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Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis*

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Summary

The transcription factor Spx is the master regulator of the disulfide stress response in *Bacillus subtilis*. Intriguingly, the activation of Spx by diamide relies entirely on posttranslational regulatory events in spite of the complex transcriptional control of the *spx* gene. Here, we show that cell wall stress, but not membrane stress, also results in induction of the Spx regulon. Remarkably, two major differences were found regarding the mechanism of induction of Spx under cell wall stress in comparison to disulfide stress. First, transcriptional induction of the *spx* gene from a σ^M -dependent promoter is required for accumulation of Spx in response to cell wall stress. Second, activation of the Spx regulon during cell wall stress is not accompanied by oxidation of the Spx disulfide switch. Finally, we demonstrate that cells lacking Spx have increased sensitivity towards antibiotics inhibiting both early and late steps in peptidoglycan synthesis, suggesting that the Spx regulon plays an important adaptive role in the cell wall stress response. This study expands the functional role of the Spx regulon and reveals novel regulatory mechanisms that result in induction of Spx in *B. subtilis*.

Graphical abstract

Spx regulates the expression of genes induced by disulfide stress. Here, the Spx transcription factor is also shown to be induced by cell wall stress through activation of the σ^M alternative sigma factor, and this induction contributes to cell survival. In contrast with disulfide stress, Spx remains largely in its reduced state after cell wall stress.

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Introduction

Spx is a pleiotropic transcription factor that controls the disulfide-stress response in *Bacillus subtilis* (Nakano *et al.*, 2003), and other low-GC Gram-positive bacteria including major pathogens (Pamp *et al.*, 2006; Veiga *et al.*, 2007; Kajfasz *et al.*, 2010; Barendt *et al.*, 2013). An *spx* null mutant is sensitive to diamide, an electrophilic agent that selectively leads to the formation of intramolecular and intermolecular disulfide bonds (Nakano *et al.*, 2003). Spx belongs to the ArsC family of transcription factors, and controls gene expression by direct binding to the C-terminal domain of the alpha subunit of the RNA polymerase (Newberry *et al.*, 2005). Activation of Spx results in the specific induction or repression of more than 140 transcriptional units, including genes involved in the synthesis of bacillithiol and cysteine, redox homeostasis, and proteolytic control (Rochat *et al.*, 2012).

The production of Spx is tightly regulated at several levels. At the transcriptional level, *spx* expression is affected by four promoters responsive to different sigma factors: σ^A , $\sigma^{M/X/W}$, σ^M , and σ^B (Antelmann *et al.*, 2000; Leelakriangsak and Zuber, 2007; Jervis *et al.*, 2007; Luo and Helmann, 2012). Two repressors, PerR and YodB, also control the expression of *spx* (Leelakriangsak *et al.*, 2007). At the post-translational level, the cytoplasmic concentration of Spx is tightly controlled by proteolysis. Under non-stress conditions, the Spx protein is translated but actively unfolded and degraded via the ATP-dependent protease ClpXP in a process dependent on the adaptor protein YjbH (Nakano *et al.*, 2002; Larsson *et al.*, 2007; Zhang and Zuber, 2007; Garg *et al.*, 2009). Conversely, upon disulfide treatment the proteolytic activity of ClpXP is reduced and YjbH aggregates, allowing for Spx accumulation (Zhang and Zuber, 2007; Garg *et al.*, 2009; Engman and von Wachenfeldt, 2015). The ability of Spx to regulate gene expression is also influenced by the oxidation state of its redox-sensing switch. The N-terminal domain of Spx contains a CXXC motif that becomes oxidized under conditions that increase disulfide bond formation (disulfide stress). *In vitro* studies have shown that the formation of this disulfide bond in Spx provokes a conformational change in the RNA polymerase-Spx-DNA promoter ternary complex that stimulates the expression of *trxA* and *trxB* (Nakano *et al.*, 2005). Recent evidence, however, suggests that oxidation of the redox-sensing switch is not essential for induction of all Spx-controlled genes (Rochat *et al.*, 2012).

Bacteria respond to cell envelope damage through multiple regulatory systems (Jordan *et al.*, 2008; Helmann, 2016), often involving extracytoplasmic (ECF) σ factors (σ^{ECF}). The specific molecular signals to which ECF σ factors respond remain largely unknown. One notable exception is σ^V , which responds strongly and specifically to lysozyme (Guariglia-Oropeza and Helmann, 2011; Ho *et al.*, 2011), and this response involves a direct protein interaction between lysozyme and the anti- σ factor (Hastie *et al.*, 2016). Although the precise molecular signals remain unknown, σ^M , σ^W and σ^X respond to inhibitors of peptidoglycan (PG) synthesis, membrane-active compounds, and antimicrobial cationic peptides, respectively (Helmann, 2016). Interestingly, the *yjbC-spx* operon contains two promoters that appear to be regulated by ECF σ factors (Jervis *et al.*, 2007; Eiamphungporn and Helmann, 2008), and cell envelope antibiotics induce *spx* and Spx-dependent genes (Cao *et al.*, 2002b; Eiamphungporn and Helmann, 2008; Wecke *et al.*, 2011; Kawai *et al.*, 2015).

Studies employing diamide to generate disulfide stress have provided the foundation for our understanding of the Spx regulon. In contrast, activation of the Spx regulon in response to other signals is poorly understood. Here, we show that Spx is required for a robust cell wall stress response and, unlike disulfide stress, transcriptional induction of *spx* from a σ^M -regulated promoter is required to ensure full induction of the Spx regulon. We also show that oxidation of the Spx redox-sensing switch under cell wall stress plays a more limited role compared to diamide stress in induction of Spx-controlled genes, and that induction of the Spx regulon confers protection against antibiotics.

Results

Cell wall stress leads to upregulation of *spx*

The *spx* gene is located in a bicistronic operon with *yjbC*, which encodes a putative acetyltransferase. Several promoters appear to be responsible for the transcription of *spx*, including one σ^A promoter (P_A) located in the intergenic region between *yjbC* and *spx* (Leelakriangsak and Zuber, 2007), a σ^B -dependent promoter (P_B) located upstream of *yjbC* (Antelmann *et al.*, 2000), and two promoters controlled by σ^{ECF} factors (Cao *et al.*, 2002a; Jervis *et al.*, 2007). One putative σ^{ECF} -dependent promoter is upstream of *yjbC* (P_{M1}) and another is in the intergenic region between *yjbC* and *spx* (P_{M2}) (Fig 1A). Additionally, two repressors (i.e. PerR and YodB) bind the intergenic region of *spx* and *yjbC* (Leelakriangsak *et al.*, 2007). This complex transcriptional context suggests that induction of the *spx* gene in response to stress likely contributes to activation of the Spx regulon.

Both P_{M1} and P_{M2} were detected by 5'-RACE in cells induced for σ^M expression (Jervis *et al.*, 2007), but the contribution of these promoters to *spx* expression in response to stress has not been defined. Since σ^M responds to cell wall antibiotics, we first studied the transcriptional profile of *spx* in cells treated with various cell wall-active antibiotics. Two transcripts were identified in an RNA (northern) blot with molecular sizes that corresponded to promoters located in the upstream region of *yjbC* and the intergenic region of the operon: ~1.3 kb and ~0.5 kb, respectively (Fig 1B). There was no significant change in the level of the *yjbC-spx* mRNA after 10 min. or 40 min. of diamide treatment. The monocistronic *spx* transcript was somewhat elevated after 40 min. compared to the 10 min. sample in both the diamide treated and the control cells. In contrast, treatment with PG synthesis inhibitors resulted in a dramatic increase in the expression of the *yjbC-spx* mRNA, which was most apparent at the 40 min. timepoint. These results suggest that P_B or P_{M1} likely account for transcriptional induction of *spx* under cell wall stress.

To further evaluate the contribution of the *spx* proximal promoters to the cell wall stress response, we constructed a transcriptional fusion of the region encompassing the P_A and P_{M2} promoters to the *lacZ* gene (i.e. $P_{M2,A-lacZ}$) (Fig 1C) and monitored induction using northern blots. Compared to the untreated control, both fosfomycin and vancomycin resulted in a decrease in the activity of these intergenic promoters, suggesting that the smaller RNA transcript previously observed after treatment with PG inhibitors (Fig 1B) may arise from post-transcriptional processing. In the presence of diamide, a modest increase was observed after 10 min. (Fig 1C). We next assessed if the observed regulation occurred at P_A , which is regulated by YodB and PerR. As observed using the $P_{M2,A-lacZ}$ fusion, cell wall stress

resulted in repression of P_A -*lacZ* compared to the untreated control, while a marked induction of P_A was observed in presence of diamide (Fig 1D), as previously reported (Leelakriangsak *et al.*, 2007). Because the antibiotics tested inhibited different steps in the biosynthesis of peptidoglycan, we conclude that cell wall stress, rather than recognition of any one antibiotic, accounts for the induction of *spx* and that this induction originates from promoter(s) upstream of *yjbC*.

The P_{M1} promoter drives the expression of *spx* under cell wall stress

To dissect the contribution of the various promoters upstream of *spx* to induction in response to cell wall stress, we used a GFP reporter fusion expressed ectopically from a DNA fragment containing the entire *yjbC* gene together with upstream and downstream promoters. This fusion is significantly induced after 40 min. of antibiotic treatment, but this induction is eliminated by point mutations designed to inactivate P_{M1} (Fig 2A, 2B, 2D, 2E). In contrast, mutations in the predicted P_B or P_{M2} promoters had no effect on induction, which is consistent with the fact that *sigB* cells are still able to upregulate *yjbC-spx* (Fig 2E) and that the intergenic, *spx* proximal promoters do not contribute to induction of *spx* (Fig 1C) in response to cell wall stress. The location of P_{M1} upstream of *yjbC* is in agreement with the size of the induced transcript as observed using northern blot analysis (Fig 1B). We conclude that induction of the *spx* gene is driven by P_{M1} in response to cell wall stress.

To determine which ECF sigma factor(s) were responsible for induction of P_{M1} , we first monitored the activation of the major ECF σ factors (i.e. σ^M , σ^W , and σ^X) in response to cell wall stress (Helmann, 2016). Since the genes encoding σ^M , σ^W , and σ^X are positively autoregulated, the measurement of their mRNA levels reflects their activity in response to different environmental conditions. Fosfomycin and ampicillin resulted in induction of *sigM* and *sigW*, whereas *sigX* was unresponsive (Fig 2C). This suggests that σ^M and/or σ^W are likely responsible for induction of *yjbC-spx*. In a *sigM* null mutant, the level of antibiotic induction was dramatically reduced, but not eliminated, and this phenotype was complemented by ectopic expression of σ^M (Fig 2D). The residual induction in the *sigM* null mutant was not due to σ^W since induction was largely unaffected in a *sigW* mutant, and a *sigW sigM* double mutant behaved similarly to the *sigM* single mutant. Further analysis revealed that σ^X also contributes to the expression of *yjbC-spx*: induction was absent in a *sigM sigX* mutant and expression was fully eliminated in the *sigM sigW sigX* triple mutant (Fig 2E). Together, these results indicate that σ^M is the major ECF sigma factor required for induction of *spx* in response to cell wall antibiotics.

The Spx protein accumulates under cell wall stress

The accumulation of the Spx protein in response to disulfide stress primarily occurs through reduced proteolysis (Zuber, 2009). We therefore asked whether antibiotic stress also leads to accumulation of Spx protein, and whether induction of the P_{M1} promoter is required for this accumulation. Western blot analysis using anti-Spx antiserum reveals a dramatic increase in Spx in cells following treatment with ampicillin and fosfomycin when compared to mock-treated samples (Fig 3A). Several other peptidoglycan synthesis inhibitors (vancomycin, D-cycloserine, and cefuroxime), also led to a clear increase in Spx levels, but there was

comparatively little effect after treatment with membrane active antibiotics (colistin, daptomycin, polymyxin B, and nisin) (Fig 3D). This pattern of induction is consistent with the role of σ^M in induction of P_{M1} , since σ^M is known to respond strongly to cell wall stress elicited by inhibitors of peptidoglycan synthesis (Eiamphungporn and Helmann, 2008; Czarny *et al.*, 2014), whereas membrane-active compounds strongly induce σ^W (Helmann, 2016). As expected, the accumulation of the Spx protein under cell wall stress was σ^M -dependent: the P_{M1}^* strain was unable to accumulate Spx upon fosfomycin treatment (Fig 3B), even though fosfomycin is amongst the strongest inducers of Spx accumulation (Fig 3A). Ectopic complementation of a strain harboring the P_{M1}^* promoter with an *spx* allele driven from the P_{M1} promoter (i.e. *amyE::P_{M1}-spx*) fully restored induction (Fig 3B), further validating the critical role of the P_{M1} promoter. In contrast, Spx accumulation in response to diamide was unaffected by the P_{M1}^* mutation (Fig 3C). These results suggest that, in contrast to disulfide stress, transcriptional activation of the *spx* gene is required for accumulation of Spx under cell wall stress.

Cell wall stress activates the Spx regulon

To determine whether accumulation of Spx is correlated with induction of the Spx regulon, we monitored mRNA levels for four Spx target genes: *trxB*, *trxA*, *yjbH*, and *nfrA*, after treatment with fosfomycin (Fig 4A). Fosfomycin led to an Spx-dependent induction of all four genes. To investigate the dynamics of induction of the genes in the Spx regulon, we chose the *trxB* gene whose transcription fully depends on Spx. Expression of *trxB* was transient, and peaked after 20 min. and 40 min. of treatment with ampicillin and fosfomycin, respectively (Fig 4B). These results correlate with the interruption of late and early stages in the synthesis of PG. While ampicillin prevents PG transpeptidation, fosfomycin inhibits the first cytosolic enzyme committed in the synthesis of PG (i.e. MurAA). Cell wall damage, therefore, correlates with the induction of the Spx regulon.

Next, we studied the Spx-dependent induction of *trxB* in WT and WT(P_{M1}^*) cells using a *P_{trxB}-gfp* transcriptional fusion. In the absence of antibiotic or diamide challenge, both strains displayed similar *trxB* activity. Similar induction levels were also observed in diamide-treated cells (Fig 4C), which was consistent with the observed Spx protein levels (Fig 3C), and suggests that the induction of *trxB* in response to diamide proceeds in a P_{M1} -independent fashion. In contrast, induction of *trxB* by PG synthesis inhibitors was significantly reduced, but not eliminated, in cells harboring P_{M1}^* (Fig 4C). A similar pattern emerged when we monitored the induction dynamics of *trxB*, *trxA*, and *nfrA* in response to fosfomycin: induction was dramatically reduced, but not eliminated, in cells unable to activate P_{M1} (Fig 4D). Taken together, we conclude that cell wall stress leads to induction of the Spx regulon, transcriptional induction of *spx* from P_{M1} is required for the full induction, but post-transcriptional stabilization of Spx may also contribute to induction.

The Spx protein remains primarily in its reduced state under cell wall stress

To study the redox state of Spx (Fig 5A) and its contribution to the induction of the regulon, we first monitored *trxB* induction using the *P_{trxB}-gfp* transcriptional fusion. Induction of *trxB* was substantially higher under disulfide stress than cell wall stress (Fig 5B), although comparable protein levels were observed in both conditions (Fig 3 and data not shown).

Since oxidation of the Spx redox-sensing switch increases transcription of *trxB*, we therefore hypothesized that cell wall stress might be less efficient than diamide in leading to Spx oxidation (Nakano *et al.*, 2005; Rochat *et al.*, 2012).

To further study the role of Spx oxidation, we compared *trxB* induction in cells harboring *spx* or *spx*^{C10A} (under native control). The *spx*^{C10A} allele encodes a mutant Spx protein (Cys10 to Ala substitution) unable to form the canonical intramolecular disulfide bond (Fig 5A) and therefore is generally assumed to reflect the activity of reduced Spx protein (Nakano *et al.*, 2005). We note that Spx^{C10A} only displays a slight decrease in affinity for binding to the alpha subunit of RNA polymerase compared with the wild-type protein (Lin *et al.*, 2013), and that similar Spx protein levels are observed in cells harboring the wild-type or mutant Spx protein (Fig 5D) (Nakano *et al.*, 2005). The basal level of *trxB* expression was significantly lower in cells harboring Spx^{C10A}, even in the absence of stress, suggesting that either some Spx oxidation occurs in growing cells, or that this amino acid change impacts the ability of Spx to induce gene expression (Fig 5B). Diamide treatment caused a dramatic increase in *trxB* expression in wild type cells compared to cells harboring Spx^{C10A}, further validating the importance of the Spx disulfide switch in the induction of the Spx regulon under disulfide stress (Fig 5B, 5C). Cell wall stress, as expected, also led to a significant increase in the expression of *trxB*, as previously observed (Fig 4A, 4B); however, the point mutations in the redox-sensing switch had a significantly smaller effect on the induction of *trxB* and other Spx-controlled genes (Fig 5B, 5C) by fosfomycin compared to diamide. Thus, oxidation of the Spx protein seems to play a more limited role during induction of the regulon by cell wall stress. Strikingly, further analysis of the CxxC motif showed that cells harboring a Spx mutant protein lacking both cysteines (i.e. Spx^{C10AC13A}) displayed reduced induction of Spx-controlled genes compared to Spx^{C10A} in response to cell wall stress, but increased induction in response to diamide (Fig 5C). While unexpected, we note that the increased activity of an Spx^{C10S,C13S} double mutant compared to a single Spx^{C10S} mutant has been seen previously (Birch, 2017). These seemingly incongruous results highlight the complexity of the regulation of Spx, and seem to indicate that other factors likely influence Spx activity.

To further explore changes in Spx oxidation during cell wall stress, we used *in vivo* 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) alkylation experiments. This assay relies on the ability of the AMS maleimide group to covalently modify reduced cysteine residues at neutral pH, which results in an increase in the apparent molecular weight of the protein (~0.5 kDa per AMS molecule attached) as observed by SDS-PAGE. Conversely, disulfide bonds and other oxidized thiol species are resistant to modification. In agreement with previous findings, diamide treatment led to the formation of a non-modified Spx protein band (i.e. lower molecular mass band), consistent with the presence of an intramolecular disulfide bond. By contrast, in untreated cells, we observed the presence of two well-defined protein bands: one faint band that corresponds to Spx + 0 AMS, the oxidized species, as well as a dominant protein band that corresponds to Spx + 2 AMS, the reduced species (Fig 5D, 5E). Thus, in actively growing cells Spx is mostly reduced, although some oxidized protein is present. Treatment with ampicillin, fosfomycin, and vancomycin resulted in a dramatic accumulation of Spx, and this newly synthesized protein was present largely in the reduced form (i.e. Spx + 2 AMS) (Fig 5D). Under these

conditions, the intensity of the faint band corresponding to oxidized protein was actually decreased. The present evidence thus suggests that in contrast to disulfide stress, the induction of the Spx regulon under cell wall stress is likely driven by the reduced Spx species.

Cells lacking Spx display increased sensitivity towards cell wall active antibiotics

We next set to determine if induction of the Spx regulon in response to cell wall stress increased cell survival in the presence of inhibitors of PG synthesis. Consistent with previous findings (Nakano *et al.*, 2003), cells lacking Spx displayed reduced survival after treatment with diamide (Fig 6A). Cells lacking Spx were also more sensitive to cell wall antibiotics, with a dramatic loss of viability within the first 60 min. of treatment (Fig 6A); the same time period during which the Spx regulon is induced. Ectopic complementation of *spx* with either the entire *yjbC-spx* operon (Fig 6A) or an IPTG-controlled *spx* allele (Fig S1), as expected, restored the wild-type phenotype.

We next asked whether cells lacking the P_{M1} promoter, or those unable to form the disulfide switch, were also more sensitive to the PG inhibitors. For this, we monitored bacterial survival 90 min. after treatment. Cells harboring the mutant P_{M1}^* promoter display increased sensitivity to fosfomycin and D-cycloserine, but not ampicillin or diamide. Since the Spx regulon is active throughout exponential phase and slightly induced even in the absence of σ^M control (Fig 4C, 4D), it is likely that this partial activity suffices to provide ampicillin resistance. Fosfomycin and D-cycloserine resistance, by contrast, required full upregulation of the Spx regulon. As expected, no differences between WT and WT(P_{M1}^*) were observed upon diamide challenge (Fig 6B), which is consistent with the fact that both strains display similar Spx protein and *trxB* induction levels under disulfide stress (Fig 3C, 4C). Finally, we note that cells unable to form the disulfide switch (i.e. Spx^{C10A}) were significantly more sensitive to all three antibiotics studied (Fig 6B). It is not yet clear if this is indicative of a requirement for Spx oxidation during cell wall stress or if this reflects the fact that these cells display overall reduced Spx activity (Fig 5B).

Discussion

Spx controls the expression of a large number of genes in response to disulfide stress (Nakano *et al.*, 2003; Rochat *et al.*, 2012); a stress condition that results in the formation of nonnative disulfide bonds protein in the cytoplasm and therefore leads to protein misfolding and aggregation. Under disulfide stress, activation of the Spx regulon relies on a decrease in Spx proteolysis, as well as oxidation of the Spx redox-sensing switch (Larsson *et al.*, 2007; Zhang and Zuber, 2007; Garg *et al.*, 2009; Engman and von Wachenfeldt, 2015). The decrease in Spx proteolysis occurs due to the aggregation of the adaptor protein YjbH through a mechanism that does not involve its cysteine residues (Engman and von Wachenfeldt, 2015), and a decrease in the activity of the ATP-dependent ClpXP protease (Garg *et al.*, 2009). Other stress conditions that result in formation of protein aggregates, such as heat shock, also cause Spx accumulation (Runde *et al.*, 2014; Engman and von Wachenfeldt, 2015). Remarkably, no changes in the expression of the *spx* gene were observed when the cells were treated with diamide (Fig 1B), suggesting that the complex

transcriptional architecture of the *spx* gene (Fig 1A) plays a minimal role in the induction of the regulon under disulfide stress (Rochat *et al.*, 2012).

In most bacteria, the PG layer is essential for growth and development. Due to its essentiality, bacteria possess specific transcription factors that allow cells to respond to physical or chemical threats that can compromise its integrity (Jordan *et al.*, 2008). Among these transcription factors, the ECF σ factor σ^M plays a central role, as it coordinates the expression of genes involved in cell wall biosynthesis and cell division (Helmann, 2016). In addition, σ^M mediates transcriptional induction of the *spx* gene under conditions in which the integrity of the cell wall is compromised. Induction of the *yjbC-spx* operon, for example, has been observed in transcriptomic studies of *B. subtilis* cells treated with vancomycin (Cao *et al.*, 2002b; Eiamphungporn and Helmann, 2008), enduracidin and bacitracin (Rukmana *et al.*, 2009), and ramoplanin and moenomycin (Salzberg *et al.*, 2011). *B. subtilis* protoplasts also display significant *spx* upregulation compared to either walled cells or L-forms (Kawai *et al.*, 2015). We here confirmed the upregulation of *spx* by treatment with early and late PG synthesis inhibitors and further show that the P_{M1} promoter, which is primarily controlled by σ^M , is the major driver of this transcriptional induction (Fig 2). Membrane antibiotics that also target the cell envelope did not lead to Spx accumulation (Fig 3D). Remarkably, in contrast to disulfide stress, transcriptional induction of *spx* in response to cell wall stress is required for both accumulation of Spx protein (Fig 3) and induction of Spx-controlled genes (Fig 4).

Cells lacking σ^M are significantly more sensitive to drugs that interrupt essential steps in the construction of PG, which is at least partially due to an inability to induce genes involved in PG homeostasis (Mascher *et al.*, 2007; Luo and Helmann, 2012). Although the roles of many genes in the σ^M regulon have been well defined (Cao *et al.*, 2005; Eiamphungporn and Helmann, 2008; Meeske *et al.*, 2015; Meeske *et al.*, 2016; Zhao *et al.*, 2016), the contributions of each σ^M target operon to antibiotic resistance are incompletely understood. Here, we show that antibiotic-dependent induction of the Spx regulon protects cells against killing by cell wall antibiotics (Fig 6). Although *spx* transcription is up-regulated from the P_{M1} promoter upstream of the *yjbC-spx* operon, a clean deletion of the *yjbC* gene does not affect antibiotic resistance (data not shown), and the role of the YjbC protein remains unclear. Our results extend previous findings where artificial induction of Spx conferred high level resistance to the beta-lactam antibiotic cefuroxime (Luo and Helmann, 2012). The Spx-regulated genes that protect against various cell wall antibiotics are not yet known, and likely vary depending on the antibiotic.

The functional role of the redox-sensing switch in the induction of the Spx regulon is complex. *In vitro* and *in vivo* experiments have shown that diamide induction of the canonical Spx-controlled gene *trxB* is largely dependent on the oxidized form of Spx (Nakano *et al.*, 2005; Rochat *et al.*, 2012; Gaballa *et al.*, 2013). The present results support those observations as diamide treatment only led to a comparatively modest increase in *trxB* expression in cells harboring the *spx*^{C10A} allele (Fig 5B, 5C). Under cell wall stress, by contrast, several Spx-controlled genes were strongly induced (Fig 4A) even when the protein was present primarily in the reduced state (Fig 5D); additionally, relatively small differences in induction of *trxB* were observed in cells harboring Spx or Spx^{C10A} (Fig 5). Oxidation of

the redox-sensing switch, therefore, seems to play a limited contribution in induction of the Spx regulon under cell wall stress. Indeed, it has been previously shown that both reduced and oxidized Spx have similar affinity for the α -CTD domain of RNA polymerase (Nakano *et al.*, 2005). These results suggest that although induction of *trxB* by diamide requires oxidation of Spx, the sustained high level of reduced Spx seen under cell wall stress conditions also suffices (Fig 3, Fig 7).

Interestingly, recent evidence suggests that the contribution of the redox-sensing switch may be promoter specific. Spx-dependent expression of the bacillithiol biosynthesis genes, for example, does not require the formation of an intramolecular disulfide (Gaballa *et al.*, 2013). Furthermore, Rochat *et al.* (2012) also identified genes that displayed a large variation in their requirement for the oxidized Spx species using *in vitro* transcription. Some genes, for instance, were activated by either oxidized or reduced Spx, whereas other genes were predominantly activated by oxidized Spx. These observations raise the possibility that the Spx regulon under cell wall stress (where Spx is largely reduced) may differ from that observed under diamide treatment (where Spx is largely oxidized).

A link between Spx and the cell wall stress response has been directly or indirectly observed in other Gram-positive species. In *Lactococcus lactis*, for example, deletion of an Spx paralog called SpxB renders the cells sensitive to lysozyme (Veiga *et al.*, 2007); SpxB controls the expression of an *O*-acetylase that modifies the peptidoglycan subunits and protects the cells against lysozyme. In *Staphylococcus aureus*, deletion of YjbH, which causes accumulation of Spx, renders the cells more resistant to diverse cell wall antibiotics (Gohring *et al.*, 2011). In this species, the Spx-dependent induction of the *trfA* gene, a *B. subtilis* *mecA* homologue, has been associated with increased antibiotic resistance (Jousselin *et al.*, 2013). In *Enterococcus faecalis*, cells lacking Spx display increased sensitivity towards ampicillin and vancomycin, two inhibitors of the synthesis of PG (Kajfasz *et al.*, 2012). Notably, in *B. anthracis* and *S. mutants*, the Spx proteins are also potential regulator of genes involved in PG biosynthesis (Veiga *et al.*, 2007; Barendt *et al.*, 2013), suggesting a direct effect of Spx on cell wall homeostasis. In *B. subtilis*, interestingly, Spx is capable of binding the promoters of *murAA*, *ponA*, and *mreB*, that encode proteins involved in synthesis of PG; their expression, however, is Spx-independent in response to diamide (Rochat *et al.*, 2012) as well as fosfomycin (Fig S2). The conditions in which those genes are induced by Spx remain to be determined. The functional roles of Spx are broad and not limited to disulfide and cell wall stress; virulence, biofilm formation, and resistance to oxidative stress also require the activity of Spx (Pamp *et al.*, 2006; Veiga *et al.*, 2007; Wang *et al.*, 2010; Kajfasz *et al.*, 2012; Barendt *et al.*, 2013; Zheng *et al.*, 2014).

The results presented here support a model in which cell wall damage leads to the induction of the Spx regulon in a σ^M -dependent fashion. This induction is critical for the cell wall stress response, since cells lacking it are more sensitive than WT against antibiotics interrupting various steps in the peptidoglycan biosynthesis pathway. In response to cell wall stress, two notable differences were observed in the regulation of Spx activity in comparison to diamide (Fig. 7). First, σ^M was required for transcriptional induction of the *spx* gene, and therefore induction of the Spx regulon. Second, Spx primarily accumulated in the reduced form and this form sufficed to induce the regulon. It is likely that down-regulation of ClpXP

proteolysis also contributes to induction of the Spx regulon under cell wall stress, as suggested by the observation that cells unable to induce P_{M1} still display a modest increase in Spx accumulation and activity. The present results not only expand the conditions known to induce the Spx regulon, but also highlight the diversity of regulatory mechanisms that can lead to induction of Spx.

Experimental procedures

Bacterial strains and culture conditions

All bacterial strains are listed in Table 1. The primers used are listed in Tables S1-S3. *Bacillus subtilis* strains (all based on the *B. subtilis* 168 wild-type) were grown under standard conditions: lysogeny broth (LB) (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) at 37 °C with vigorous shaking, unless otherwise stated. *Escherichia coli* DH5a was used for plasmid construction. Antibiotics were added to the growth medium when appropriate: 100 µg ml⁻¹ ampicillin for *E. coli*, and 1 µg ml⁻¹ erythromycin plus 25 µg ml⁻¹ of lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 µg ml⁻¹ chloramphenicol, 100 µg ml⁻¹ spectinomycin, 5 µg ml⁻¹ tetracycline and 10 µg ml⁻¹ kanamycin for *B. subtilis*. For monitoring gene induction and cell survival in response to cell envelope active antibiotics the standard concentrations employed (corresponding to 2× MIC) were 2 µg ml⁻¹ ampicillin, 200 µg ml⁻¹ fosfomycin, 250 µg ml⁻¹ D-cycloserine, 1 µg ml⁻¹ vancomycin, and 0.5 mM diamide (unless otherwise stated).

Strain constructions

For construction of the complementation strains, HB18907 and HB18923, the mutagenic primers for P_{M1}^* and *spx*^{C10A} were DR150/DR151 and DR84/DR85, respectively (Table S1). The external primers were DR51/DR52, and genomic DNA was used as template. The pGFPStar* vector was amplified using DR28/DR29. The assembled constructs were transformed into an *spx::kan* mutant strain. For construction of the GFP promoter-reporter fusions the primers for mutagenesis for the P_{M1}^* , P_{M2}^* , and P_B^* promoters were DR150/DR151, DR152/DR153, and DR154/DR155 (reverse and forward), respectively. The external primers for the insert were DR51/DR181. Genomic DNA was used as template. The vector was PCR amplified using DR29/DR180. For construction of the P_{trxB} -GFP, the *trxB* promoter was PCR amplified from chromosomal DNA using DR132/DR133, and the pGFPStar vector using DR130/DR131. In all cases, the PCR-amplified vectors were digested using DpnI for 1 h at 37°C. All fragments were then purified and used for Gibson assembly. The ligation product was transformed into *E. coli* DH5a. Then the plasmids were extracted, and 1 µg of plasmid used to transform *Bacillus subtilis* 168. The insert was verified by PCR and sequencing.

For construction of the *yjbc-spx* mutants at locus (HB18697 and HB18698) we used the same mutagenic primers as for HB18907 and HB18923. Overlap PCR was used to construct the *B. subtilis* strains, which became resistant to kanamycin. Chromosomal DNA was used as template. The external primers for the *yjbc-spx* fragment were DR70/DR81. Then, the fragment containing the mutations was fused with a fragment containing the kanamycin cassette, which was amplified using primers 1295/1296; as well as a DNA region

encompassing the *yjbE* and *mecA* genes downstream *yjbC-spx*, which was amplified using DR82/DR71. The ~5 kb fragment was transformed into *B. subtilis*, and confirmed by PCR and sequencing. For construction of a WT strain harboring the kanamycin cassette at the same position, the protocol was identical except for the fact that mutagenic primers were not included.

For construction of the *sigM* complementation strain (HB18646), the *sigM* gene and its promoter were amplified using DR194/DR198, and cloned by Gibson Assembly into pDG1663, which was amplified using DR192/DR193. The chosen primers for the vector removed the *lacZ* gene. The DNA was transformed into HB18640. The *sigW::ery* strain was obtained from the Bacillus Genetic Stock Center, and backcrossed into 168.

For construction of the $P_{M2,A}$ -*lacZ* and P_A -*lacZ* fusions, the promoter regions were amplified using DR325/DR327 and DR326/DR327, respectively, and cloned into pDG1663 using the BamHI and EcoRI restriction sites. The promoters $P_{M2,A}$ and P_A included the regions -170 to +121 and -80 to +121, respectively, relative to the P_A transcription start site as described by Leelakriangsak *et al.* (2007). The vector containing the fusion was transformed into *B. subtilis* 168. The P_{M1} -*spx* fusion at the *amyE* site was constructed using PCR to fuse the region including the P_{M1} promoter and the *spx* coding sequence. For this, the primers DR333/DR334 and DR332/DR335 were used for PCR amplification of the promoter and coding region, respectively. The resulting fragment was cloned into pDG1662 using the HindIII and EcoRI restriction enzymes. The vector containing the fusion was transformed into HB18905. The inserts and vectors were fused with T4 ligase, and cloned into *E. coli* DH5a. The integrity of all constructs was verified by sequencing.

RNA isolation

Cells were grown under standard conditions until OD₆₀₀ reached ~0.5, and then treated or not with cell wall antibiotics or diamide. RNA was isolated using the hot phenol-chloroform method. Briefly, five ml of cells are collected, centrifuged at 5000 rpm, and resuspended in 400 µl of TE buffer (pH 8.0) supplemented with 20 mg ml⁻¹ of lysozyme and 10 µl of proteinase K. The mixture was incubated at room temperature for 10 min., and then transferred to a 1.7 microfuge tube. Next, 23 µl of 2 M sodium acetate buffer (pH 5.2), 45 µl of 10% SDS, and 400 µl of acid phenol was added, the mixture was vigorously vortexed, and the tubes were incubated at 65 °C for 10 min. Next, the tubes were placed on ice for 5 min., and then centrifuged at top speed for 15 min. at 10°C. The upper phase was carefully removed, mixed with equal parts of chloroform, and then vortexed to form an emulsion. The mixture was centrifuged at 14000 rpm for 10 min., and the upper phase was removed. The fraction containing the RNA (~400 µL) was adjusted to 0.3 M sodium acetate buffer (pH 5.2), and mixed with a similar volume of isopropanol for RNA precipitation. The precipitated RNA was centrifuged at 15°C for 30 min. at 14000 rpm, and the supernatant was carefully decanted. The RNA was washed with chilled 70% ethanol, incubated for 10 min. at RT to dissolve salts, and centrifuged at 14000 rpm at 4 °C for 10 min. The supernatant was carefully removed, and the samples were air-dried for 10 min. at 50°C. The RNA was resuspended in 70 µl of 1 mM sodium citrate (pH 6.2). RNA concentration and

purity was determined using spectrophotometry (Nanodrop), while RNA integrity was checked using an RNA formaldehyde gel.

Northern Blot

A total of 1-10 µg of RNA were loaded per lane in an RNA denaturing agarose gel, and separated at 70 V for about 1.5 h. RNA was transferred to a nylon membrane by the downward capillarity technique using Transfer Buffer (Bio-Rad, US), and cross-linked using a UV 302 nm transilluminator (Spectroline, US) for 3 min. The blots were maintained at 4°C in conical tubes until ready to analyze. Probes were synthesized using *in vitro* transcription and ³²P-radiolabeled alpha-UTP. The T7 promoter was included in the reverse primer. Probe sizes ranged between 150-250 nt. Hybridization was performed at 68°C overnight using the UltraHyb buffer and following manufacturer's instructions. Then the membrane was washed twice in 2× SSC buffer + 0.1% SDS at 68°C for 5 min. each, and then twice in 0.1 × SSC buffer + 0.1% SDS at 68°C for 15 min. The blotted membrane was wrapped in a plastic sheet, and exposed to a phosphorimaging cassette overnight prior to visualization using a Typhoon 7000 machine (GE, US).

Western Blot

Samples were collected before and after treatment with the antibiotics or diamide. A total of 5 ml were collected, washed in PBS, and resuspended in 150 µl of Buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 5% glycerol) supplemented with complete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche). Then cells were disrupted by sonication, and centrifuged for 15 min. at top speed at 4°C. The soluble fraction was collected and quantified using the Bradford Assay, with BSA as standard. Reducing sample buffer was added to the protein extract, and 10 µg of protein were loaded in a 4-20% SDS-PAGE. Proteins were transfer onto a PVDF membrane using the TransBlot Turbo Transfer System (Bio-Rad, USA). The membrane was blocked using 5% protein blotting blocker dissolved in TTBS for 1 h at RT. Then, the primary antibodies were resuspended in 0.5% protein blotting blocker dissolved in TTBS and incubated overnight at 4°C. Finally, an anti-rabbit HRP-conjugated secondary antibody was added and incubated for 1 h at RT. The membrane was washed four times in TTBS, once in TBS, and then visualized using the Clarity Western ECL substrate (Bio-Rad, USA).

Time-dependent killing assays

The cells were grown until OD₆₀₀ reached ~0.5. Then, cells were treated or not with different chemicals, and incubated at 37°C with agitation. At specific time points, samples were taken, washed in saline, and serially diluted in 0.15 M NaCl. Ten-µl aliquots of each dilution were plated on LB plates, incubated for 16 h at 28°C, and the resulting colonies enumerated.

Flow cytometry

The fluorescence of cells harboring the transcriptional fusions with *gfp* was determined using flow cytometry. Briefly, cells were grown until OD₆₀₀ reached ~0.5, and then were mock-treated or treated with ampicillin, fosfomycin, or diamide. Forty-seven minutes after

treatment, the cells were collected, washed twice in PBS, and finally resuspended in PBS buffer. Fluorescence was read in a BD FACS Aria using a 488 nm excitation laser. A total of ~50,000 cells were sampled per event.

AMS alkylation

To determine the oxidation state of the cysteine residues in Spx, cells were grown under standard conditions until OD₆₀₀ reached ~0.5, and then treated or not with cell wall antibiotics or diamide. At specific time points, 1.8 mL samples were collected and precipitated using 0.2 mL of 100% TCA, and then rapidly incubated on ice. The amount of protein was normalized by resuspending the cells to a final concentration of OD₆₀₀=1.0. The samples were then washed in ice-cold 100% acetone, and the pellet was allowed to dry for 10 min. at room temperature. Next, the precipitate was resuspended in 100 µl of denaturing buffer (2% SDS, 1 mM EDTA, 250 mM Tris-HCl, pH 6.8), and then supplemented or not with AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid disodium salt, ThermoFisher) to produce a ~20 fold molar excess relative to cell proteins (assuming an average size of 30 kDa for cytoplasmic proteins). Cells were sonicated until the lysate cleared and the alkylation reaction was incubated in the dark at 37°C for 2 h. For the fully reduced and alkylated samples, after sonication the samples were pre-treated with TCEP (in a ~20 molar excess to protein), and then alkylated using AMS as described. Non-reducing sample buffer was then added to the alkylation reactions, and 4.0 µg were loaded in a 4-20% SDS-PAGE gel for western blot.

RT-qPCR

Samples were taken as previously described, and the RNA was isolated using the RNeasy Kit (Qiagen) following manufacturer's instructions. The isolated RNA was treated with DNase I for 20 min. at 37 °C in order to eliminate genomic DNA contamination. Then, RNA was re-extracted using acidic phenol-chloroform followed by ethanol precipitation. The RNA was resuspended in DEPC-treated water, and the purity and quality were verified using a spectrophotometer (Nanodrop). The integrity of the RNA was monitored in native agarose gels. A total of 200 ng of RNA were reverse transcribed using TaqMan™ Reverse Transcription Reagents (Thermo-Fisher Scientific) following manufacturer's instructions. qPCR was carried out using the iTaq Universal SYBR Green Supermix (Bio-Rad) according to the instructions of the manufacturer. Primers for the 23S rRNA and *gyrA* gene were used as reference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

The *spx* gene is induced in response to cell wall stress. A) Organization of the *yjbC-spx* operon and location of the *spx* RNA probe. B) Northern blot analysis using an *spx* RNA probe and 5 μ g of total RNA per lane. Samples were taken 10 min. and 40 min. after treatment with 2 μ g ml⁻¹ ampicillin, 200 μ g ml⁻¹ fosfomycin, 250 μ g ml⁻¹ D-cycloserine, 1 μ g ml⁻¹ vancomycin, and 0.5 mM diamide (standard concentrations corresponding to 2 $\times$ MIC, unless otherwise stated). C) Analysis of the small transcript using a *lacZ* fusion. The intergenic promoters (i.e. P_{M2} and P_A) were fused to the *lacZ* gene, and their induction in response to fosfomycin, vancomycin, and diamide was studied by northern blot. Untreated cells were used as control. The position of the *lacZ* RNA probe is depicted. D) The contribution of the P_A promoter to induction of the *spx* gene was studied as described in Fig 1C, with the exception that only the region encompassing the P_A promoter was chosen for the transcriptional fusion (see Experimental Procedures). Each blot is representative of at least two biological replicates.

Fig 2.

Analysis of the *spx* promoter in response to cell wall stress. A) Sequences of the promoters in the *yjbC-spx* operon as stated in Fig 1A; additionally, the sequences of the mutant promoters are presented with an asterisk. The DNA bases in red color indicate the sites at which point mutations were introduced, and the underlined bases in the consensus sequence indicate the most conserved residues for the mentioned sigma factors. The -10 and -35 boxes are also displayed. B) The importance of the *spx* promoters was tested using transcriptional fusions of the promoters listed in Fig 2A with a *gfp* reporter. Cells were left untreated, or

treated with ampicillin ($2 \mu\text{g ml}^{-1}$) or fosfomycin ($200 \mu\text{g ml}^{-1}$), and fluorescence was measured after 47 min of treatment using flow cytometry (7 min. were allowed for maturation of GFPMut3). Error bars indicate standard error of the mean (SEM), $n=3$. One, two, and three asterisks indicate significant differences with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, as estimated using the Dunnett Test and comparing the treated samples against the control. NS indicates no significant differences. C) Induction of the ECF sigma factors under cell wall stress. The induction profile of *sigM*, *sigW*, and *sigX* was determined using RT-qPCR. Error bars indicate SEM ($n=3$). As observed, only the σ^M and σ^W regulons were induced under the conditions studied. D) The contribution of the ECF sigma factors for the induction of the P_{M1} promoter was tested by northern blot using an *spx* RNA probe. Samples were collected after 40 min of treatment or not with fosfomycin. A total of $5 \mu\text{g}$ of RNA was loaded per well. E) Analysis of the remaining transcript using northern blot and an *spx* RNA probe. The same conditions as in Fig 2D were used.

Fig 3.

Accumulation of the Spx protein under cell wall stress requires P_{M1} . A) The Spx protein accumulates upon treatment with ampicillin and fosfomycin as studied using western blot. B) Cells unable to induce the P_{M1} promoter were unable to accumulate Spx in the presence of fosfomycin. Complementation with an ectopic copy of *spx* driven from P_{M1} restored the wild-type phenotype. C) Accumulation of Spx in cells harboring P_{M1} or P_{M1}^* after treatment with diamide display no significant differences, suggesting that induction of P_{M1} is not required for the disulfide stress response. D) PG synthesis inhibitors, but not membrane antibiotics, induce the Spx regulon as monitored using western blot. Each blot is representative of at least three biological replicates.

Fig 4.

The Spx regulon is induced under cell wall stress. A) Northern blot analysis shows that *trxB*, *trxA*, *yjbH*, and *nfrA* are induced in response to 200 $\mu\text{g ml}^{-1}$ fosfomycin treatment in an Spx-dependent fashion. The RNA was isolated after 40 min. of treatment. B) Time-course experiment, using RT-qPCR, shows the dynamics of *trxB* expression. C) The expression of *trxB* was also studied in cells unable to activate the P_{M1} promoter using a P_{trxB} -*gfp* transcriptional fusion. Fluorescence was measured by flow cytometry 47 min. after treatment or not with ampicillin (2 $\mu\text{g ml}^{-1}$), fosfomycin (200 $\mu\text{g ml}^{-1}$), or diamide (0.5 mM). Each

experiment corresponds to at least three independent experiments. Error bars indicate SEM. One, two, and three asterisks indicate significant differences with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, as estimated using the Dunnett Test and comparing the mutant strains against WT. NS indicates no significant differences. D) Time-course experiment shows the dynamics of induction of three Spx-controlled genes in response to fosfomycin in WT, WT(P_{M1}^*), and *spx*.

Fig 5.

Analysis of the oxidation of Spx following cell wall stress. A) The CXXC redox-sensing switch is located at the N-terminal part of the Spx protein. Formation of a disulfide bond in presence of oxidants such as diamide fully activates the protein. B) A transcriptional fusion of the *trxB* promoter and *gfp* was used to monitor the activity of the Spx and *Spx^{C10A}* proteins. Fluorescence was measured using flow cytometry. One, two, and three asterisks indicate significant differences with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, as estimated using the T-test. C) Evaluation of the induction of four Spx-dependent genes in response to either fosfomycin (RNA harvested after 40 min. of treatment) or diamide (RNA harvested after 10 min. of treatment) in cells harboring either the *spx*, *spxC^{10A}*, or *spxC^{10AC13A}* allele by using northern blot. D) AMS alkylation experiments were carried out to determine the oxidation state of the Spx protein *in vivo*. 0.5 mM Diamide was included as positive control for oxidation of Spx, and vancomycin ($1 \mu\text{g ml}^{-1}$) was to monitor Spx following cell wall stress. Appropriate controls were included to estimate the molecular weight of alkylated Spx with zero, one, or two AMS molecules linked. A total of $4 \mu\text{g}$ of protein were loaded in each well. The concentrations of antibiotic were used as listed in Fig 1B. E) Both protein bands correspond to the same protein samples as in Fig 5C at time zero, but overexposed. The amounts listed below the gel correspond to the total amount of protein loaded in each well.

Fig 6.

Cells lacking Spx are sensitive to cell wall antibiotics. A) Time-killing experiments in WT, *spx* null mutant, and the complementation strain. Cells were grown until OD₆₀₀ reached ~0.5, and treated with ampicillin, fosfomycin, D-cycloserine, and diamide. Samples were taken each hour and survival was monitored by plating serial dilutions on plain LB plates. B) The survival of cells harboring the P_{M1}* promoter or *spx*^{C10A} allele was measured after 90 min of 2 $\mu\text{g ml}^{-1}$ ampicillin, 200 $\mu\text{g ml}^{-1}$ fosfomycin, 250 $\mu\text{g ml}^{-1}$ D-cycloserine, or 0.5 mM diamide treatment. One, two, and three asterisks indicate significant differences with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, as estimated using the Dunnett Test and comparing the mutant strains against WT. NS indicates no significant differences. The experiments are the result of at least five biological replicates.

Fig 7.

Model of Spx regulation under disulfide and cell wall stress. Under disulfide stress, the expression of *spx* is unchanged; reduced proteolysis due to YjbH aggregation and ClpXP oxidation results in Spx accumulation. Disulfide stress then leads to formation of an intramolecular disulfide bond in Spx, which results in high levels of induction of the Spx regulon. In comparison, cell wall stress leads to transcriptional induction of the *spx* gene through activation of the P_{M1} promoter in a σ^M -dependent fashion, and this induction is required for Spx accumulation. In addition, Spx may be stabilized against proteolysis under cell wall stress conditions, a process under investigation. Spx then accumulates primarily in its reduced form, and activates the Spx regulon. In both cases, induction of the Spx regulon is required for survival against stress.

Table 1

Strains used in this study

Strain	Genotype	Reference/Construction
168	<i>trpC2</i>	Lab strain
HB18801	<i>168 spx::kan</i>	Lab strain
HB18816	<i>168 spx::kan amyE::P_{spx}-spx-gfp (cm)</i>	This study
HB18907	<i>168 spx::kan amyE::P_{spx(PM1*)}-spx-gfp (cm)</i>	This study
HB18923	<i>168 spx::kan amyE::P_{spx}-spxC10A-gfp (cm)</i>	This study
HB18926	<i>168 amyE::P_{spx}-GFP (cm)</i>	This study
HB18650	<i>168 amyE::P_{spx(PM1*)}-GFP (cm)</i>	This study
HB18651	<i>168 amyE::P_{spx(PM2*)}-GFP (cm)</i>	This study
HB18652	<i>168 amyE::P_{spx(PB)}-GFP (cm)</i>	This study
HB18634	<i>168 amyE::P_{empty}-GFP (cm)</i>	This study
HB18640	<i>168 sigM::markerless</i>	This study
HB18646	<i>168 sigM::markerless thrC::sigM (ery)</i>	This study
HB18824	<i>168 sigW::mls</i>	BKE strain -> 168
HB18914	<i>168 sigW::mls sigM::tet</i>	BKE strain -> <i>sigM::tet</i>
HB18696	<i>168 spx::P_{spx}-spx (kan) amyE::P_{trxB}-gfp (cm)</i>	This study
HB18697	<i>168 spx::P_{spx}-spxC10A (kan) amyE::P_{trxB}-gfp (cm)</i>	This study
HB18698	<i>168 spx::P_{spx(PM1*)}-spx (kan) amyE::P_{trxB}-gfp (cm)</i>	This study
HB18699	<i>168 spx::kan amyE::P_{trxB}-gfp (cm)</i>	This study
HB18900	<i>168 sigX::spec</i>	Lab strain
HB18915	<i>168 sigX::spec sigM::tet</i>	Lab strain
HB18917	<i>168 sigX::spec sigM::tet sigW::mls</i>	Lab strain
HB13551	<i>168 sigB::cm</i>	Lab strain
HB23013	<i>168 spx::P_{spx(PM1*)}-spx (kan) amyE::P_{M1}-spx (cm)</i>	This study
HB23010	<i>168 thrC::P_{M2A}-lacZ (ery)</i>	This study
HB23011	<i>168 thrC::P_A-lacZ (ery)</i>	This study
HB18905	<i>168 spx::P_{spx(PM1*)}-spx (kan)</i>	This study
HB18805	<i>168 spx::kan amyE::P_{ITG}-spx (spec)</i>	Lab strain