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A σ^W-dependent stress response in *Bacillus subtilis* that reduces membrane fluidity

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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 *Bacillussubtilis* 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.

Keywords

homeoviscous adaptation; membrane; detergent; ECF σ; fatty acid biosynthesis; condensing enzymes

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 (Jordan *et al.*, 2008). *B. subtilis* encodes seven ECF σ factors, several of which are induced by, and confer resistance to, antibiotics targeting the cell envelope (Helmann, 2002). 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

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to modify membrane lipid composition to acclimatize to membrane stress (Zhang and Rock, 2008). Some modifications affect the net membrane charge, while others adjust fluidity by changing the fatty acid (FA) composition (Denich *et al.*, 2003; Rivera-Milla *et al.*, 2006). 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 (Los and Murata, 2004). A clear example of homeoviscus adaptation is the response of *B. subtilis* to cold-temperature stress (Lu *et al.*, 2004; Aguilar and de Mendoza, 2006; Zhang and Rock, 2008). 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 (Mansilla *et al.*, 2004; Cybulski *et al.*, 2010).

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 (Luo *et al.*, 2010). The σ^X regulon includes the phosphatidylethanolamine synthesis genes *pssA* and *psd* (Cao and Helmann, 2004) while the σ^M regulon includes the phosphatidylglycerol hydrolysis enzyme YtpA (Tamehiro *et al.*, 2002; Eiamphungporn and Helmann, 2008) and numerous proteins involved in cell wall synthesis and cell division (Eiamphungporn and Helmann, 2008). Both the σ^M and σ^V stress responses are activated when the phosphatidylglycerol content of the membrane is reduced (Hashimoto *et al.*, 2009). The σ^W regulon includes numerous membrane-localized proteins (Huang *et al.*, 1999) and is activated under conditions of membrane stress, such as the presence of detergents or when membrane proteins are overproduced (Eiamphungporn and Helmann, 2008; Zweers *et al.*, 2009).

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.

Results and discussion

Identification of an active σ^W-dependent promoter within *fabHa*

Because ECF σ promoters are, as a class, highly conserved (Helmann, 2002; Koo *et al.*, 2009), 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 (Huang and Helmann, 1998; Huang *et al.*, 1999). 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) (Cao *et al.*, 2002a; Cao *et al.*, 2002b; Cao and Helmann, 2004; Eiamphungporn and Helmann, 2008). In the σ^M regulon, several promoter sites are located within genes (Eiamphungporn and Helmann, 2008), a finding consistent with whole genome chromatin immunoprecipitation studies in other systems (Wade *et al.*, 2006). We therefore searched 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 contains a perfect match

to the σ^W consensus in both the -35 (TGAAAC) and -10 (CGTA) elements (Helmann, 2002; Mascher *et al.*, 2007) (Fig. 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.

Several of the seven ECF σ factors in *B. subtilis* overlap in their promoter recognition properties (Mascher *et al.*, 2007; Luo *et al.*, 2010). 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 the σ^W-regulon such as polymyxin B, vancomycin, cephalosporin C, D-cycloserine, and triton X-100 (Table 1, Fig. S1) (Cao *et al.*, 2002b). 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.

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 (Wiegert *et al.*, 2001). P_5 displayed a low basal activity that increased ~3-fold in response to alkaline shock (Fig. 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 (Cao *et al.*, 2002a; Helmann, 2002), and as predicted this P_5*-*lacZ* strain expressed no detectable β-galactosidase activity either with or without alkaline shock.

The *fabHAF* operon is expressed from a promoter, P_{fabHaF}, negatively regulated by FapR, a repressor of genes involved in membrane lipid biosynthesis (Schujman *et al.*, 2003; Schujman *et al.*, 2006; Schujman and de Mendoza, 2008). β-galactosidase activity in strain HB13001 containing an ectopically located P_{fabHaF} -P_5-*lacZ* fusion (Fig. 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. P_5 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 (Fig. 1C). In contrast, expression was unaffected in control strains lacking the P_{xyl}-*sigW* fusion (Fig. 1C) or containing the inactive P_5* promoter (Fig. S2A). 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% (Fig. S2B). 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 (Fig. 1D): both enzymes utilize branched-chain primers but FabHb is significantly more capable of utilizing the straight chain precursor acetyl-CoA (Choi *et al.*, 2000). 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) with a concomitant increase in 31:0-carbon phosphatidylethanolamine species (Fig. 2A & 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 (Fig. 2A & 2C) similar to strain W168, although there was a small decrease in the proportion of straight-chain FA. These data are consistent with the 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; Fig. 2D & 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). 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 (Fig. 2D) that reflects a significant increase in 18 carbon FA (Table 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, Fig. 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). 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°C to 25°C (Beranova *et al.*, 2010), 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 (Mascher *et al.*, 2007). 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 (Fig. 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 (Fig. S3).

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 (Fig. 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 (Fig. 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 (Butcher and Helmann, 2006). We used a spot-on-lawn assay in which an 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 (Butcher and Helmann, 2006). 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* (Fig. 4). No significant P_5-dependent differences in sensitivity were observed with *B. subtilis ssp. spizizenii 2A9* (Fig. 4), *B. licheniformis*, or *B. atrophaeus NRS-213* (Fig. S4) 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 (Fig. 4), consistent with the previous demonstration that other σ^W-dependent operons contribute to antimicrobial resistance (Butcher and Helmann, 2006).

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 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 (White *et al.*, 2005). The two *B. subtilis* FabH isozymes, FabHa and FabHb (Lu *et al.*, 2004), catalyze the same reaction although FabHb has a higher specific activity for straight chain FA substrates than FabHa (Choi *et al.*, 2000). Thus, downregulation of FabHa by σ^W increases the proportion of straight chain FAs by increasing the cell's reliance on FabHb (Fig. 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 (White *et al.*, 2005). As the only elongation condensing enzyme in *B. subtilis*, FabF activity plays a determining role in the final chain length of the acyl-ACP (de Mendoza *et al.*, 2001; Schujman *et al.*, 2008). 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 (Mohedano *et al.*, 2005). However, the physiological role for this homeoviscous adaptation system is not yet clear. 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 (Singh *et al.*, 2009).

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 (Cybulski *et al.*, 2010). 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.

Experimental Procedures

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; 15μg ml^{-1}), chloramphenicol (cat; 10μg 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 (Harwood and Cutting, 1990). 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 (Guerout-Fleury *et al.*, 1996). 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 (Guerout-Fleury *et al.*, 1996) 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 (Vagner *et al.*, 1998) 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 (Mascher *et al.*, 2003). To create HB13118 (*fabHb::kan*), 800 bp DNA fragments flanking *fabHb* were amplified using primers 5059 and 5060 for the upstream (Kuhry *et al.*) 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* (Bhavsar *et al.*, 2001) 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) (Schramm *et al.*, 2000). 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 °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 (Mascher *et al.*, 2007). 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 (Miller, 1972).

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 °C with aeration. Cells were lysed and subjected to Western blot analysis using anti-FLAG antibodies to detect the fusions as described (Soonsanga *et al.*, 2008). 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 (Butcher and Helmann, 2006). 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 (Svobodová and Svoboda, 1988). 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 (Kuhry *et al.*, 1985).

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 $MnCl_2$, 10μM $FeCl_3$, 0.5% glucose and 2% xylose. Cells were harvested and lipids extracted as described by Bligh and Dyer (Bligh and Dyer, 1959). 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 (Choi *et al.*, 2000). 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 (Leonardi *et al.*, 2009). 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 performedby the use of unpaired Student's *t* tests. A value of $P \leq 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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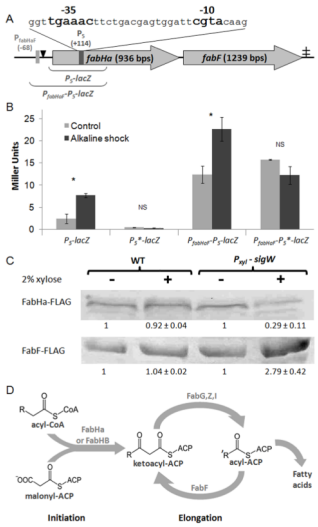

Figure 1.

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 ≥ 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 ≤ 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; Schujman and de Mendoza, 2008; and this work).

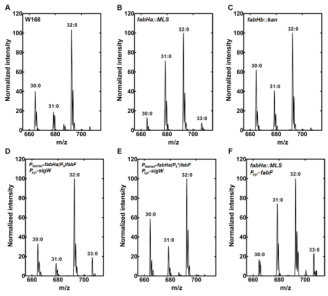

Figure 2.

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).

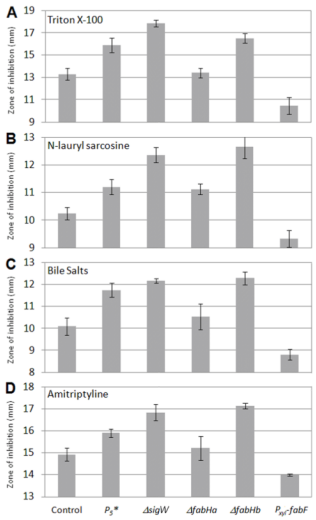

Figure 3.

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 ≤ 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

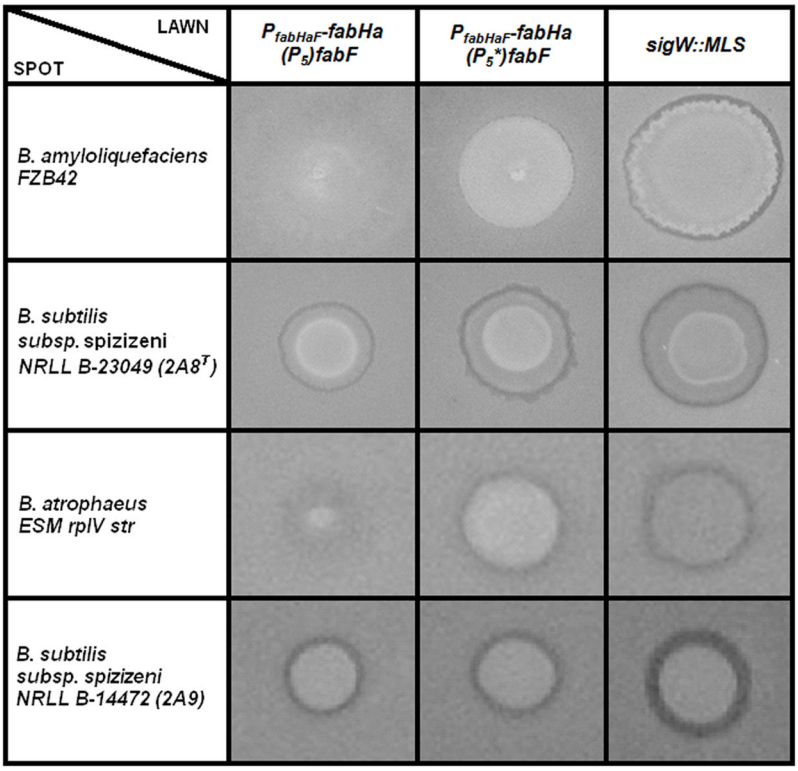

Figure 4.

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.

Table 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 (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.

Table 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 ≤ 0.05) from each other.