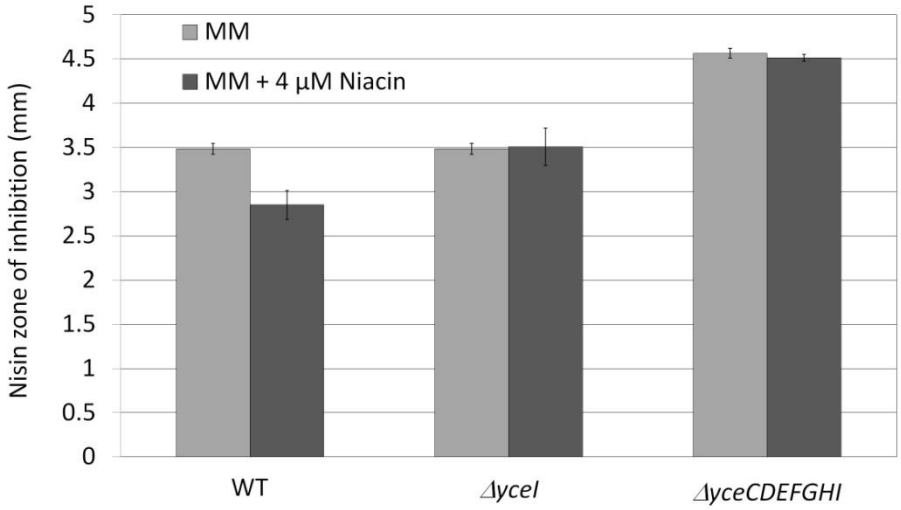


Figure S3.5: *yceI*-mediated nisin resistance requires nicotinate. Nisin disk diffusion assays of WT (168), *ΔyceI* (HB13385) and *ΔyceCDEFGHI* (HB13281) strains grown on minimal media supplemented with and without 4 µM nicotinate. Nisin sensitivity is significantly reduced by nicotinate treatment in the WT strain, but not in the *ΔyceI or ΔyceCDEFGHI* strains.

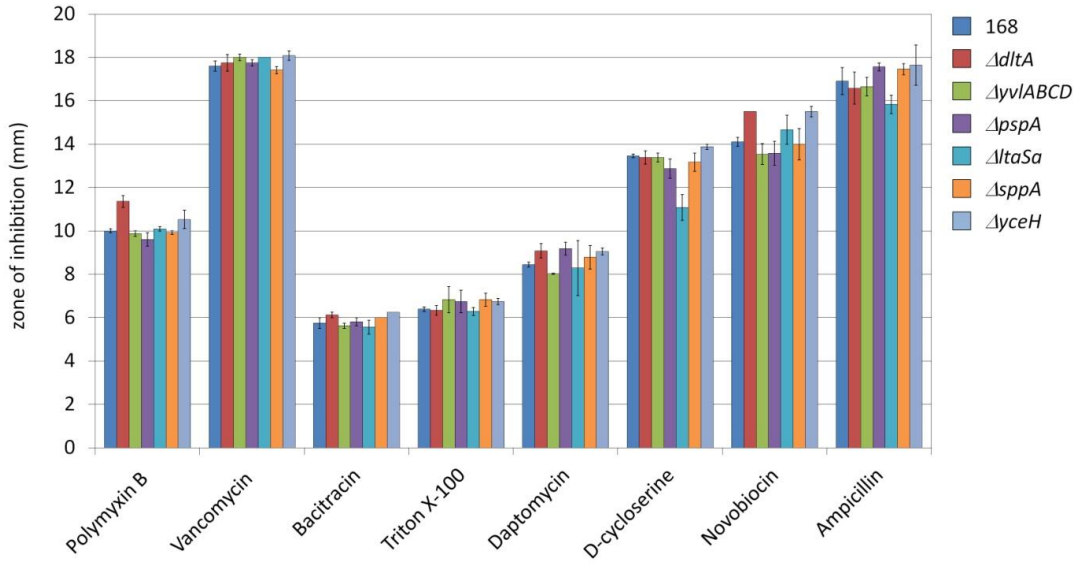


Figure S3.6: Sensitivity of nisin resistance gene knockout strains to various stresses. The WT (168), Δ*pspA* (HB13243), Δ *yvlABCD* (HB13242), Δ*sppA* (HB13251), Δ*ltaSa* (HB13210), Δ*dltA* (HB12084), and Δy*ceH* (HB13281) strains were assessed for sensitivity to polymyxin B, vancomycin, bacitracin, triton X-100, daptomycin, D-cycloserine, novobiocin, and ampicillin. The only significant differences between a WT and mutant strain are observed for dltA::MLS to polymyxin B and novobiocin, for Δ*pspA* to daptomycin, for Δy*ceH* to novobiocin, and Δ*ltaSa* to D-cycloserine, but in all cases these differences account for less than 10% of total zone of inhibition.

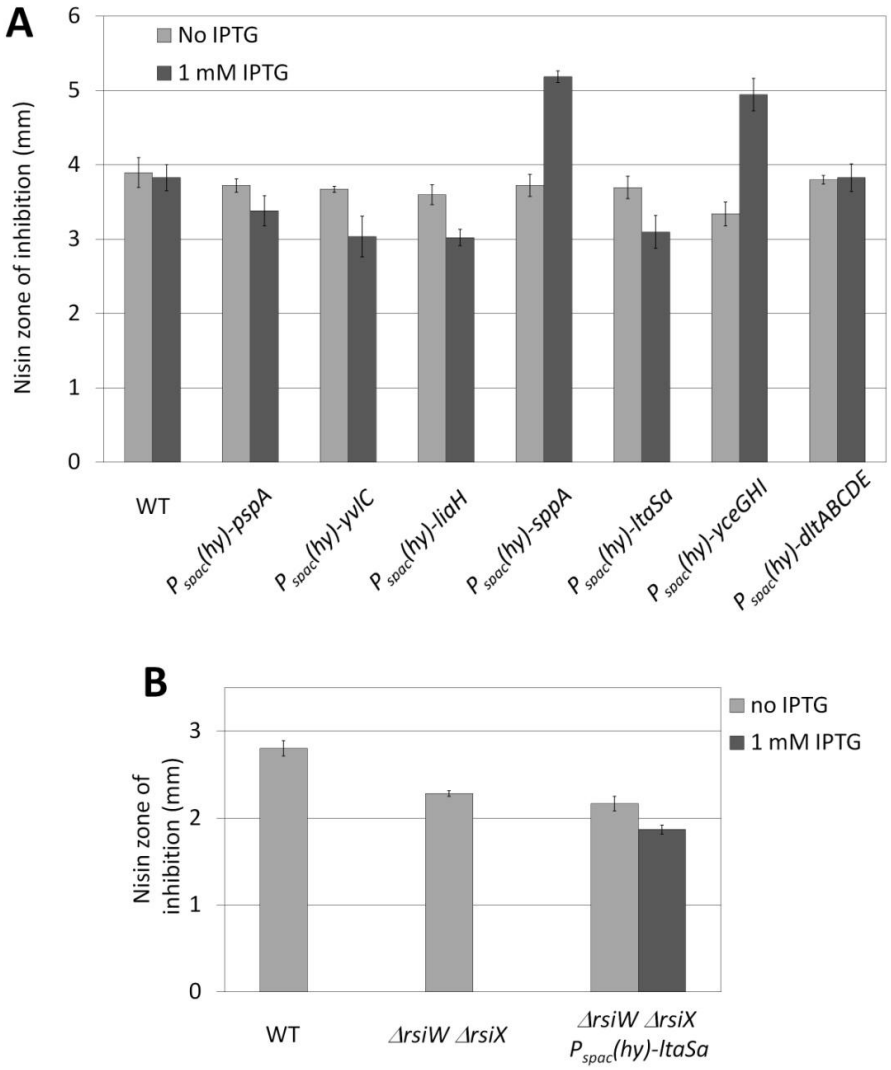
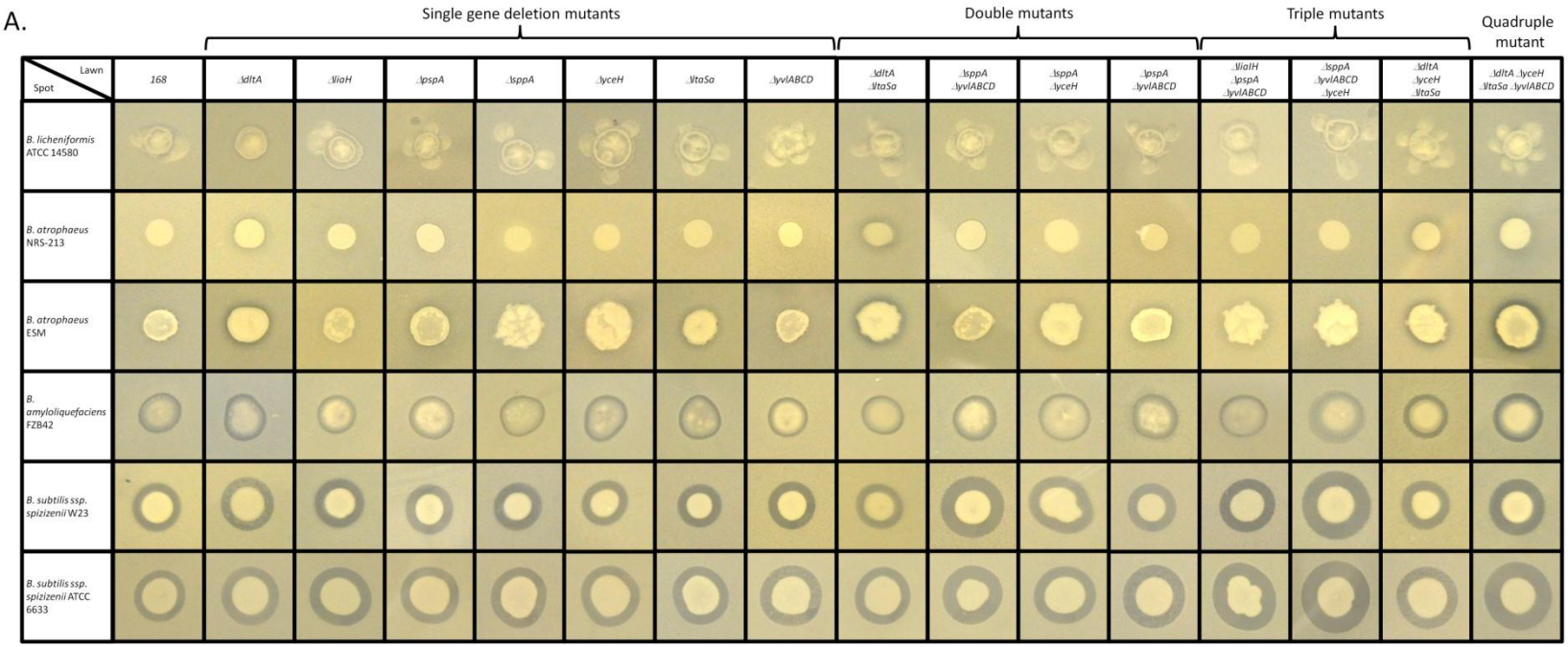


Figure S3.7: Creating a nisin resistant *B. subtilis* strain. A. Nisin disk diffusion assays for WT (168), P_{spac}*(hy)-pspA* (13328), P_{spac}*(hy)-yvlC* (HB13269), P_{spac}*(hy)-liaH* (13327), P_{spac}*(hy)-sppA* (13329), P_{spac}*(hy)-ltaSa* (HB13249), P_{spac}*(hy)-yceGHI* (HB13334), and P_{spac}*(hy)-dltABCDE* (HB13326) strains on media with and without 1 mM IPTG. Each disk contained 25 µg nisin instead of the usual 20 µg. In Media without IPTG, none of the strains exhibit significant differences in nisin sensitivity. In 1 mM IPTG media, nisin sensitivity was significantly lower in P_{spac}*(hy)-yvlC*, P_{spac}*(hy)-liaH*, and P_{spac}*(hy)-ltaSa* strains, significantly higher in P_{spac}*(hy)-sppA* and P_{spac}*(hy)-yceGHI* strains, and showed no significant difference in P_{spac}*(hy)-pspA* and P_{spac}*(hy)-dltABCDE* strains compared to WT. B. Nisin disk diffusion assays for WT (168), *ΔrsiW ΔrsiX* (HB13393), and *ΔrsiW ΔrsiX* P_{spac}*(hy)-ltaSa* (HB13394) strains on media with and without 1 mM IPTG. The nisin sensitivity was significantly lower in *ΔrsiW ΔrsiX* compared to WT and in *ΔrsiW ΔrsiX* P_{spac}*(hy)-ltaSa* + IPTG compared to *ΔrsiW ΔrsiX*.

A.


Single gene deletion mutants
Double mutants
Triple mutants
Quadruple mutant
Lawn
Spot
168
ΔdltA
ΔliaH
ΔlspA
ΔsppA
ΔlyceH
ΔltaSa
ΔlyvlABCD
ΔdltA ΔltaSa
ΔsppA ΔlyvlABCD
ΔsppA ΔlyceH
ΔlspA ΔlyvlABCD
ΔliaIH ΔlspA ΔlyvlABCD
ΔsppA ΔlyvlABCD ΔlyceH
ΔdltA ΔlyceH ΔltaSa
ΔdltA ΔlyceH ΔltaSa ΔlyvlABCD
B. licheniformis ATCC 14580
B. atrophaeus NRS-213
B. atrophaeus ESM
B. amyloliquefaciens FZB42
B. subtilis ssp. spizizenii W23
B. subtilis ssp. spizizenii ATCC 6633

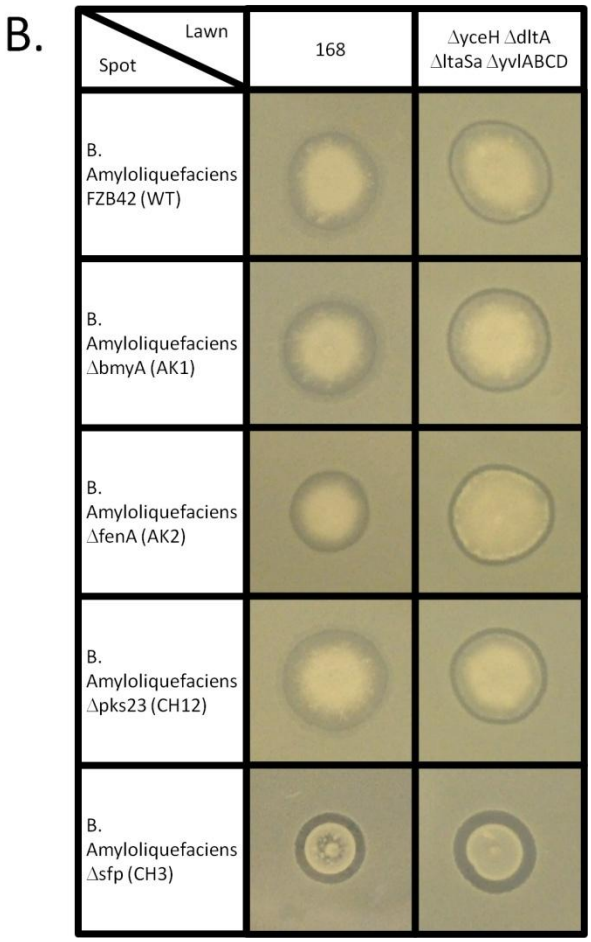


Figure S3.8: A representative set of spot on lawn data for the complete set of mutant strains. A. Spot on lawn assays of WT (168), *ΔdltA* (HB12084), *ΔliaH* (HB13245), *ΔpspA* (HB13243), *ΔsppA* (HB13251), *ΔyceH* (HB13281), *ΔltaSa* (HB13210), *ΔyvlABCD* (HB13242), *ΔltaSa ΔdltA* (HB13216), *ΔsppA ΔyvlABCD* (HB13253), *ΔsppA ΔyceH* (HB13352), *ΔpspA ΔyvlABCD* (HB13244), *ΔliaIH ΔpspA ΔyvlABCD* (HB13248), *ΔsppA ΔyceH ΔyvlABCD* (HB13289), *ΔyceH ΔdltA ΔltaSa* (HB13287), and *ΔyceH ΔdltA ΔltaSa* ΔyvlABCD (HB13335) lawn strains to spots of *B. licheniformis* ATCC 14580*, B. atrophaeus* NRS-213*, B. atrophaeus* ESM*, B. amyloliquefaciens* FZB42*,* and *B. subtilis ssp. spizizenii* W23*,* and *B. subtilis ssp. spizizenii* ATCC 6633. B. Spot on lawn assays of WT (168) and *ΔyceH ΔdltA ΔltaSa ΔyvlABCD* (HB13335) lawn strains to spots of *B. amyloliquefaciens* FZB42 (WT), *ΔbmyA* (AK1), *ΔfenA* (AK2), *Δpks23* (CH12), and *Δsfp* (CH3). The relative sensitivity of the lawn strains to each spotted strain is reflected by the size of the spot and the zone of inhibition surrounding it after 18 h growth at 37 ^{o}C. A larger spot size and zone of inhibition represents increased sensitivity of the lawn strains to the metabolites produced by the spotted strains. Pictures are representative of at least three assays performed with three independent clones of each strain.

REFERENCES

1. **Abriouel, H., C. M. A. P. Franz, N. B. Omar, and A. Galvez.** 2011. Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiol Rev **35:**201–232.

2. **Anantharaman, V., L. M. Iyer, and L. Aravind.** 2012. Ter-dependent stress response systems: novel pathways related to metal sensing, production of a nucleoside-like metabolite, and DNA-processing. Mol BioSyst **8:**3142–3165.

3. **Asai, K., K. Ishiwata, K. Matsuzaki, and Y. Sadaie.** 2008. A viable *Bacillus subtilis* strain without functional extracytoplasmic function sigma genes. J Bacteriol **190:**2633-2636.

4. **Bendtsen, J. D., H. Nielsen, G. v. Heijne, and S. Brunak.** 2004. Improved Prediction of Signal Peptides: SignalP 3.0. J Mol Biol **340:**783–795.

5. **Bierbaum, G., and H.-G. Sahl.** 2009. Lantibiotics: Mode of Action, Biosynthesis and Bioengineering. Curr Pharm Biotechno **10:**2-18.

6. **Bolhuis, A., A. Matzen, H.-L. Hyyrylainen, V. P. Kontinen, R. Meima, J. Chapuis, G. Venema, S. Bron, R. Freudl, and J. M. v. Dijl.** 1999. Signal peptide peptidase- and ClpP-like proteins of *Bacillus subtilis* required for efficient translocation and processing of secretory proteins. J Biol Chem **247:**24585–24592.

7. **Breukink, E., and B. d. Kruijff.** 2006. Lipid II as a target for antibiotics. Nat Rev Drug Discov **5:**321-332.

8. **Butcher, B. G., and J. D. Helmann.** 2006. Identification of *Bacillus subtilis* σ^W-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli. Mol Microbiol **60:**765-782.

9. **Cao, M., and J. D. Helmann.** 2004. The *Bacillus subtilis* extracytoplasmic-function σ^X factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides. J Bacteriol **186:**1136-1146.

10. **Cao, M., P. A. Kobel, M. M. Morshedi, M. Fang, and W. Wu.** 2002. Defining the *Bacillus subtilis* σ^W regulon: A comparative analsis of promoter consensus search, run-off transcription/macroarrray analysis (ROMA), and transcriptional profiling approaches. J Mol Biol **315:**443-457.

11. **Cao, M., T. Wang, R. Ye, and J. D. Helmann.** 2002. Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ^W and σ^M regulons. Mol Microbiol **45:**1267-1276.

12. **Chen, X. H., A. Koumoutsi, R. Scholz, K. Schneider, J. Vater, R. Süssmuth, J. Piel, and R. Borriss.** 2009. Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. J Biotechnol **140:**27-37.

13. **Collins, B., S. Joyce, C. Hill, P. D. Cotter, and R. P. Ross.** 2010. TelA contributes to the innate resistance of *Listeria monocytogenes* to nisin and other cell wall-acting antibiotics. Antimicrob Agents Ch **54:**4658–4663.

14. **Cotter, P. D., C. Hill, and R. P. Ross.** 2005. Bacteriocins: Developing innate immunity for food. Nat Rev Microbiol **3:**777-788.

15. **Darwin, A. J.** 2005. The phage-shock-protein response. Mol Micro **57:**621-628.

16. **Dawson, M. J., and R. W. Scott.** 2012. New horizons for host defense peptides and lantibiotics. Curr Opin Pharmacol **12:**545–550.

17. **Delves-Broughton, J.** 2005. Nisin as a food preservative. Food Aust **57:**525-527.

18. **Eiamphungporn, W., and J. D. Helmann.** 2008. The *Bacillus subtilis* σ^M regulon and its contribution to cell envelope stress responses. Mol Microbiol **67:**830-848.

19. **Eldholm, V., B. Gutt, O. Johnsborg, R. Brückner, P. Maurer, R. Hakenbeck, T. Mascher, and L. S. Håvarstein.** 2010. The Pneumococcal cell envelope stress-sensing system LiaFSR Is activated by murein hydrolases and lipid II-interacting antibiotics. J Bacteriol **192:**1761-1773.

20. **Field, D., M. Begley, P. M. O'Connor, K. M. Daly, F. Hugenholtz, P. D. Cotter, C. Hill, and R. P. Ross.** 2012. Bioengineered nisin A derivatives with enhanced activity against both Gram positive and Gram negative pathogens. PLOS One **7:**e46884.

21. **Field, D., P. M. O. Connor, P. D. Cotter, C. Hill, and R. P. Ross.** 2008. The generation of nisin variants with enhanced activity against specific Gram-positive pathogens. Mol Micro **69:**218-230.

22. **Gravesen, A., B. Kallipolitis, K. Holmstrøm, P. E. Høiby, M. Ramnath, and S. Knøchel.** 2004. *pbp2229*-mediated nisin resistance mechanism in *Listeria monocytogenes* confers cross-protection to class IIa bacteriocins and affects virulence gene expression. Appl Environ Microbiol **70:**1669–1679.

23. **Guariglia-Oropeza, V., and J. D. Helmann.** 2011. Bacillus subtilis σ^V

Confers Lysozyme Resistance by Activation of Two Cell Wall Modification Pathways, Peptidoglycan O-Acetylation and D-Alanylation of Teichoic Acids. J Bacteriol **193:**6223-6232.

24. **Guerout-Fleury, A.-M., N. Frandsen, and P. Stragier.** 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57-61.

25. **Gut, I. M., S. R. Blanke, and W. A. van der Donk.** 2011. Mechanism of inhibition of *Bacillus anthracis* spore outgrowth by the lantibiotic nisin. ACS Chem Biol **6:**744–752.

26. **Haas, R., H. U. Koch, and W. Fischer.** 1984. Alanyl turnover from lipoteichoic acid to teichoic acid in *Staphylococcus aureus*. FEMS Microbiol Lett **21:**27–31.

27. **Hachmann, A. B., E. R. Angert, and J. D. Helmann.** 2009. Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. Antimicrob Agents Ch **53:**1598-1609.

28. **Hansen, M. E., R. Wangari, E. B. Hansen, I. Mijakovic, and P. R. Jensen.** 2009. Engineering of *Bacillus subtilis* 168 for Increased Nisin Resistance. Appl Environ Microbiol **75:**6688–6695.

29. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for Bacillus. John Wiley & Sons Ltd, Chichester, United Kingdom.

30. **Hasper, H. E., N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. d. Kruijff, and E. Breukink.** 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science **313:**1636-1637.

31. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol **46:**47-110.

32. **Horstman, N. K., and A. J. Darwin.** 2012. Phage shock proteins B and C prevent lethal cytoplasmic membrane permeability in *Yersinia enterocolitica*. Mol Micro **85:**445-460.

33. **Huang, X., A. Decatur, A. Sorokin, and J. D. Helmann.** 1997. The Bacillus subtilis σ^X Protein Is an Extracytoplasmic Function σ Factor Contributing to Survival at High Temperature. J Bacteriol **79:**2915–2921.

34. **Huang, X., and J. D. Helmann.** 1998. Identification of target promoters for the *Bacillus subtilis* sigma X factor using a consensus-directed search. J Mol Biol **279:**165-173.

35. **Hurdle, J. G., A. J. O'Neill, I. Chopra, and R. E. Lee.** 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol **9:**62-75.

36. **Huvet, M., T. Toni, X. Sheng, T. Thorne, G. Jovanovic, C. Engl, M. Buck, J. W. Pinney, and M. P. H. Stumpf.** 2011. The evolution of the phage shock protein response system: interplay between protein function, genomic organization, and system function. Mol Biol Evol **28:**1141-1155.

37. **Islam, M. R., J.-i. Nagao, T. Zendo, and K. Sonomoto.** 2012. Antimicrobial mechanism of lantibiotics. Biochem Soc T **40:**1528-1533.

38. **Jeanguenin, L., A. Lara-Núñez, D. A. Rodionov, A. L. Osterman, N. Y. Komarova, D. Rentsch, J. F. G. III, and A. D. Hanson.** 2012. Comparative genomics and functional analysis of the NiaP family uncover nicotinate transporters from bacteria, plants, and mammals. Funct Integr Genomics **12:**25-34.

39. **Joly, N., C. Engl, G. Jovanovic, M. Huvet, T. Toni, X. Sheng, M. P. H. Stumpf, and M. Buck.** 2010. Managing membrane stress: the phage shock protein (Psp) response, from molecular mechanisms to physiology. FEMS Microbiol Rev **34:**797–827.

40. **Jordan, S., M. I. Hutchings, and T. Mascher.** 2008. Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol Rev **32:**107-146.

41. **Kawai, Y., J. Marles-Wright, R. M. Cleverley, R. Emmins, S. Ishikawa, M. Kuwano, N. Heinz, N. K. Bui, C. N. Hoyland, N. Ogasawara, R. J. Lewis, W. Vollmer, R. A. Daniel, and J. Errington.** 2011. A widespread family of bacterial cell wall assembly proteins. EMBO J **30:**4931-4941.

42. **Kim, A. C., D. C. Oliver, and M. Paetzel.** 2008. Crystal structure of a bacterial signal peptide peptidase. J Mol Biol **376:**352–366.

43. **Kobayashi, R., T. Suzuki, and M. Yoshida.** 2007. *Escherichia coli* phage-shock protein A (PspA) binds to membrane phospholipids and repairs proton leakage of the damaged membranes. Mol Micro **66:**100-109.

44. **Kovács, M., A. Halfmann, I. Fedtke, M. Heintz, A. Peschel, W. Vollmer, R. Hakenbeck, and R. Brückner1.** 2006. A functional *dlt* operon, encoding proteins required for incorporation of D-alanine in teichoic acids in Gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*. J Bacteriol **188:**5797-5805.

45. **Kramer, N. E., H. E. Hasper, P. T. C. v. d. Bogaard, S. Morath, B. d.**

Kruijff, T. Hartung, E. J. Smid, E. Breukink, J. Kok, and O. P. Kuipers. 2008. Increased D-alanylation of lipoteichoic acid and a thickened septum are main determinants in the nisin resistance mechanism of *Lactococcus lactis*. Microbiology **154:**1755-1762.

46. **Lee, Y. H., A. W. Kingston, and J. D. Helmann.** 2012. Glutamate dehydrogenase affects resistance to cell wall antibiotics in *Bacillus subtilis*. J Bacteriol **194:**993–1001.

47. **Luo, Y., K. Asai, Y. Sadaie, and J. D. Helmann.** 2010. Transcriptomic and phenotypic characterization of a *Bacillus subtilis* strain without extractyoplasmic function σ factors. J Bacteriol **192:**5736-5745.

48. **Luo, Y., and J. D. Helmann.** 2012. Analysis of the role of *Bacillus subtilis* σ^M in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. Mol Micro **83:**623-639.

49. **Luo, Y., and J. D. Helmann.** 2009. Extracytoplasmic function σ factors with overlapping promoter specificity regulate sublancin production in *Bacillus subtilis*. J Bacteriol **191:**4951-4958.

50. **M.Willey, J., and W. A. v. d. Donk.** 2007. Lantibiotics: Peptides of Diverse Structure and Function. Annu Rev Microbiol **61:**477–501.

51. **Mantovani, H. C., and J. B. Russell.** 2001. Nisin resistance of *Streptococcus bovis*. Appl Environ Microbiol **67:**808-813.

52. **Martínez, B., A. L. Zomer, A. Rodríguez, J. Kok, and O. P. Kuipers.** 2007. Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. Mol Micro **64:**473–486.

53. **Mascher, T., A. B. Hachmann, and J. D. Helmann.** 2007. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. J Bacteriol **189:**6919-6927.

54. **Mascher, T., N. G. Margulis, T. Wang, R. W. Ye, and J. D. Helmann.** 2003. Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. Mol Microbiol **50:**1591-1604.

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

56. **McBride, S. M., and A. L. Sonenshein.** 2011. The *dlt* operon confers

resistance to cationic antimicrobial peptides in *Clostridium difficile*. Microbiology **157:**1457-1465.

57. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
58. **Nam, S.-E., A. C. Kim, and M. Paetzel.** 2012. Crystal structure of *Bacillus subtilis* signal peptide peptidase A. J Mol Biol **419:**347-358.

59. **Neuhaus, F. C., and J. Baddiley.** 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in Gram-positive bacteria. Microbiol Mol biol R **67:**686–723.

60. **O'Keeffe, T., C. Hill, and R. P. Ross.** 1999. Characterization and heterologous expression of the genes encoding enterocin A production, immunity, and regulation in *Enterococcus faecium DPC1146*. Appl Environ Microbiol **65:**1506–1515.

61. **Parisot, J., S. Carey, E. Breukink, W. C. Chan, A. Narbad, and B. Bonev.** 2008. Molecular mechanism of target recognition by subtilin, a class I lanthionine antibiotic. Antimicrob Agents Ch **52:**612–618.

62. **Perego, M., P. Glaser, A. Minutello, M. A. Strauch, K. Leopold, and W. Fischer.** 1995. Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. Identification of genes and regulation. J Biol Chem **270:**15598-15606.

63. **Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Gotz.** 1999. Inactivation of the *dlt* Operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem **274:**8405-8410.

64. **Peschel, A., and H.-G. Sahl.** 2006. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol **4:**529-536.

65. **Pietiäinen, M., M. Gardemeister, M. Mecklin, S. Leskela, M. Sarvas, and V. P. Kontinen.** 2005. Cationic antimicrobial peptides elicit a complex stress response in *Bacillus subtilis* that involves ECF-type sigma factors and two-component signal transduction systems. Microbiol **151:**1577–1592.

66. **Potempa, J., and R. N. Pike.** 2009. Corruption of innate immunity by bacterial proteases. J Innate Immun **1:**70-87.

67. **Quisel, J. D., W. F. Burkholder, and A. D. Grossman.** 2001. *In vivo* effects of sporulation kinases on mutant Spo0A proteins in *Bacillus subtilis*. J Bacteriol **183:**6573-6578.

68. **Reichmann, N. T., C. P. Cassona, and A. Gründling.** 2013. Revised mechanism of D-alanine incorporation into cell wall polymers in Gram-positive bacteria. Microbiol**:**mic.0.069898-0.

69. **Reichmann, N. T., and A. Gründling.** 2011. Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. FEMS Microbiol Lett **319:**97–105.

70. **Rodionov, D. A., X. Li, I. A. Rodionova, C. Yang, L. Sorci, E. Dervyn, D. Martynowski, H. Zhang, M. S. Gelfand, and A. L. Osterman.** 2008. Transcriptional regulation of NAD metabolism in bacteria: genomic reconstruction of NiaR (YrxA) regulon. Nuc Acids Res **36:**2032-2046.

71. **Saar-Dover, R., A. Bitler, R. Nezer, L. Shmuel-Galia, A. Firon, E. Shimoni, P. Trieu-Cuot, and Y. Shai.** 2012. D-alanylation of lipoteichoic acids confers resistance to cationic peptides in group B *Streptococcus* by increasing the cell wall density. Plos Pathog **8:**e1002891.

72. **Sahl, H.-G., U. Pag, S. Bonness, S. Wagner, N. Antcheva, and A. Tossi.** 2005. Mammalian defensins: structures and mechanism of antibiotic activity. J Leukocyte Biol **77:**466-475.

73. **Schirner, K., J. Marles-Wright, R. J. Lewis, and J. Errington.** 2009. Distinct and essential morphogenic functions for wall- and lipo-teichoic acids in *Bacillus subtilis*. EMBO J **28:**830–842.

74. **Schneider, T., and H.-G. Sahl.** 2010. An oldie but a goodie – cell wall biosynthesis as antibiotic target pathway. Int J Med Microbiol **300:**161–169.

75. **Scholz, R., K. J. Molohon, J. Nachtigall, J. Vater, A. L. Markley, R. D. Süssmuth, D. A. Mitchell, and R. Borriss.** 2011. Plantazolicin, a novel microcin B17/streptolysin S-like natural product from *Bacillus amyloliquefaciens* FZB42. J Bacteriol **193:**215-224.

76. **Shi, Y., X. Yang, N. Garg, and W. A. van der Donk.** 2011. Production of lantipeptides in *Escherichia coli*. J Am Chem Soc **133:**2338–2341.

77. **Sieprawska-Lupa, M., P. Mydel, K. Krawczyk, K. Wójcik, M. Puklo, B. Lupa, P. Suder, J. Silberring, M. Reed, J. Pohl, W. Shafer, F. McAleese, T. Foster, J. Travis, and J. Potempa.** 2004. Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. Antimicrob Agents Ch **48:**4673-4679.

78. **Smaldone, G. T., O. Revelles, A. Gaballa, U. Sauer, H. Antelmann, and J.**

D. Helmann. 2012. A global investigation of the *Bacillus subtilis* iron-sparing response identifies major changes in metabolism. J Bacteriol **194:**2594-2605.

79. **Staron, A., D. E. Finkeisen, and T. Mascher.** 2011. Peptide antibiotic sensing and detoxification modules of *Bacillus subtilis*. Antimicrob Agents Ch **55:**515–525.

80. **Stein, T., S. Heinzmann, I. Solovieva, and K.-D. Entian.** 2003. Function of *Lactococcus lactis* nisin immunity genes *nisI* and *nisFEG* after coordinated expression in the surrogate host *Bacillus subtilis*. J Biol Chem **278:**89-94.

81. **Steinmetz, M., and R. Richter.** 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through *in vivo* recombination. Gene **142:**79-83.

82. **Sun, Z., J. Zhong, X. Liang, J. Liu, X. Chen, and L. Huan.** 2009. Novel mechanism for nisin resistance via proteolytic degradation of nisin by the nisin resistance protein NSR. Antimicrob Agents Ch **53:**1964-1973.

83. **Swoboda, J. G., J. Campbell, T. C. Meredith, and S. Walker.** 2010. Wall teichoic acid function, biosynthesis, and inhibition. ChemBioChem **11:**35-45.

84. **Vrancken, K., L. V. Mellaert, and J. Anné.** 2008. Characterization of the *Streptomyces lividans* PspA response. J Bacteriol **190:**3475-3481.

85. **Weidenmaier, C., and A. Peschel.** 2008. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat Rev Microbiol **6:**279-287.

86. **Wenzel, M., B. Kohl, D. Münch, N. Raatschen, H. B. Albada, L. Hamoen, N. Metzler-Nolte, H.-G. Sahl, and J. E. Bandow.** 2012. Proteomic response of *Bacillus subtilis* to lantibiotics reflects differences in interaction with the cytoplasmic membrane. Antimicrob Agents Ch **51:**5749-5757.

87. **Wolf, D., F. Kalamorz, T. Wecke, A. Juszczak, U. Mäder, G. Homuth, S. Jordan, J. Kirstein, M. Hoppert, B. Voigt, M. Hecker, and T. Mascher.** 2010. In-depth profiling of the LiaR response of *Bacillus subtilis*. J Bacteriol **198:**4680-4693.

88. **Wörmann, M. E., R. M. Corrigan, P. J. Simpson, S. J. Matthews, and A. Gründling.** 2011. Enzymatic activities and functional interdependencies of *Bacillus subtilis* lipoteichoic acid synthesis enzymes. Mol Micro **79:**566-583.

89. **Wright, G. D.** 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. Nat Rev Microbiol **5:**175-186.

90. **Yamaguchi, S., E. Gueguen, N. K. Horstman, and A. J. Darwin.** 2010. Membrane association of PspA depends on activation of the phage-shock-protein response in *Yersinia enterocolitica*. Mol Micro **78:**429-443.

91. **Zeigler, D. R.** 2011. The genome sequence of *Bacillus subtilis subsp. spizizenii* W23: insights into speciation within the *B. subtilis* complex and into the history of *B. subtilis* genetics. Microbiol **157:**2033–2041.

92. **Zweers, J. C., P. Nicolas, T. Wiegert, J. M. v. D. mail, and E. L. Denham.** 2012. Definition of the σ^W regulon of *Bacillus subtilis* in the absence of stress. PLOS One **7:**e48471.

CHAPTER 4

CONCLUSIONS AND PERSPECTIVES

This work identified and characterized several physiologically relevant CESR pathways in *Bacillus subtilis*. These pathways all contribute to, or are indirectly related to, ECF σ factor-mediated responses to membrane stress which serves as a unifying theme that binds this thesis together.

In Chapter 2, I investigated P_5, an internal σ^W-dependent promoter within *fabHa* and upstream of *fabF* that could influence membrane fluidity. This promoter was able to employ an unusual regulatory mechanism whereby its activation could simultaneously reduce the expression of the gene it was within while increasing downstream gene expression. Although this unique method of regulation was intriguing, I chose to study the physiological function of the promoter instead. Since this promoter affected the expression of genes involved in fatty acid biosynthesis, I hypothesized that it was a membrane stress adaptation and focused my research in that direction. My hypothesis proved to be correct, and I was eventually able to show that P_5 reduces membrane fluidity with fluorescence anisotropy. Our collaborators were able to further show that this reduction in membrane fluidity is associated with specific changes in membrane composition using FAME and ESI-MS. Previous studies in the literature were extremely helpful as they provided sufficient information on the functions and characteristics of FabHa and FabF to explain how their regulation could affect membrane fluidity (1, 5, 6). I finally showed that P_5 contributed to resistance against detergents and competing *Bacilli* to confirm that this membrane

stress adaptation was physiologically relevant.

The goal of Chapter 3 was to uncover all of the ECF σ factor mediated resistance mechanisms employed by *B. subtilis* to protect the cell against lantibiotics. To accomplish this goal, I used the prototypical lantibiotic nisin which binds lipid II and forms pores in the phospholipid bilayer. I discovered that the phage shock proteins, which had been thought to only function in Gram negative bacteria, resist lantibiotic pore forming activity in the Gram positive *B. subtilis (4)*. I also showed that the membrane bound SppA (signal peptide peptidase) protects against lantibiotics that integrate into the membrane and proposed a novel hypothesis that SppA is directly degrading lantibiotics that it comes in contact with. *yceGHI* was discussed as well, and although I demonstrated that this operon prevents nisin from binding lipid II and may encode NAD-dependent enzymes, I was unable to determine a specific resistance mechanism. Finally, I characterized the roles of teichoic acids in preventing lantibiotics from reaching the cell membrane. This study had a very broad focus and included components of the lantibiotic resistome that were not directly related to ECF σ factors, such as the liaRS TCS and the primary LTA synthase LtaS. It was also necessary to acknowledge the fact that lantibiotics inhibit cell wall synthesis by binding to lipid II, and some of the resistance mechanisms protect against this type of stress as well.

Several strategies were employed to emphasize the importance of my findings in Chapter 3. I used the knowledge obtained from this study to generate a nisin-resistant *B. subtilis* strain. Although the resistance of this strain was not particularly dramatic, it could potentially be further modified to be used for industrial nisin

production. Furthermore, I showed that these nisin resistance mechanisms contributed to resistance against other lantibiotics (gallidermin & mersacidin) and to competing bacteria commonly found in the soil. The chapter also contains numerous references that connect the resistance mechanisms I characterized to those in known pathogens. For example, *B. subtilis* YceH is a homolog of TelA *Listeria monocytogenes* which contributes to innate nisin resistance as well (2).

Interestingly chapters 2 and 3 represent two distinct strategies for studying CESR. Chapter 2 analyzes a single resistance mechanism in extensive detail. It uses a wide range of assays, from basic experiments like western blots to specialized techniques like fluorescence anisotropy, to fully understand the P_5-mediated CESR. On the other hand, Chapter 3 looks at a single stress and characterizes all of the resistance mechanisms that confer resistance to that stress. Since multiple mechanisms are analyzed simultaneously, they individually receive less attention than the P_5 mechanism in Chapter 2. Despite these differences, both chapters make significant contributions to the field. For future studies, I would like to investigate several of the lantibiotic resistance mechanisms in more detail. My hypothesis that SppA inactivates lantibiotics through direct interaction is quite intriguing and should be confirmed with additional experiments. In addition, it would be worthwhile to further analyze the resistance mechanism associated with YceGHI because so little is known about it.

Appendices A, B, and C further supplement this thesis by summarizing several minor ECF σ factor-mediated mechanisms that affect the cell membrane. Appendix A confirms that some of the NfeD and SPFH protein homologs in *B. subtilis*, which have previously been shown to contribute to lipid raft formation (3), can influence

membrane fluidity. Appendix B proposes the hypothesis that the phospholipase YtpA resists daptomycin by reducing PG levels and begins to verify this hypothesis with some preliminary findings. The evidence presented in Appendix C indicates that inhibiting sesquarterpene biosynthesis accumulates a UPP mimic that increases bacitracin sensitivity while overproducing this C_{35} terpene destabilizes membranes. Both Appendix B and Appendix C represent the beginnings of a promising future project that could uncover multiple novel resistance mechanisms. In particular, the hypothesis that ytpB deletion causes the buildup of an undecaprenyl pyrophosphate mimic that interferes with bacitracin resistance mechanisms is quite intriguing and should be investigated in more detail.

Chapter 2, Chapter 3, and the appendices comprise the bulk of my work at Cornell University. They have shown that specific genes in the ECF σ factor regulons confer resistance to membrane stresses through novel resistance mechanisms that modify the cell envelope. Overall, these findings have contributed to our knowledge of CESR in *B. subtilis* and may help in the development of membrane targeting antibiotics.

REFERENCES

1. **Choi, K. H., R. J. Heath, and C. O. Rock.** 2000. β-ketyacyl-acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. J Bacteriol **182:**365-370.

2. **Collins, B., S. Joyce, C. Hill, P. D. Cotter, and R. P. Ross.** 2010. TelA contributes to the innate resistance of *Listeria monocytogenes* to nisin and other cell wall-acting antibiotics. Antimicrob Agents Ch **54:**4658–4663.

3. **Donovan, C., and M. Bramkamp.** 2009. Characterization and subcellular localization of a bacterial flotillin homologue. Microbiology **155:**1786-1799.

4. **Huvet, M., T. Toni, X. Sheng, T. Thorne, G. Jovanovic, C. Engl, M. Buck, J. W. Pinney, and M. P. H. Stumpf.** 2011. The evolution of the phage shock protein response system: interplay between protein function, genomic organization, and system function. Mol Biol Evol **28:**1141-1155.

5. **Mendoza, D. d., P. Aguilar, and G. Schujman.** 2001. Biosynthesis and function of membrane lipids, p. 43-55. *In* J. A. Hoch, A. L. Sonenshein, and R. Losick (ed.), *Bacillus subtilis* and Its Closest Relatives: From Genes to Cells. American Society for Microbiology, Washington D.C.

6. **Schujman, G. E., S. Altabe, and D. de Mendoza.** 2008. A malonyl-CoA-dependent switch in the bacterial response to a dysfunction of lipid metabolism. Mol Microbiol **68:**987-996.

APPENDIX A

THE *yuaFGI* OPERON REDUCES MEMBRANE FLUIDITY WHEN ACTIVATED BY σ^W [1]

A.1 Summary

In *Bacillus subtilis*, a survey of σ^W-controlled genes for effects on CEF resistance identified both the NfeD protein YuaF and the flotillin homologue YuaG (FloT). The flotillin has been previously shown to localize to defined lipid microdomains, and we show here that the *yuaFGI* operon contributes to a σ^W-dependent decrease in membrane fluidity.

[1]The results of this experiment were published in Lee YH, Kingston AW, and Helmann JD. Molecular Microbiology. 2011 Jul;81(1):69-79. The experiment described here was performed by A.W.K. Y.H.L, A.W.K, and J.D.H wrote the original manuscript.

A.2 Introduction

Cefuroxime (CEF) belongs to the group of broad-spectrum β-lactam cephalosporin antibiotics which are reviewed in section 1.2.1. The extracytoplasmic function (ECF) sigma (σ) factor σ^W of *B. subtilis* has been shown to confer resistance against CEF (14). Of the ~60 genes in the σ^W regulon, we identified the *yuaFGI* operon as playing a pivotal role in CEF resistance (14). This operon encodes both the NfeD protein YuaF and the flotillin YuaG (FloT) which are reviewed in section 1.1.1. Here, we show that σ^W dependent activation of the *yuaFGI* operon reduces membrane fluidity in tandem with the P_5 promoter (Chapter 2). This reduction in membrane fluidity may directly or

indirectly increase CEF resistance.

A.3 Materials and methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table A.1. Cells were routinely cultured in Luria-Bertani (LB) broth at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bacto agar (Difco). Difco sporulation medium (DSM) agar was used for spore formation and maintenance of *B. subtilis* strains. The following antibiotics were used when appropriate: Kanamycin (Kan) (15 μg/ml), chloramphenicol (Cat) (10 μg/ml), or macrolide-lincosamide-streptogramin B (MLS) (contains 1 μg/ml erythromycin and 25 μg/ml lincomycin).

Fluorescence anisotropy. Fluorescence anisotropy analysis of *B. subtilis* strains treated with 1,6-diphenyl-1,3,5-hexatriene (DPH) was performed as described previously (26) with slight modifications. Strains were grown to mid-log phase (OD_{600} of 0.4 ± 0.01) in LB supplemented with 2% xylose. A 0.5-mL sample of each culture was then washed once and suspended in 2 mL of phosphate buffer (100 mM, pH 7.0) containing 5 μM DPH. After a 30-min incubation at room temperature, fluorescence anisotropy measurements (λ_{ex} = 358 nm, slit width = 10 nm; λ_{em} = 428 nm, slit width = 15 nm) were taken with a Perkin-Elmer LS55 luminescence spectrometer. The correction for the fluorescence intensity of nonlabeled cells was calculated as described by Kuhry et al. (13).

Table A.1: Bacterial strains used in Appendix A.[a]

Strain	Genotype or description	Source or reference[a]
W168	*trpC2*	BGSC 1A1
HB6156	CU1065 *yuaFGI*::*kan*	(4)
HB13159	W168 *yuaFGI*::*kan*	HB6156 chr DNA → W168
HB5331	CU1065 *yqeZ-yqfAB*::*kan*	(4)
HB13566	W168 *yqeZ-yqfAB*::*kan*	HB5331 chr DNA → W168
HB13042	W168 *amyE*::P_{xyl}-*sigW* (*cat*)	(11)
HB13122	W168 P_{fabHAF} *fabHA*(P5*)-*fabF amyE*::P_{xyl}-*sigW* (*cat*)	(11)
HB13160	W168 *yuaFGI*::*kan amyE*::P_{xyl}-*sigW* (*cat*)	HB6156 chr DNA → HB13042
HB13226	W168 P_{fabHAF} *fabHA*(P5*) *fabF yuaFGI*::*kan amyE*::P_{xyl}-*sigW* (*cat*)	HB6156 chr DNA → HB13122
HB13236	W168 *yqeZ-yqfAB*::*kan amyE*::P_{xyl}-*sigW*(*cat*)	HB13566 chr DNA → HB13042

[a] Abbreviations used: → indicates transformation; chrDNA indicates chromosomal DNA.

Statistical analysis. All experiments were performed with a minimum of three biological replicates. Unless otherwise noted, data is presented as mean ± standard error. Statistical evaluation of the data was performed by the use of unpaired Student's *t* tests. A value of $P \leq 0.05$ was considered statistically significant.

A.3 Results and discussion

The *yuaFGI* operon reduces membrane fluidity under σ^W-inducing conditions. To better understand how *yuaFGI* and *yqeZ-yqfAB* contribute to intrinsic CEF resistance, we investigated the influence of these genes on membrane fluidity. Both of these operons encode a NfeD protein (YuaF and YqeZ) and a putative flotillin-like protein, (YuaG and YqfA) that are believed to organize the cell membrane into functional microdomains (2, 15). In addition, σ^W overexpression has previously been shown to reduce membrane fluidity by altering expression of fatty acid biosynthesis genes (11). The σ^W-dependent activation of a promoter (P_5) within the *fabHAF* operon leads to an increase in the proportion of straight-chain fatty acids and an increase in overall chain length. Since activation of P_5 accounts for some, but not all, of the σ^W-dependent decrease in membrane fluidity (11), we reasoned that upregulation of *yuaFGI* and/or *yqeZ-yqfAB* might alter membrane fluidity.

Membrane fluidity was assessed by measuring the fluorescence anisotropy of *B. subtilis* cells labeled with DPH (Figure A.1). Under normal growth conditions, both wild-type and *yuaFGI* knockout cells exhibited similar anisotropy levels. However, when *sigW* was overexpressed with a xylose-inducible promoter (P_{xyl}-*sigW*), the

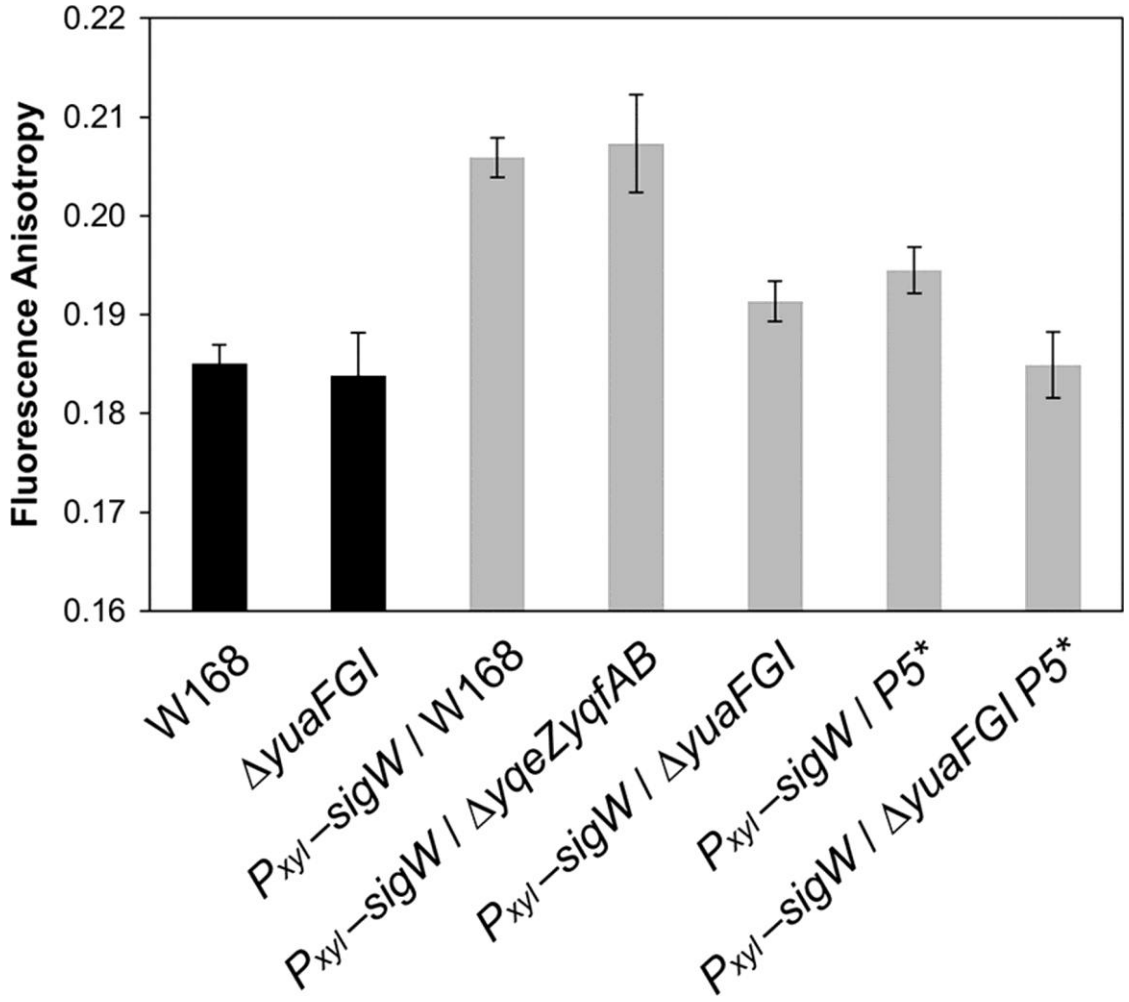


Figure 1.A: Inactivation of *yuaFGI* prevents the decrease in membrane fluidity induced by overexpression of σ^W. Cells were grown in LB medium with xylose (2%) to an OD_{600} of 0.4 and then incubated in phosphate buffer (100 mM, pH 7.0) with DPH (5 μM) at 25°C for 30 min. In strains containing the P_{xyl}-*sigW* construct, σ^W was expressed under the control of a xylose-inducible promoter. The membrane fluidity of each strain was determined via fluorescence anisotropy measurements. Data are presented as the average of at least three trials, and the standard error is indicated by error bars.

resulting increase in anisotropy was significantly lower in the *yuaFGI* knockout strain than in control cells. Since a higher anisotropy is indicative of a less-fluid membrane, these results indicate that expression of the *yuaFGI* operon reduces membrane fluidity when activated by σ^W. In contrast, deleting *yqeZ-yqfAB* had no effect on anisotropy levels, even under *sigW* overexpression conditions. The effect of *yuaFGI* on membrane fluidity is comparable to that of the σ^W-dependent promoter (P_5) within the *fabHAF* operon (11). In a σ^W overexpression strain both lacking *yuaFGI* and containing a mutation (P_5*) that abolishes P_5 activity (P_{xyl}-*sigW yuaFGI* P_5*), anisotropy levels were the same as in wild-type cells. This demonstrates that both P_5 and *yuaFGI* function to reduce membrane fluidity and that they are the primary components of the σ^W regulon to do so.

The effect of *yuaFGI* on membrane fluidity might explain how this operon contributes to CEF resistance. Adjustments in membrane fluidity can influence numerous properties of the lipid bilayer, such as permeability, protein mobility, and protein-protein interactions (16). However, not all changes in membrane fluidity result in CEF resistance, since the P_5-inactive strain was not any more susceptible to CEF than the wild-type strain (data not shown). YuaG (FloT) has also been linked to the formation of lipid domains, which have been shown to regulate sporulation, biofilm formation, and other signal transduction pathways (6, 15).

A.4 Conclusion

We show that expression of the *yuaFGI* operon reduces membrane fluidity under σ^W-inducing conditions and that this protein-based mechanism is additive with a

previously described lipid-based pathway (11). These findings suggest that YuaFG influences CEF resistance by altering the physical properties of the membrane, but the origins of this effect are presently unclear. YuaFG are thought to help organize membrane microdomains (6, 15), and this could affect the assembly or activity of cell wall biosynthetic complexes known to be targeted by CEF.

APPENDIX B

THE σ^M-INDUCED PHOSPHOLIPASE YtpA MAY RESIST DAPTOMYCIN BY REDUCING MEMBRANE PHOSPHATIDYLGLYCEROL LEVELS

B.1 Summary

Preliminary evidence indicates that the phospholipase YtpA resists daptomycin by reducing phosphatidylglycerol levels in the cell membrane.

B.2 Introduction

In *B. subtilis*, the σ^M-dependent gene *ytpA* encodes a phospholipase that hydrolyzes fatty acids from the two position of phosphatidylglycerol (PG) (27). This function suggests that YtpA may play a role in phospholipid turnover and could specifically be activated to reduce the PG content of the plasma membrane. Daptomycin is a lipopeptide antibiotic (section 1.2.2) that interacts with PG, and previous studies have shown that a reduction in PG levels could lead to increased daptomycin resistance (8). Here, we show that *ytpA* makes a minor contribution to daptomycin resistance and may be able to reduce PG levels.

B.3 Materials and methods

Strains, plasmids, and growth conditions. All *B. subtilis* strains used in this study are listed Table B.1. Bacteria were grown in liquid Luria-Bertani (LB) medium at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bacto agar (Difco) with appropriate selection. Plasmids were amplified in *Escherichia coli* DH5α

Table B.1: Bacterial strains used in Appendix B.[a]

Strains	Genotype or description	Source or reference
168	*trpC2*	Lab stock
CU1065	trpC2 attspβ	Lab Stock
HB0031	CU1065 *sigM::kan*	(5)
HB13129	168 *ytpA::MLS*	LFH PCR → 168
HB13130	168 *amyE::*P_{xyl}*-ytpA (cat)*	pTK048 → 168
HB13185	168 *sigM::kan*	HB0031 chrDNA → 168
HB13188	168 *amyE::ytpA (cat)*	pTK074 → 168
HB13189	168 *ytpA::MLS amyE::ytpA (cat)*	HB13188 chrDNA → HB13129
HB13209	168 *ytpa::MLS sigM::kan*	HB 0031 chrDNA → HB13129

[a] Abbreviations used: → indicates transformation; chrDNA indicates chromosomal DNA.

before transformation of *B. subtilis* strains. Ampicillin (amp; 100 μg ml^{-1}) was used to select *E. coli* transformants. For *B. subtilis*, antibiotics used for selection were: Kanamycin (kan; 15 μg ml^{-1}), chloramphenicol (cat; 10 μg ml^{-1}), and macrolide-lincosoamide-streptogramin B (MLS; contains 1 μg ml^{-1} erythromycin and 25 μg ml^{-1} lincomycin).

Genetic techniques. Chromosomal and plasmid DNA transformations were performed as described previously (9). Unless otherwise stated, all PCR products were generated using W168 chromosomal DNA as a template and all strains were verified by sequence analysis (Cornell University Life Sciences Core Laboratories Center).

Disk diffusion assays. Disk diffusion assays were performed as described (18). Briefly, strains were grown to an OD_{600} of 0.4. A 100 μl aliquot of these cultures was mixed with 4 ml of 0.7% LB soft agar (kept at 50°C and supplemented with 50 g/L Ca^{2+}) and directly poured onto LB plates (containing 15 ml of 1.5% LB agar and supplemented with 50 g/L Ca^{2+}). The plates were dried for 20 min in a laminar airflow hood. Filter paper disks containing 100 μg, daptomycin were placed on the top of the agar and the plates were incubated at 37°C overnight. The distances between the edge of the inhibition zones and the edge of the filter paper disks were measured.

Lipid extraction and thin-layer chromatography. Protocol adapted from (23). Ten-mL aliquots cultures treated with 1 μg/mL daptomycin at OD_{600} ~0.1 for 1 hour were centrifuged for 10 min at 4,500 × *g*. The cell pellet was resuspended in 100 μL ddH_2O with the addition of perchloric acid to a final concentration of 1 M. The cell suspension was then incubated at 0 °C for 30 min, after which lipids were extracted by the addition of 1 mL methanol-chloroform-water (12:6:2 [vol/vol]) 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°C and then centrifuged for 5 min at 720 × *g* at 4 °C. The organic phase was then removed and dried under nitrogen. The lipids were resuspended in 20 μL of chloroform-methanol (2:1 [vol/vol]), spotted to silica gel 60 plates (VWR), and separated using the solvent mixture chloroform-methanol-water (65:25:4 [vol/vol]). Phospholipids were detected using molybdenum blue spray reagent (Sigma-Aldrich). PG and PE standards were obtained from Sigma-Aldrich. Densitometry analysis with ImageJ was used to compare the relative levels of PE and PG in a cell culture. This assay was performed 3 separate times with biological replicates.

Statistical analysis. All experiments were performed with a minimum of three biological replicates. Unless otherwise noted, data is presented as mean ± standard error. Statistical evaluation of the data was performed by the use of unpaired Student's *t* tests. A value of $P \leq 0.05$ was considered statistically significant.

B.3 Results and discussion

We became interested in *ytpA* because it is known to be activated by σ^M (7), and its PG hydrolysis activity (27) had the potential to affect the cell membrane. Previous studies have shown a connection between decreasing PG levels and increased daptomycin resistance (8), so we tested the sensitivity of a *ytpA* knockout strain to daptomycin using a disk diffusion assay (Figure B.1). Deleting *ytpA* significantly increased daptomycin sensitivity, introducing an ectopic copy of this gene into the Δ*ytpA* background restored daptomycin sensitivity to WT levels, and overexpressing

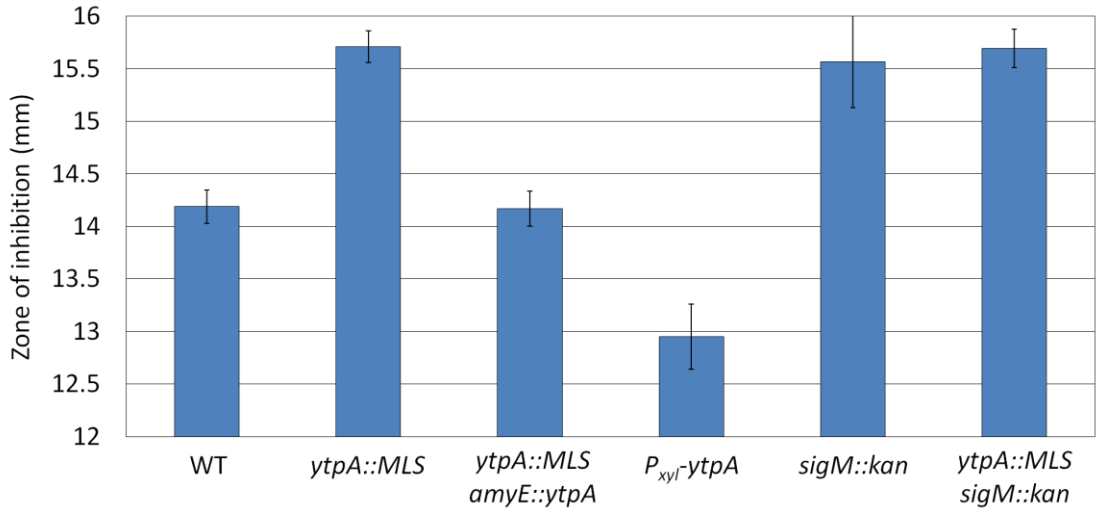


Figure B.1: *ytpA* contributes to daptomycin resistance. Daptomycin disk diffusion assays of the WT (168), *ytpA::MLS* (HBHB13129), *ytpA::MLS amyE::ytpA* (HB13189), P_{xyl}*-ytpA* (HB13130), *sigM::kan* (HB13185), and *ytpA::MLS sigM::kan* (HB13209) strains. When compared to WT, daptomycin sensitivity was significantly higher in the ytpA::MLS strain and significantly lower in the P_{xyl}*-ytpA* strain. There was no significant difference between the *ytpA::MLS, sigM::kan,* and *ytpA::MLS sigM::kan* strains.

ytpA with a xylose inducible promoter increased daptomycin resistance. These results, when taken together, confirm that *ytpA* contributes to daptomycin resistance. We also showed that a Δ*sigM* strain exhibited daptomycin sensitivity comparable to that of the Δ*ytpA* strain, but a double mutant lacking both Δ*sigM* and Δ*ytpA* was not more sensitive than either single gene deletion strain. Thus, σ^M primarily resists daptomycin by activating *ytpA* expression.

To determine whether or not YtpA is capable of significantly altering PG levels in the cell membrane, we performed thin layer chromatography analysis on lipids extracted from daptomycin treated WT and Δ*ytpA* cells (Figure B.2). TLC is not as quantitative as assays like ESI-MS, but this assay allows for the comparison of relative PE and PG levels in a group of cells. Through densitometry analysis, we determined that the PG/PE ratio was higher in the ΔytpA strain, but this difference only trended towards significance.

B.4 Conclusion

Taken together, the results from this study suggest that YtpA resists daptomycin by reducing PG content in the membrane under daptomycin stress conditions. However, additional assays are required definitively confirm this hypothesis.

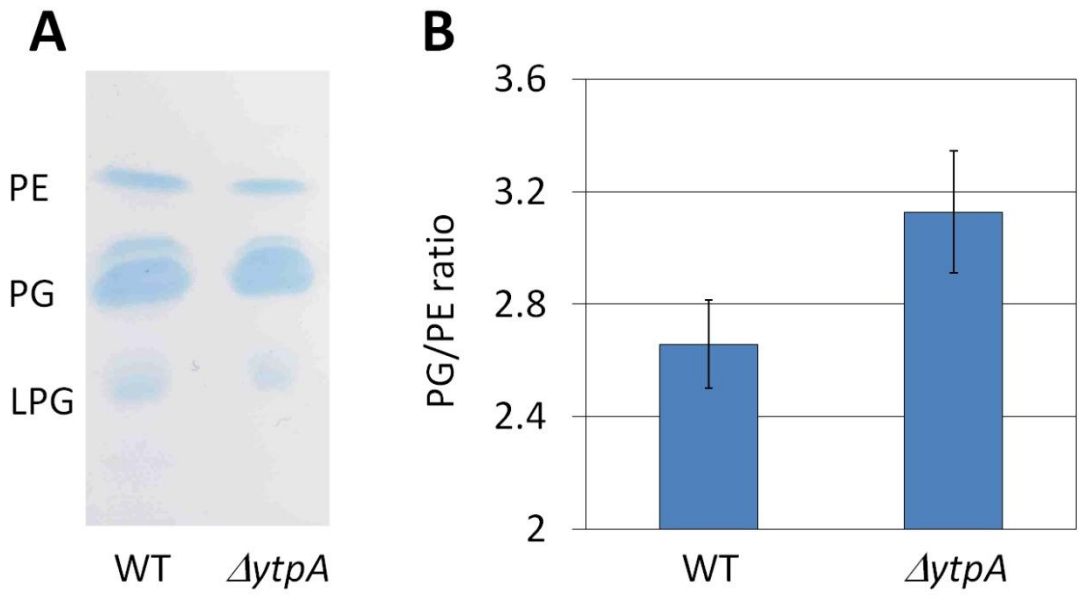


Figure B.2: TLC analysis of WT and *ΔytpA* membranes. Membrane lipids were extracted from exponential cultures of WT or the *ΔytpA* (HB13129) mutant grown in LB. Daptomycin (1 µg/mL) was added for one hour prior to extraction. Extracted lipids were spotted to silica TLC plates and detected with molybdenum blue (Sigma). Bands indicating PE and PG were identified by comparison with standards. A. Representative image of TLC plate. B. PG/PE ratio of the two strains as determined by comparing the relative densities of the two bands with ImageJ.

APPENDIX C

BACITRACIN AND MEMBRANE STRESS RESISTANCE MECHANISMS ASSOCIATED WITH *ytpB*

C.1 Summary

The σ^M regulated *ytpB* encodes a tetraprenyl-β-curcumene synthase involved in the production of sesquarterpene. Deleting this gene increases bacitracin resistance, possibly due to the buildup of an undecaprenyl pyrophosphate mimic. Overexpressing *ytpB* increases detergent sensitivity which could be caused by the over-production of an unconventional membrane lipid.

C.2 Introduction

B. subtilis is known to make long polycyclic carbons that are derived from squalene (12). These squalene derivatives have been implicated in sporulation and oxidative stress resistance. The compounds (which are also known as sporulenes, terpenes, and isoprenoids, among other names) are believed to integrate into the cell membrane and affect its organization/fluidity (10). Sesquarterpene is one such C_{35} terpene produced by *B. subtilis* (Figure C.1) (24). We were particularly interested in this compound because the tetraprenyl-β-curcumene synthase YtpB required for its synthesis is regulated by σ^M (7). Here, I show that deleting the gene encoding this enzyme increases bacitracin sensitivity while overexpressing it increases detergent sensitivity and propose hypotheses to explain these phenotypes.

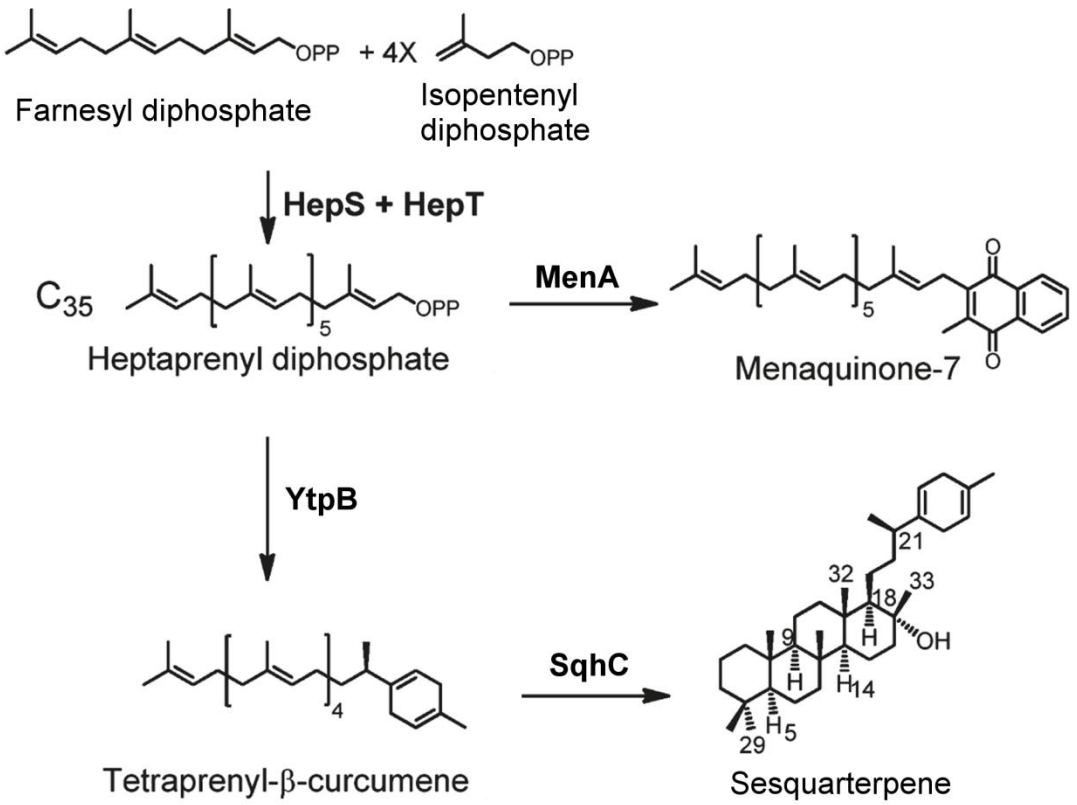


Figure C.1: Proposed pathway for the biosynthesis of sesquarterpenes in *B. subtilis*. Adapted from (24).

C.3 Materials and methods

Strains, plasmids, and growth conditions. All *B. subtilis* strains used in this study are listed Table C.1. Bacteria were grown in liquid Luria-Bertani (LB) medium at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bacto agar (Difco) with appropriate selection. Plasmids were amplified in *Escherichia coli* DH5α before transformation of *B. subtilis* strains. Ampicillin (amp; 100 μg ml^{-1}) was used to select *E. coli* transformants. For *B. subtilis*, antibiotics used for selection were: Chloramphenicol (cat; 10 μg ml^{-1}) and macrolide-lincosoamide-streptogramin B (MLS; contains 1 μg ml^{-1} erythromycin and 25 μg ml^{-1} lincomycin).

Genetic techniques. Chromosomal and plasmid DNA transformations were performed as described previously (9). Unless otherwise stated, all PCR products were generated using W168 chromosomal DNA as a template and all strains were verified by sequence analysis (Cornell University Life Sciences Core Laboratories Center).

Disk diffusion assays. Disk diffusion assays were performed as described (18). Briefly, strains were grown to an OD_{600} of 0.4. A 100 μl aliquot of these cultures was mixed with 4 ml of 0.7% LB soft agar (kept at 50°C) and directly poured onto LB plates (containing 15 ml of 1.5% LB agar). The plates were dried for 20 min in a laminar airflow hood. Filter paper disks containing the chemicals to be tested were placed on the top of the agar and the plates were incubated at 37°C overnight. The distances between the edge of the inhibition zones and the edge of the filter paper disks were measured. The following chemicals and quantities were used in the disk diffusion assays: Triton X-100 10 μl of a 25% solution, N-lauryl-sarcosine 10 uL of a

Table C.1: Bacterial strains used in Appendix C.[a]

Strains	Genotype or description	Source or reference
168	*trpC2*	Lab stock
HB13320	168 *amyE::P_{spac}(hy)-ytpB (cat)*	pTK083 → 168
HB13321	168 *ytpB::MLS*	LFH PCR → 168
HB13346	168 *ytpB::MLS amyE::P_{spac}(hy)-ytpB (cat)*	HB13321 chrDNA → HB13320

[a] Abbreviations used: → indicates transformation; chrDNA indicates chromosomal DNA.

10% solution, sodium dodecyl sulfate 500 μg, colistin 100 μg, clofazimine 1 mg, bile salts 1 mg, poly-L-lysine 5 μg, ethylenediaminetetraacetic acid (EDTA) 365 μg, polymyxin B 50 μg, vancomycin 50 μg, daptomycin 100 μg, D-cycloserine 500 μg, cefuroxime 5 μg, novobiocin 50 μg, nisin 20 μg, bacitracin 200 μg, cerulenin 5 μg, moenomycin 5 μg, amitriptyline 200 μg, and dodecyltrimethylammonium bromide (DTAB) 100 μg. For Nisin assays, a 2.5 mg ml^{-1} nisin stock solution was prepared by dissolving a 2.5% nisin mixture balanced with sodium chloride and denatured milk solids (Sigma-Aldrich Co. St. Louis, MO USA) in 0.02 M HCl. For daptomycin assays, the media was supplemented with 1.25 mM $CaCl_2$.

MIC determination. We analyzed the growth of *B. subtilis* strains in bacitracin media using a variation of the broth dilution assay described previously (17). Briefly, strains were grown to an OD_{600} of 0.4, then diluted 1:200 in MH broth. 200 μl of the diluted culture was dispensed in a Bioscreen 100-well microtitre plate. Each strain was grown in Bacitrain concentrations ranging from 0 to 300 μg/mL. Growth was measured spectrophotometrically (OD_{600}) every 15 min for 48 h using a Bioscreen C incubator (Growth Curves USA, Piscataway, NJ) at 37°C with continuous shaking.

Statistical analysis. All experiments were performed with a minimum of three biological replicates. Unless otherwise noted, data is presented as mean ± standard error. Statistical evaluation of the data was performed by the use of unpaired Student's *t* tests. A value of $P \leq 0.05$ was considered statistically significant.

C.4 Results and discussion

Sensitivity phenotypes arising from *ytpB* overexpresion or deletion. *ytpB* initially

attracted our interest as a potential CESR contributor because it is both activated by an ECF σ factor (7), and it encodes a protein that can influence the composition of the cell membrane (24). To determine if *ytpB* plays a role in the CESR of *B. subtilis*, we analyzed the effect of deleting or overexpressing *ytpB* on sensitivity to cell envelope stresses with disk diffusion assays (Figure C.2). We used a variety of stresses, including detergents (triton X-100, bile salts, SDS, amitriptyline, DTAB, N-lauryl-sarcosine, EDTA, and poly-L-lysine) cell wall synthesis inhibitors (moenomycin, vancomycin, D-cycloserine, cefuroxime, and bacitracin), membrane active agents (daptomycin, polymyxin B, colistin, and clofazimine), a fatty acid synthesis inhibitor (cerulenin), and a DNA gyrase inhibitor (novobiocin). The most notable phenotype from this assay was that deleting ytpB resulted in a substantial increase in bacitracin zone of inhibition, but had little effect on sensitivity to other stresses. We also found that overexpressing *ytpB* with an IPTG induicible promoter slightly increased sensitivity to triton X-100, cefuroxime, cerulenin, poly-L-lysine, DTAB, EDTA, and clofazamine.

Bacitracin induced early-onset cell cell death in ΔytpB cells may be caused by the accumulation of a UPP mimic. The bacitracin sensitivity phenotype of the Δ*ytpB* strain is unusual and needed to be analyzed in more detail. WT cells treated with a bacitracin disk exhibit a clear albeit small zone of inhibition surrounding the disk representing complete inhibition of growth or cell death (Figure C.3A). In the Δ*ytpB* strain, the zone of death is only slightly bigger than that of WT cells, but a large halo of diminished cell density can be observed around the disk as well. The edge of this

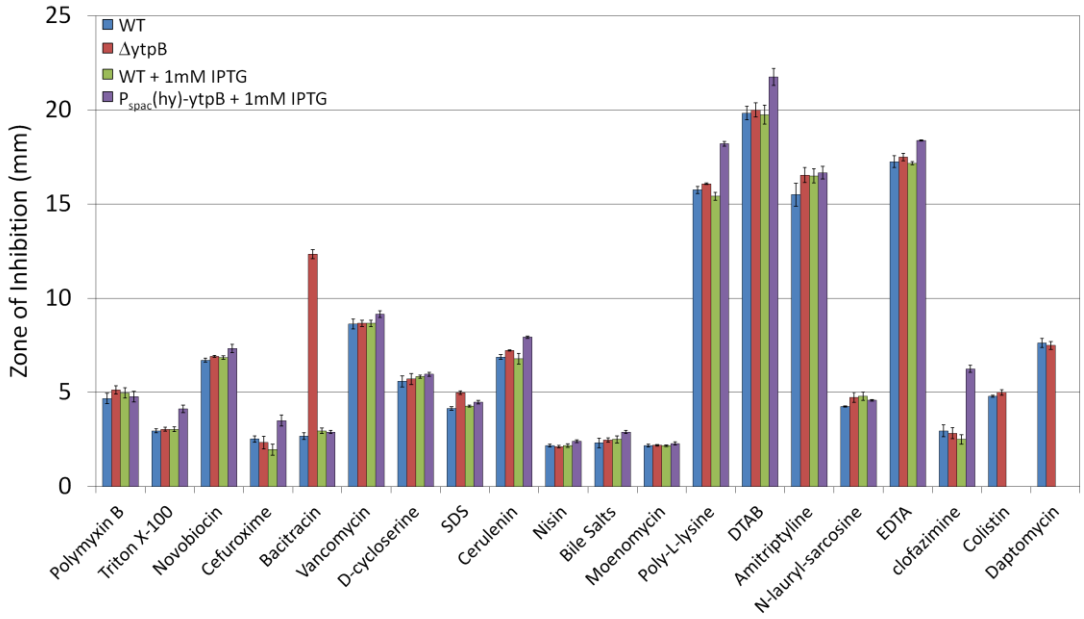
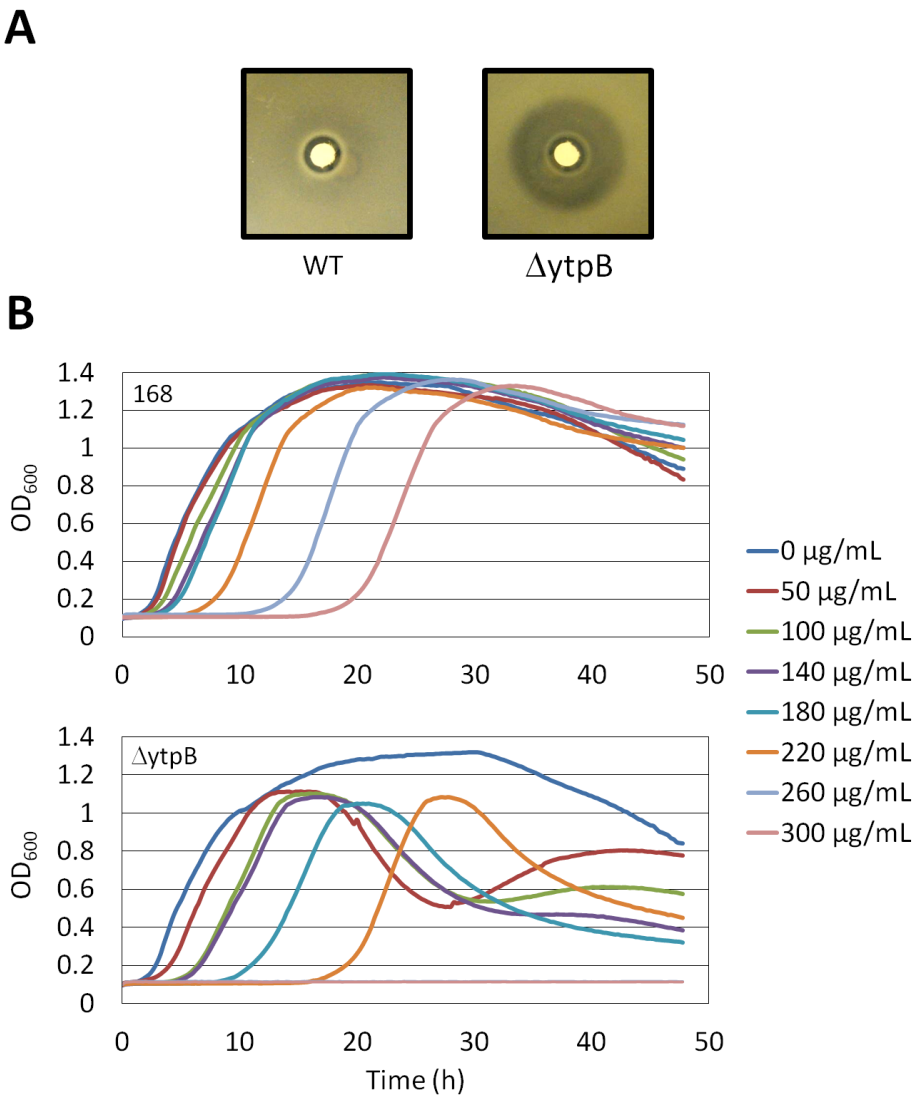


Figure C.2: Disk diffusion assays displaying the sensitivities of WT (168), Δ*ytpB* (HB13321), and *P*$_{spac}$*(hy)-ytpB* (HB13320) strains to various stresses. WT was tested in media with and without 1 mM IPTG, *ΔytpB* was tested in media without IPTG, and *P*$_{spac}$*(hy)-ytpB* was tested in media with 1 mM IPTG. The *ΔytpB* strain exhibited a large sensitivity phenotype to bacitracin, but was not significantly more sensitive to the other stresses tested. Small but significant sensitivity phenotypes were observed for the *P*$_{spac}$*(hy)-ytpB* strain to triton X-100, cefuroxime, cerulenin, poly-L-lysine, DTAB, EDTA, and clofazamine.

Figure C.3: Analyzing the bacitracin sensitivity phenotype exhibited by the Δ*ytpB* strain. A. Representative image of a bacitracin disk diffusion assay with WT and Δ*ytpB* (HB13321) cells. B. Representative growth curve assay of WT and Δ*ytpB* (HB13321) cells in various concentrations of bacitracin. C. Bacitracin sensitivity of the WT, Δ*ytpB* (HB13321), P_{spac}*(hy)-ytpB* (HB13320), and P_{spac}*(hy)-ytpB* Δ*ytpB* (HB13346) strains as measured with a disk diffusion assay. Bacitracin sensitivity in the Δ*ytpB* background reverts to wild type upon the introduction of the P_{spac}*(hy)-ytpB* fusion even in the absence of IPTG induction.


A
WT
ΔytpB
B
168
ΔytpB
OD600
Time (h)
0 µg/mL
50 µg/mL
100 µg/mL
140 µg/mL
180 µg/mL
220 µg/mL
260 µg/mL
300 µg/mL
C
Bacitracin Zone of Inhibition (mm)
No IPTG
1 mM IPTG
WT
ΔytpB
Pspac(hy)-ytpB
Pspac(hy)-ytpB ΔytpB

halo was recorded as the zone of inhibition, but we were not certain as to what the halo represented. We further investigated this phenotype by comparing the growth of WT and Δ*ytpB* cells in bacitracin media over the course of 48 hours (Figure C.3B). We used bacitracin concentrations ranging from 50 μg/mL, which had little effect on WT cells, to 300 μg/mL, which was able to increase the lag phase of WT cells for over 20 hours. Bacitracin had only a minor effect on the lag phase of ΔytpB cells compared to WT cells. The major difference in growth between these two strains occurred shortly after they entered the stationary phase. Under bacitracin stress the WT strain would maintain a fairly constant stationary phase for at least 48 hours, but the *ΔytpB* strain would exhibit only a brief stationary phase followed by cell death. For all bacitracin concentrations tested, the *ΔytpB* cell death phase would reduce the OD_{600} of cells by ~50% over the course of ~10 hours. We reasoned that this early-onset death phase was responsible for the halo of reduced cell density observed in our disk diffusion assays.

We also wanted to make sure that the bacitracin sensitivity phenotype of the Δ*ytpB* strain was exclusively caused by the lack of YtpB. We introduced an IPTG-induicible copy of *ytpB* (*P_{spac}(hy)-ytpB*) into a Δ*ytpB* background and tested the sensitivity of this strain to bacitracin (Figure C.3C). The sensitivity of the Δ*ytpB* *P_{spac}(hy)-ytpB* double mutant was equivalent to that of the WT strain confirming that *ytpB* is involved in this resistance mechanism. Furthermore, this strain maintained WT levels of bacitracin resistance in the absence of IPTG indicating that even the low levels of YtpB produced by the uninduced *P_{spac}(hy)* promoter are sufficient to resist bacitracin.

A careful analysis of the literature has revealed several clues that may explain why the Δ*ytpB* strain is bacitracin sensitive. Bacitracin inhibits cell wall synthesis by binding to undecaprenyl pyrophosphate (UPP) (25). As explained in section 1.1.2, UPP is recycled to undecaprenyl phosphate (UP) which translocates peptidoglycan subunits across cytoplasmic membrane and is converted back to UPP (19). Two major bacitracin resistance mechanisms have already been discovered in *B. subtilis*. The bacitracin-induced BceRS TCS activates the expression of the ABC transporter BceAB which confers substantial bacitracin resistance, potentially by transporting UPP to the cytoplasm (20). In addition, the σ^M dependent gene *bcrC* encodes an alternative UPP phosphatase that can compete with bacitracin for UPP (3). The major connection that links bacitracin resistance and these two resistance mechanisms to YtpB is that the substrate for YtpB, C_{35}-PP, is a UPP mimic (24). We hypothesized that the C_{35}-PP accumulates in the absence of YtpB. Such accumulation may inhibit the flipping of UPP across the membrane by BceAB or the ability of BcrC to dephosphorylate UPP.

***ytpB* overexpression may negatively affect the cell membrane via sesquarterpene overproduction**. Compared to the ΔytpB strain's bacitracin sensitivity phenotype, our explanation for why ytpB overexpression slightly increased sensitivity to triton X-100, cefuroxime, cerulenin, poly-L-lysine, DTAB, EDTA, and clofazamine is relatively straightforward. The key connection between these stresses is that they all target the membrane in one way or another. Triton X-100, poly-L-lysine, and DTAB are detergents (1.2.2), cerulenin inhibits FA synthesis enzymes (22), clofazamine increases phospholipase A2 activity (1), and EDTA destabilizes membranes (21).

Since YtpB contributes to the synthesis of sesquarterpenes that integrate into the membrane, it is possible that the overproduction of this unusual membrane lipid reduces overall membrane integrity rendering the cell more susceptible to these membrane stresses.

C.5 Conclusion

The data from this study have led to the formation of two hypotheses that explain the sensitivity phenotypes observed in the *ytpB* deletion and overexpression strains. We believe that a strain lacking *ytpB* exhibits early-onset cell death in the presence of bacitracin because of the accumulation of a UPP mimic that interferes with known bacitracin resistance mechanisms. Future studies could begin to confirm this mechanism by showing that the *ytpB* strain exhibits altered expression levels of the known bacitracin resistance genes. The membrane stress sensitivity of the *ytpB*-overexpression strain could be attributed to excessive sesquarterpene levels interfering with membrane stability.

REFERENCES

1. **Arbiser, J. L., and S. L. Moschella.** 1995. Clofazimine: A review of its medical uses and mechanisms of action. J Am Acad Dermatol **32:**241–247.

2. **Bateman, A., L. Coin, R. Durbin, R. D. Finn, V. Hollich, S. Griffiths, A. K. Jones, M. Marshall, S. Moxon, E. L. L. Sonnhammer, D. J. Studholme, C. Yeats, and S. R. Eddy.** 2004. The Pfam protein families database. Nuc Acids Res **32:**D138–D141.

3. **Bernard, R., M. E. Ghachi, D. Mengin-Lecreulx, M. Chippaux, and F. Denizot.** 2005. BcrC from *Bacillus subtilis* acts as an undecaprenyl pyrophosphate phosphatase in bacitracin resistance. J Biol Chem **280:**28852-28857.

4. **Butcher, B. G., and J. D. Helmann.** 2006. Identification of *Bacillus subtilis* σ^W-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by Bacilli. Mol Microbiol **60:**765-782.

5. **Cao, M., T. Wang, R. Ye, and J. D. Helmann.** 2002. Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ^W and σ^M regulons. Mol Microbiol **45:**1267-1276.

6. **Donovan, C., and M. Bramkamp.** 2009. Characterization and subcellular localization of a bacterial flotillin homologue. Microbiology **155:**1786-1799.

7. **Eiamphungporn, W., and J. D. Helmann.** 2008. The *Bacillus subtilis* σ^M regulon and its contribution to cell envelope stress responses. Mol Microbiol **67:**830-848.

8. **Hachmann, A. B., E. Sevim, A. Gaballa, D. L. Popham, H. Antelmann, and J. D. Helmann.** 2011. Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*.

9. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for Bacillus. John Wiley & Sons Ltd, Chichester, United Kingdom.

10. **Kannenberg, E. L., and K. Poralla.** 1999. Hopanoid biosynthesis and function in bacteria. Naturwissenschaften **86:**168–176.

11. **Kingston, A. W., C. Subramanian, C. O. Rock, and J. D. Helmann.** 2011. A σ^W-dependent stress response in Bacillus subtilis that reduces membrane fluidity. Mol Micro **81:**69-79.

12. **Kontnik, R., T. Bosak, R. A. Butcher, J. J. Brocks, R. Losick, J. Clardy, and A. Pearson.** 2008. Sporulenes, heptaprenyl metabolites from *Bacillus*

subtilis spores. Org Lett **10:**3551–3554.

13. **Kuhry, J.-G., G. Duportail, C. Bronner, and G. Laustriat.** 1985. Plasma membrane fluidity measurements on whole living cells by fluorescence anisotropy of trimethylammoniumdiphenylhexatriene. Biochim Biophys Acta **845:**60-67.

14. **Lee, Y. H., A. W. Kingston, and J. D. Helmann.** 2012. Glutamate dehydrogenase affects resistance to cell wall antibiotics in *Bacillus subtilis*. J Bacteriol **194:**993–1001.

15. **López, D., and R. Kolter.** 2010. Functional microdomains in bacterial membranes. Genes Dev **24:**1893-1902.

16. **Los, D. A., and N. Murata.** 2004. Membrane fluidity and its roles in the perception of environmental signals. Biochim Biophys Acta **1666:**142-157.

17. **Luo, Y., and J. D. Helmann.** 2012. Analysis of the role of *Bacillus subtilis* σ^M in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. Mol Micro **83:**623-639.

18. **Mascher, T., A. B. Hachmann, and J. D. Helmann.** 2007. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. J Bacteriol **189:**6919-6927.

19. **Matteï, P.-J., D. Neves, and A. Dessen.** 2010. Bridging cell wall biosynthesis and bacterial morphogenesis. Curr Opin Struc Biol **20:**749-755.

20. **Ohki, R., Giyanto, K. Tateno, W. Masuyama, S. Moriya, K. Kobayashi, and N. Ogasawara.** 2003. The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*. Mol Micro **49:**1135–1144.

21. **Prachayasittikul, V., C. Isarnakura-Na-Ayudhya, T. Tantimongcolwat, C. Nantasenamat, and H.-J. Galla.** 2007. EDTA-induced membrane fluidization and destabilization: Biophysical studies on artificial lipid membranes. ACTA Biochim Biophys **39:**901-913.

22. **Price, A. C., K.-H. Choi, R. J. Heath, Z. Li, S. W. White, and C. O. Rock.** 2001. Inhibition of β-ketoacyl-acyl carrier protein synthases by thiolactomycin and cerulenin. J Biol Chem **276:**6551-6559.

23. **Salzberg, L. I., and J. D. Helmann.** 2008. Phenotypic and Transcriptomic Characterization of *Bacillus subtilis* Mutants with Grossly Altered Membrane Composition. J Bacteriol **190:**7797-7807.

24. **Sato, T., S. Yoshida, H. Hoshino, M. Tanno, M. Nakajima, and T. Hoshino.** 2011. Sesquarterpenes (C35 terpenes) biosynthesized via the cyclization of a linear C35 isoprenoid by a tetraprenyl-β-curcumene synthase and a tetraprenyl-β-curcumene cyclase: Identification of a new terpene cyclase. J Am Chem Soc **133:**9734–9737.

25. **Stone, K. J., and J. L. Strominger.** 1971. Mechanism of action of bacitracin: Complexation with metal ion and C_{55}-isoprenyl pyrophosphate. PNAS **68:**3223–3227.

26. **Svobodová, J., and P. Svoboda.** 1988. Cytoplasmic membrane fluidity measurements on intact living cells of *Bacillus subtilis* by fluorescence anisotropy of 1,6-diphenyl 1,3,5-hexatriene. Folia Microbiol (Praha) **33:**1-9.

27. **Tamehiro, N., Y. Okamoto-Hosoya, S. Okamoto, M. Ubukata, M. Hamada, H. Naganawa, and K. Ochi.** 2002. Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrob Agents Ch **46:**315-320.