Molecular Microbiology (2016) 00(00), 00–00 ■
doi: 10.1111/mmi.13304
First published online 00 Month 2016

Salt-sensitivity of σ^H and Spo0A prevents sporulation of *Bacillus subtilis* at high osmolarity avoiding death during cellular differentiation

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Summary

The spore-forming bacterium *Bacillus subtilis* frequently experiences high osmolarity as a result of desiccation in the soil. The formation of a highly desiccation-resistant endospore might serve as a logical osmostress escape route when vegetative growth is no longer possible. However, sporulation efficiency drastically decreases concomitant with an increase in the external salinity. Fluorescence microscopy of sporulation-specific promoter fusions to *gfp* revealed that high salinity blocks entry into the sporulation pathway at a very early stage. Specifically, we show that both Spo0A- and SigH-dependent transcription are impaired. Furthermore, we demonstrate that the association of SigH with core RNA polymerase is reduced under these conditions. Suppressors that modestly increase sporulation efficiency at high salinity map to the coding region of *sigH* and in the regulatory region of *kinA*, encoding one the sensor kinases that activates Spo0A. These findings led us to discover that *B. subtilis* cells that overproduce KinA can bypass the salt-imposed block in sporulation. Importantly, these cells are impaired in the morphological process of engulfment and late forespore gene expression and frequently undergo lysis. Altogether our data indicate that *B. subtilis* blocks entry into sporulation in high-salinity environments preventing commitment to a developmental program that it cannot complete.

Introduction

The soil is a challenging habitat for microorganisms. Both, the supply of nutrients and physical parameters like pH, temperature and osmolarity fluctuate frequently. *Bacillus subtilis* is a soil-dwelling bacterium (Mandic-Mulec *et al.*, 2015) that is well adapted to life in the upper layers of the soil (Earl *et al.*, 2008; Belda *et al.*, 2013). It counteracts various challenges through the induction of the σ^B-controlled general stress regulon (Hecker *et al.*, 2007; Price, 2011) and the engagement of stress-specific adaptation mechanisms (Sonenshein *et al.*, 2002). Furthermore, in response to nutrient limitation, *B. subtilis* can differentiate into a dormant and stress-resistant endospore (Errington, 2003; Higgins and Dworkin, 2012; Setlow, 2014). Here, we have investigated how *B. subtilis* simultaneously responds to two common challenges that it encounters in its native habitat: starvation and high salinity (Bremer and Krämer, 2000; Sonenshein, 2000; Bremer, 2002; Higgins and Dworkin, 2012).

In response to starvation, *B. subtilis* initiates a highly orchestrated developmental program involving hundreds of genes (Errington, 2003; Eichenberger *et al.*, 2004; Steil *et al.*, 2005; Higgins and Dworkin, 2012; Nicolas *et al.*, 2012). During this process, the cell undergoes an asymmetric division resulting in two cell types, the mother cell and forespore, which follow distinct developmental programs of gene expression driven by stage- and compartment-specific transcription factors. These programs are linked to each other through cell–cell signaling pathways, ensuring that

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gene expression in one cell is kept in register with gene expression in the other. Upon maturation of the developing forespore, lysis of the mother cell releases a highly stress-resistant endospore into the environment (Stragier and Losick, 1996; Errington, 2003; Higgins and Dworkin, 2012). *B. subtilis* spores can remain dormant in a given ecosystem for extended periods of time, yet can rapidly germinate in response to specific germinants that signal conditions are conducive for vegetative growth (Setlow, 2014; Sinai *et al*., 2015; Sturm and Dworkin, 2015).

Two transcription factors govern the entry into the sporulation pathway: the response regulator Spo0A and the alternative sigma factor σ^H. These regulators control the expression of hundreds of genes involved in stationary phase adaptation in addition to those involved in the earliest stages of spore formation. Both transcription factors are under complex and interconnected regulatory control (Hoch, 1993; Grossman, 1995; Sonenshein, 2000; Phillips and Strauch, 2002; Chastanet *et al*., 2010). A set of sensor kinases (KinA – KinE) trigger activation of Spo0A, the master regulator of sporulation, via a multicomponent phosphorelay (Burbulys *et al*., 1991; Stragier and Losick, 1996). In most cases, the specific signals sensed by these kinases are incompletely understood. Phosphorylated Spo0A (Spo0A∼P) directly and indirectly regulates the transcription of over 400 genes (Molle *et al*., 2003). Depending on the flux through the phosphorelay, different levels of Spo0A∼P are generated allowing a graded adaptive response since Spo0A-controled promoters have different affinities for Spo0A∼P (Chung *et al*., 1994; Fujita *et al*., 2005). The sporulation genes under Spo0A control have low-affinity Spo0A binding sites and therefore require high concentrations of Spo0A∼P for their effective expression (Fujita *et al*., 2005). One of the genes regulated indirectly by Spo0A∼P is *sigH* (also referred to as *spo0H*), which encodes the alternative sigma factor σ^H (Haldenwang, 1995; Stragier and Losick, 1996; Errington, 2003; Higgins and Dworkin, 2012). The globally acting repressor AbrB negatively regulates transcription of *sigH*, and Spo0A, in turn, represses *abrB* transcription. It does so at low Spo0A∼P concentrations thereby resulting in an early increase in σ^H levels (Strauch *et al*., 1990; Fujita *et al*., 2005). The elevated cellular level of σ^H then promotes expression of sporulation genes, including *spo0A*, *spo0F* (encoding one of the phosphorelay components) and *kinA*, the gene for the central sporulation-specific sensor kinase KinA. In addition, Spo0A∼P activates transcription of *spo0A* and *spo0F* genes (Britton *et al*., 2002; Chastanet and Losick, 2011). These positive feedback loops, in combination with finely tuned flux through the phosphorelay, increase Spo0A and σ^H levels and activity, resulting in the expression of early sporulation genes and entry into the sporulation pathway (Predich *et al*., 1992; Britton *et al*., 2002; Chastanet and Losick, 2011).

In its soil habitat (Earl *et al*., 2008; Mandic-Mulec *et al*., 2015), *B. subtilis* not only encounters starvation (Sonenshein, 2000; Errington, 2003), but desiccation also subjects it to high salinity (osmolarity) (Bremer and Krämer, 2000; Bremer, 2002). The cell responds to such osmotic challenges through increased potassium import (Whatmore *et al*., 1990; Holtmann *et al*., 2003), the synthesis of the compatible solutes proline and glycine betaine (Whatmore *et al*., 1990; Brill *et al*., 2011; Nau-Wagner *et al*., 2012), and the uptake of various osmoprotectants (Hoffmann *et al*., 2013; Zaprasis *et al*., 2013; Bashir *et al*., 2014; Broy *et al*., 2015). These cellular adjustments prevent efflux of water, maintain turgor at physiologically adequate levels and optimize the solvent properties of the cytoplasm for vital biochemical reactions (Bremer and Krämer, 2000; Bremer, 2002; Wood, 2011). As a result, growth of *B. subtilis* can continue under otherwise osmotically unfavourable circumstances (Boch *et al*., 1994).

The pronounced desiccation-resistant properties of the *B. subtilis* endospores (Setlow, 2014) suggest that spore formation would be an effective escape route from adverse high-salinity conditions (Bremer, 2002). However, contrary to expectations, high salinity efficiently blocks sporulation (Kunst and Rapoport, 1995; Ruzal and Sanchez-Rivas, 1998; Ruzal *et al*., 1998). Work from Sanchez-Rivas and coworkers indicates that this block is imposed relatively early in the sporulation process (Ruzal *et al*., 1998). However, the specific stage at which this occurs and the underlying molecular mechanism(s) have remained elusive.

Here, we have addressed these ecologically relevant questions and show that *B. subtilis* cells that are continuously exposed to high-salt environments are blocked at the earliest possible stage of sporulation. They fail to activate genes under the control of σ^H and Spo0A∼P. Synthetic over-production of KinA resulted in a nearly complete bypass of the block in sporulation initiation in salt-stressed cells. However, these cells were impaired in engulfment, late forespore gene expression and exhibited a high frequency of lysis. Collectively, our data suggest that the molecular block averting entry into sporulation under salt stress is not an accident. Rather, it appears to serve a physiologically important role by preventing osmotically stressed cells from committing to a developmental program they cannot complete. This trait is conserved among domesticated and non-domesticated strains of *B. subtilis*.

Results

High salinity drastically impairs spore formation

During the course of our studies on how *B. subtilis* copes with osmotic stress (Bremer and Krämer, 2000; Bremer, 2002), we found that cells grown for more than

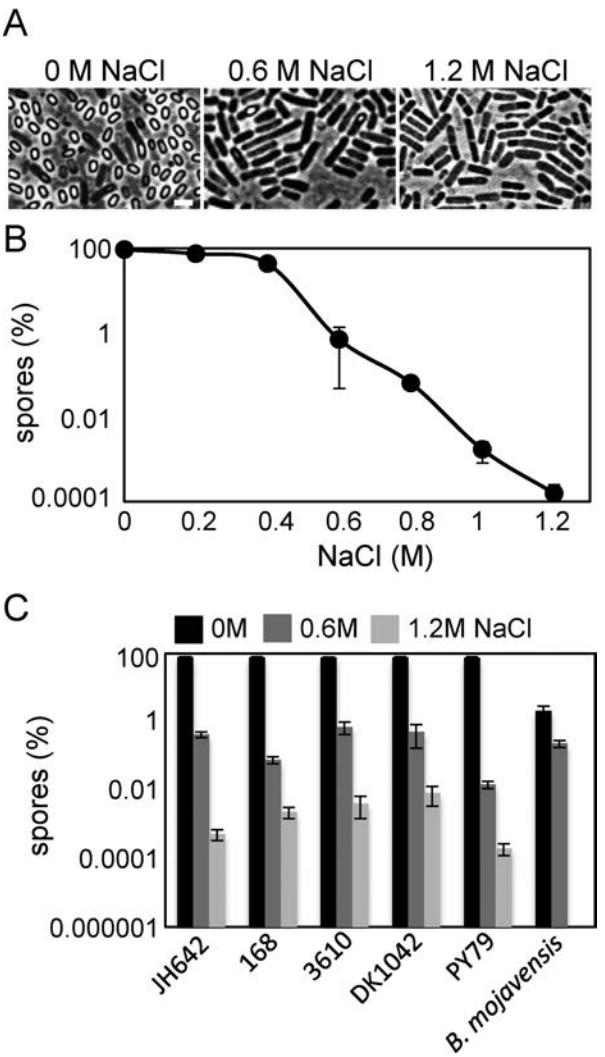

Fig. 1. Sporulation is inhibited by high salinity.
A. Representative phase-contrast images of cells of wild-type *B. subtilis* cells (JH642) sporulated by nutrient exhaustion for 36 h in the presence of the indicated concentrations of NaCl. The scale bar indicates 2 μm.
B. Quantitative assessment of increasing salt concentration on the formation of heat-resistant spores. Sporulation efficiency (spores %) indicates that number of heat-resistant CFU in the indicated concentration of NaCl compared to heat-resistant CFU in the absence of NaCl.
C. Bar graph showing the negative effects of 0.6 M and 1.2 M NaCl on sporulation of the indicated domesticated and nondomesticated *B. subtilis* strains and of *B. mojavensis*.

36 h in Difco Sporulation Medium (DSM) containing 1.2 M NaCl failed to produce phase bright spores (Fig. 1A). This was in stark contrast to the same culture conditions in medium lacking salt (0 M NaCl), in which almost all of the cells formed spores (Fig. 1A). To quantitatively assess the negative effect of high salinity on sporulation, we grew cells of the *B. subtilis* wild-type strain JH642 in DSM in the presence of various concentrations of NaCl and determined the frequency of heat-resistant spores using a standard heat-kill assay. In this assay, the starved cultures are incubated at 80°C for 20 min to kill vegetative and sporulation deficient cells and the number of heat-resistant colony-forming units (CFUs) that result from the outgrowth of the surviving spores are compared to total CFUs (and separately to heat-resistant CFUs in the absence of salt). Increasing salinity reduces growth rate (Boch *et al*., 1994) (Supporting Information Fig. S1) but the impact was less than twofold at the highest concentration of NaCl (1.2 M) used here. Cells grown in DSM in the absence of salt had a sporulation efficiency of ~90%. A systematic increase in the salt concentration (0.2 M, 0.4 M, 0.6 M NaCl) decreased this value to approximately 70%, 40% and 1%, respectively (Fig. 1B). An additional increase in the salinity led to an even further reduction in sporulation efficiency. Only about one in a million cells grown in the presence of 1.2 M NaCl formed a heat-resistant spore (Fig. 1B). For the remainder of this study, we focused our analysis on media containing either 0.6 M or 1.2 M NaCl.

To investigate whether the inhibition of sporulation by high salinity was specific to the wild-type strain JH642 (Smith *et al*., 2014), we examined the sporulation efficiency of both domesticated and undomesticated *B. subtilis* isolates. Although sporulation efficiency in the presence of salt was somewhat variable, all strains tested displayed salt sensitivity (Fig. 1C). We also investigated the sporulation efficiency of *Bacillus mojavensis*, a species that is indigenous to the Mojave Desert (Earl *et al*., 2012). We anticipated that this *Bacillus* species would have an increased tolerance to high salinity as a result of its adaptation to the drought cycles that it experiences in its desert habitat. However, sporulation of *B. mojavensis* was even more sensitive to high salinity than *B. subtilis*, as the presence of 1.2 M NaCl in the DS medium abolished sporulation entirely (Fig. 1C). Altogether, we conclude that the sporulation process is highly sensitive to even modest increases in the salinity of the environment and that this is a common trait of both domesticated and undomesticated *B. subtilis* strains.

High salinity blocks sporulation at a very early stage

Previous studies using electron microscopy to assess the stage at which sporulation is blocked in high salinity suggested that differentiation is inhibited after polar division (Ruzal *et al*., 1998). To examine a larger number of cells and multiple time points, we used fluorescence microscopy and visualized the morphological stages of sporulation with the membrane dye TMA-DPH. In the same experiment, we monitored the activity of the first forespore-specific transcription factor σ^F (Higgins and

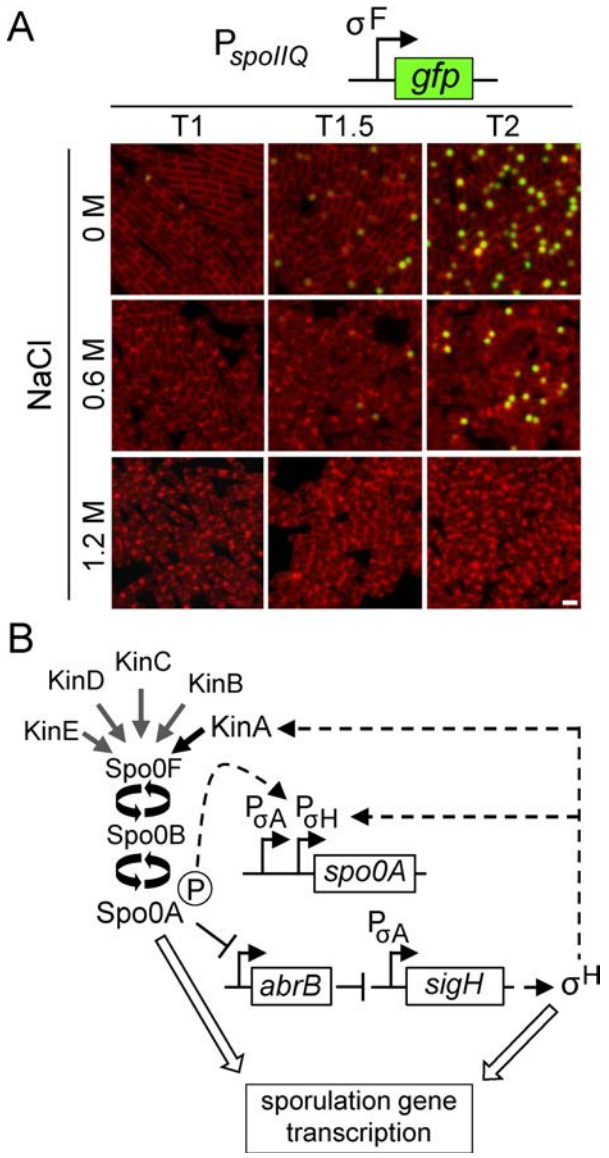

Fig. 2. High salinity inhibits sporulation prior to asymmetric cell division.
A. Representative images of cells (strain BDR1048; Supporting Information Table S1) harbouring a transcriptional reporter (P_{spoIIQ}-*gfp*) for the first forespore-specific transcription factor σ^F induced to sporulate in the indicated concentrations of NaCl. Membranes (false-coloured in red) were stained with TMA-DPH and σ^F-dependent expression is shown in green. Hour 1 (T1), 1.5 (T1.5) and 2 (T2) are shown. Additional time points can be found in Supporting Information Fig. S2. Scale bar is 2 μm.
B. Diagram illustrating part of the complex genetic circuitry involved in the activation of the transcriptional regulators σ^H and Spo0A for sporulation initiation.

Dworkin, 2012) using a P_{spoIIQ}-*gfp* promoter fusion (Londono-Vallejo *et al.*, 1997). We imaged the cells during a sporulation time-course at 30 min intervals after the onset of sporulation (T0) in cultures grown in the presence or absence of NaCl (Fig. 2A and Supporting Information Fig. S2). As reported previously, in the absence of salt >60% of the cells had a polar septum by hour 2 (T2) and most had initiated the process of engulfment (Rodrigues *et al.*, 2013). Furthermore, virtually all the sporulating cells exhibited σ^F-dependent GFP fluorescence in the forespore compartment (Fig. 2A). In the presence of 0.6 M NaCl, 21% ($n > 700$) of the cells had a polar septum and displayed expression of the σ^F reporter. However, in media with 1.2 M NaCl, virtually no cells exhibited asymmetric septation or GFP fluorescence even after 3 h of sporulation (Fig. 2A and Supporting Information Fig. S2). Thus, under our assay conditions, high salinity inhibits sporulation prior to polar division and before the activation of the first compartment-specific transcription factor, σ^F.

Spo0A and σ^H activity are impaired in the presence of high salt

Based on our cytological analysis, we sought to investigate the effects of high salinity on steps in the sporulation process that occur prior to the activation of σ^F. Accordingly, we examined the activity of the transcription factors Spo0A and σ^H that together govern entry into sporulation (Fig. 2B). First, we monitored the expression of a Spo0A-responsive reporter (P_{spoIIE}-*gfp*). The P_{spoIIE} promoter is recognized by σ^A but also requires active Spo0A (Spo0A~P) for its expression (York *et al.*, 1992; Molle *et al.*, 2003). In the absence of salt, Spo0A activity could be detected with this reporter during the first hour (T1) of sporulation and it continued to rise during asymmetric cell division and engulfment (Fig. 3A and Supporting Information Fig. S3). In media containing 0.6 M NaCl, a subset of cells displayed Spo0A-dependent transcription that was similar to the activity observed in the absence of salt. Furthermore, these 'Spo0A high' cells frequently went on to form polar septa (Fig. 3A). However, the majority of cells grown in the presence of 0.6 M NaCl exhibited low Spo0A-dependent transcription (Fig. 3A and Supporting Information Fig. S3). In the presence of 1.2 M NaCl, all cells in the field had virtually undetectable Spo0A activity (Fig. 3A and Supporting Information Fig. S3) consistent with the absence of polar septa (Supporting Information Figs S2B and S4). Thus, salt-stressed *B. subtilis* cells are unable to accumulate sufficient levels of phosphorylated Spo0A to promote the initiation of sporulation. Importantly, high salt did not impair GFP fluorescence as a σ^A-responsive promoter (P_{veg}) fused to *gfp* had strong fluorescence in the presence and absence of NaCl during sporulation (Supporting Information Fig. S5). This result also indicates that high salinity does not globally impair transcription in sporulation medium.

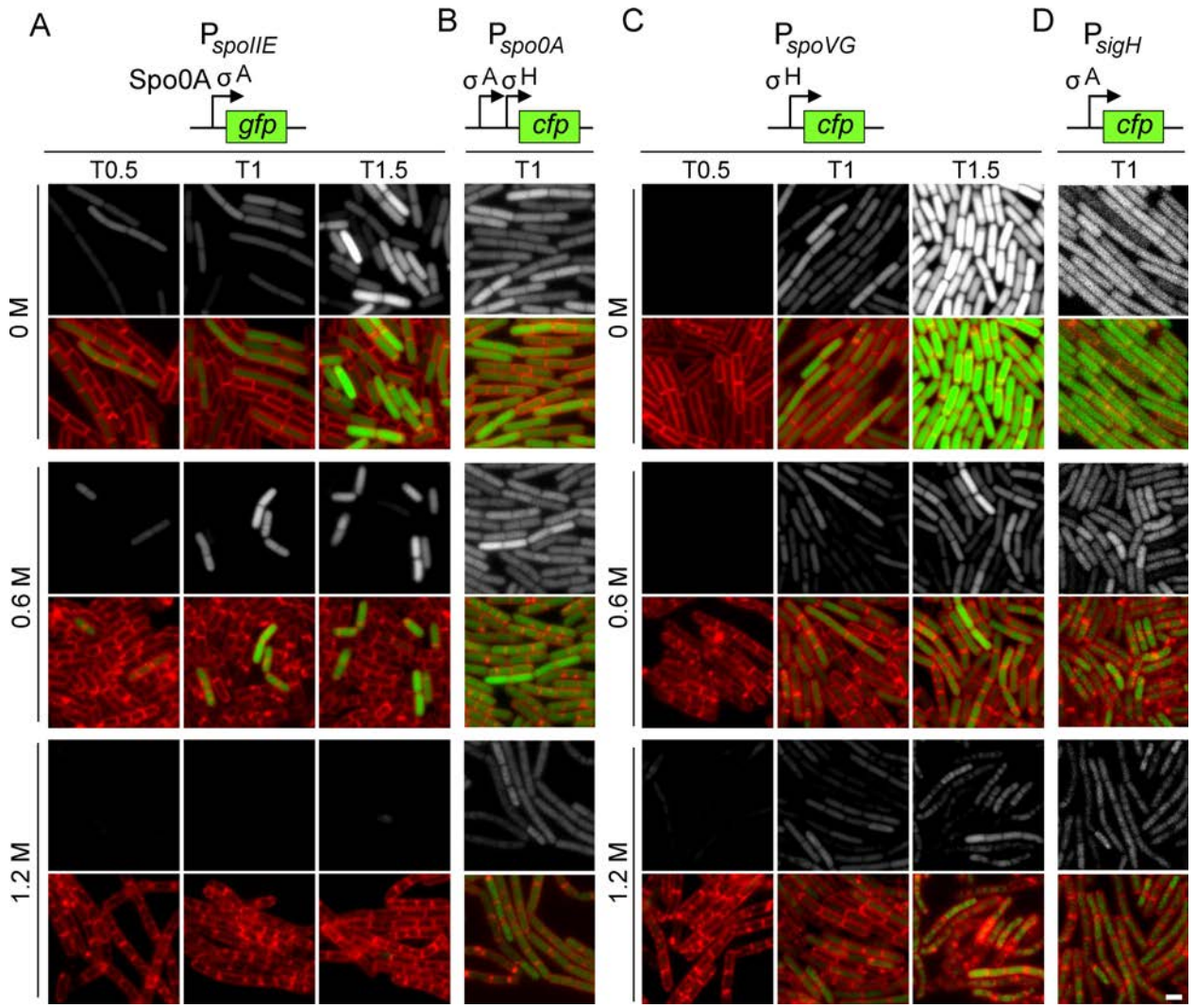

Fig. 3. Spo0A and σ^H activity are impaired in *B. subtilis* cells induced to sporulate at high salinity.
A. Representative images of cells (strain BDR2128; Supporting Information Table S1) harbouring a transcriptional reporter (P_{spoIIE}-*gfp*) monitoring Spo0A-responsive activity that were induced to sporulate in the indicated concentrations of NaCl. For each condition and time point (in hours), GFP fluorescence is shown in black/white and a merged image of membranes (red) and GFP (green) is shown below. The relevant transcription factors that act on the P_{spoIIE} promoter are indicated.
B. Representative images of cells (strain BDR3080; Supporting Information Table S1) harbouring a fusion of the *spo0A* promoter to *cfp* (P_{spo0A}-*cfp*) in the indicated concentrations of NaCl at hour 1 (T1) of sporulation.
C. Representative images of cells (strain BDR3064; Supporting Information Table S1) harbouring a transcriptional reporter (P_{spoVG}-*cfp*) for σ^H-dependent activity that were induced to sporulate in the indicated concentrations of NaCl. (D) Representative images of cells (strain BDR3090; Supporting Information Table S1) harbouring a fusion of the *sigH* promoter to *cfp* (P_{sigH}-*cfp*) at hour 1 of sporulation in the presence of the indicated concentrations of NaCl. For each reporter, all images were scaled identically. Scale bar indicates 2 μm.

To investigate whether transcription of the *spo0A* gene itself was reduced in high salinity, we examined the activity of the *spo0A* promoter using a P_{spo0A}-*cfp* reporter (Fig. 3B). *spo0A* transcription was modestly reduced in the presence 0.6 M NaCl and more severely at 1.2 M. The *spo0A* gene contains two promoters that are inversely controlled by Spo0A. The first promoter (P_v) is recognized by σ^A and negatively regulated by Spo0A∼P while the second promoter (P_s) is recognized by σ^H and activated by Spo0A∼P (Predich *et al.*, 1992; Strauch *et al.*, 1992; Eymann *et al.*, 2001; Chastanet and Losick, 2011). Thus, the reduction in *spo0A* expression could be due to the salt sensitivity of Spo0A or σ^H.

To investigate whether σ^H activity was influenced by high salt, we analyzed a σ^H-dependent promoter fusion (P_{spoVG}-*cfp*) (Segall and Losick, 1977; Zuber and Losick, 1987). The P_{spoVG} promoter is negatively regulated by AbrB, which is itself repressed by Spo0A at the transcriptional level. However, the extent of AbrB-mediated repression is relatively modest compared to the absolute

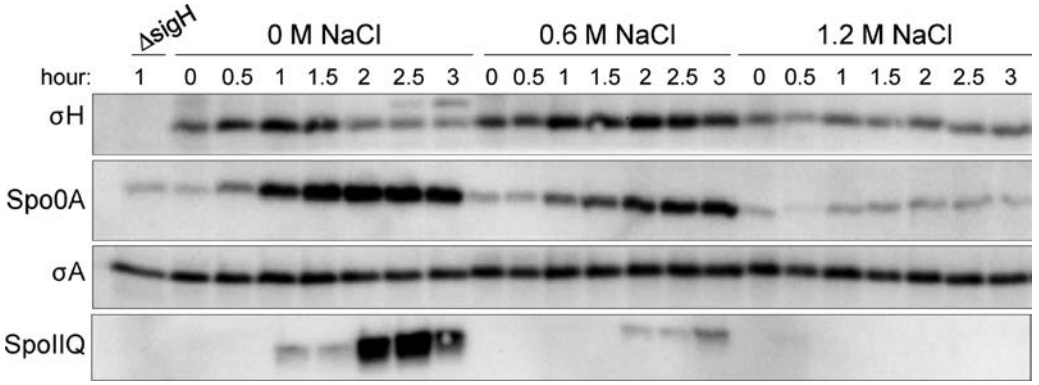

Fig. 4. Spo0A and σ^H protein levels are affected by high salinity. Immunoblot analysis assessing the levels of Spo0A, σ^H and σ^A in cells of the wild-type strain PY79 that were induced to sporulate in the presence of the indicated concentrations of NaCl. Forespore-specific σ^F activity was assessed by the accumulation of SpoIIQ. A *sigH* null mutant (strain BDR3057; Supporting Information Table S1) was included to show the requirement for σ^H activity on the accumulation of Spo0A.

requirement for σ^H (Zuber and Losick, 1987; Liu and Zuber, 2000) (Supporting Information Fig. S6). Accordingly, P_{spoVG}-*cfp* serves as a reliable reporter for σ^H activity. In cells grown in the absence of salt, σ^H-dependent gene expression was detectable at hour 1 (T1) of sporulation and increased significantly over the next half hour leading up to the formation of asymmetric septa (Fig. 3C). In the presence of 0.6 M and 1.2 M NaCl, the level of σ^H activity was slightly reduced at hour 0.5 and failed to increase over the next half hour (Fig. 3C). At hour 1.5, σ^H activity in cells grown in the presence of NaCl was significantly lower than the pre- and post-divisional cells at hour 1.5 that were sporulated in the absence of salt. Similar results were obtained using the P_{spoIIA} promoter fused to GFP (Supporting Information Fig. S7). However, since this promoter is under the control of both σ^H and Spo0A, the impact of high salinity was even more pronounced. Finally, transcription of the gene (*sigH*) encoding the σ^H sigma factor, which is indirectly controlled by Spo0A, was modestly reduced in the presence 0.6 M NaCl and more severely at 1.2 M (Fig. 3D). Collectively, these data suggest that σ^H and Spo0A activity and/or levels are reduced in the presence of high salinity resulting in a failure of *B. subtilis* cells to efficiently enter the sporulation pathway.

Spo0A and σ^H levels are differentially affected by high salinity

To directly assess the levels Spo0A, σ^H and σ^A we examined all three proteins by immunoblot analysis in a sporulation time course. Wild-type cells were induced to sporulate in the presence and absence of NaCl and samples were taken every half hour (Fig. 4). The levels of σ^A remained unchanged in all conditions throughout the entire sporulation time course. As previously reported (Fujita and Sadaie, 1998b,c; Liu and Zuber, 2000), in the absence of NaCl, σ^H levels began to increase within 30 min after the initiation of sporulation and achieved maximum levels 30–60 min later (Fig. 4). By hour 2, σ^H had returned to pre-sporulation levels. In the absence of salt, Spo0A levels closely followed the increase in σ^H, consistent with the requirement of σ^H for *spo0A* expression during sporulation (Predich *et al.*, 1992; Strauch *et al.*, 1992). However, unlike σ^H, Spo0A protein levels remained high for the 3 h time course. This is in line with the observation that Spo0A remains active in the mother cell at later times during the sporulation process (Fujita and Losick, 2003; 2005) (Supporting Information Fig. S3). In the presence of 0.6 M NaCl, σ^H levels increased in a manner similar to the no-salt condition (Fig. 4). This result and our analysis of σ^H activity using P_{spoVG}-*cfp* (Fig. 3C) suggest that σ^H activity is sensitive to high salinity. Interestingly, σ^H levels remained high for the rest of the time course in the presence of 0.6 M NaCl. The nature of this stabilization is currently unknown. However, comparing σ^H activity (assessed by P_{spoVG}-*cfp* fluorescence) and σ^H protein level in the presence and absence of 0.6 M NaCl at hour 2 reinforces the conclusion that σ^H activity is impaired in high salinity (Supporting Information Fig. S8). In contrast to σ^H, Spo0A protein levels increased more slowly in cells grown in the presence of 0.6 M NaCl, and never achieved as high levels as the no salt growth condition (Fig. 4). This is consistent with the salt-sensitivity of σ^H and the reduced activity Spo0A (Fig. 3A and Supporting Information Fig. S3). A comparison of *spo0A* transcription (Fig. 3B) and Spo0A accumulation (Fig. 4) suggests that Spo0A stability is also influenced by high salinity. Finally, both σ^H and Spo0A levels were

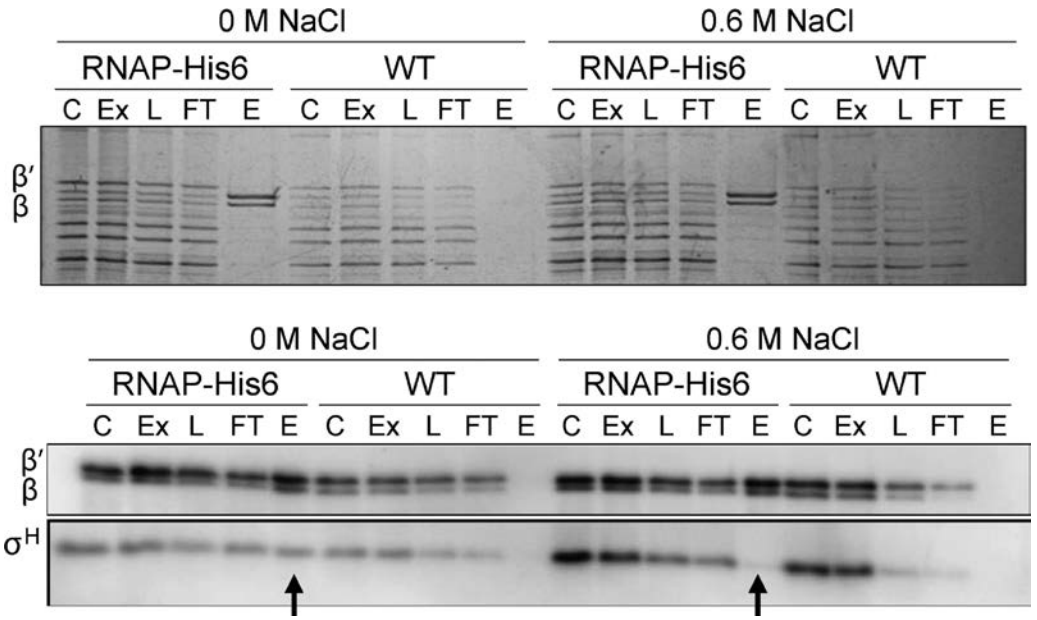

Fig. 5. The association of σ^H with core-RNA-polymerase is affected by high-salinity. Copurification of σ^H with core RNA polymerase from cells induced to sporulate in the indicated concentrations of NaCl. Top panel: Ni^{2+}-chelate affinity purification of β and β'-his6 (RNAP-His6) subunits from sporulating cells at hour 1.5. Whole-cell lysate [C], crude extract [Ex], load [L], flow-through [FT] and eluate [E] from the wild-type (WT) strain PY79 and from strain BDR485 (Supporting Information Table S1; RNAP-His6) were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Bottom panel: Immunoblot analysis of the same fractions used in the top panel using anti-core and anti-σ^H antibodies. Arrows highlight the relevant elution fractions for comparison.

significantly reduced and failed to accumulate in medium containing 1.2 M NaCl. Collectively, our data indicate that σ^H activity and Spo0A accumulation and activity are sensitive to high salinity and prevent cells from committing to the sporulation pathway.

The interaction between σ^H and core RNA polymerase is impaired in high salinity

We investigated whether the salt-sensitivity of σ^H *in vivo* was related to its inability to stably associate with core RNA-polymerase. To do this, we took advantage of a strain harbouring a functional hexa-histidine fusion to the beta prime subunit of RNA-polymerase (Fujita and Sadaie, 1998b,c). Cells were sporulated by resuspension in the presence and absence of 0.6 M NaCl. At hour 1.5 the cells were harvested by centrifugation and lysed in the presence of 300 mM NaCl followed by Ni^{2+}-NTA affinity purification of RNA-polymerase (see Materials and methods). The amount of copurified σ^H was then determined by immunoblot analysis (Fig. 5). In support of the idea that σ^H is impaired in its ability to associate with core RNA-polymerase in cells grown at high salinity, the amount of copurified σ^H was reduced in cells sporulated in medium containing 0.6 M NaCl compared to cells sporulated in the absence of salt. Importantly, the amount of core RNA-polymerase purified from the two extracts was similar and the level of σ^H in the lysate from the salt-sporulated cells was higher than from the no-salt cells (Fig. 5). We conclude from this set of experiments that the association of σ^H with core RNA polymerase is impaired during sporulation under high salinity conditions.

Enrichment for suppressors with increased sporulation at high salinity identifies mutations in kinA and sigH

On the basis of the results described above, we wondered whether it might be possible to isolate *B. subtilis* mutants that bypass the salt-imposed block in sporulation resulting in increased sporulation efficiency at high salinity. To this end, we used a genetic suppressor enrichment strategy. The wild-type *B. subtilis* strain JH642 was sporulated by nutrient exhaustion in DSM containing 1.2 M NaCl. After eliminating vegetative and sporulation-impaired cells by heat-kill (80°C for 20 min), serial dilutions were plated on LB-agar plates. The few heat-resistant colonies that emerged were then reinoculated into fresh DSM containing 1.2 M NaCl and the cycle was repeated. Mutants showing an increased sporulation phenotype in comparison to the JH642 wild type were isolated. Although the sporulation efficiencies of the 11 independently isolated suppressor strains were only moderately (threefold to eightfold) increased (Tables 1 and 2), we mapped the mutations in two of them by whole genome resequencing. Strikingly, in each

Table 1. Suppressor strains harbouring mutations in the *kinA* promoter region.

Strain	*kinA* promoter region sequence	Spores (%)	Fold increase
WT	TAGAAGGAGAATACTCATTTTCTAGCGAATCATACTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt – AAAGGAGGGATTCT**GTG** …	0.00016	-
NWB6	TAGAAGGAGAATACTCATTTTCTAGCGAATCATTCTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt – AAAGGAGGGATTCT**GTG** …	0.00086	5
NWB11	TAGAAGGAGATTACTCATTTTCTAGCGAATCATACTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt - AAAGGAGGGATTCT**GTG** …	0.00132	8
NWB13	TAGAAGGAGAAAACTCATTTTCTAGCGAATCATACTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt - AAAGGAGGGATTCT**GTG** …	0.00104	7
NWB16	TAGAAGGAGATTACTCATTTTCTAGCGAATCATACTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt - AAAGGAGGGATTCT**GTG** …	0.00129	8
NWB17	TAGAAGGAGAATACTCATTTTCTA-CGAATCATACTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt - AAAGGAGGGATTCT**GTG** …	0.00078	5
NWB19	TAGAAGGAGAATACTCATTTTCTAGCGAATCATACTAGGTAAAAGTCAATCTGTATTTGTCGAAA - 14 nt - AAAGGAGGGATTCT**GTG** …	0.00097	6
NWB22	TAGAAGGAGAATACTCATTTTCTAGCGAATCAAACTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt - AAAGGAGGGATTCT**GTG** …	0.00065	4
NWB24	TAGAAGGAGAATACTCATTTTCT-GCGAATCATACTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt - AAAGGAGGGATTCT**GTG** …	0.00081	5
NWB25	TAGAAGGA TAATACTCATTTTCTAGCGAATCATACTAGGTAAAAGTCAATCTGTATATGTCGAAA - 14 nt - AAAGGAGGGATTCT**GTG** …	0.00113	7

Suppressor strains that partially overcome the strong sporulation defect exhibited by the *B. subtilis* wild-type strain JH642 carry mutations in the promoter region of *kinA*. Mutations are highlighted in red. Predicted σ^H- (grey) and Spo0A- (black) recognition sites are indicated. Bold letters represent the start codon (GTG) of the *kinA* coding sequence and the ribosome-binding site is underlined. The spore titers and fold increases in sporulation efficiency of the suppressor strains are indicated.

Table 2. Suppressor strains harbouring mutations in the coding region of *sigH*.

Strain	Mutation	Spores (%)	Fold increase
WT	–	0.00016	–
NWB7	E146A [GAA→GCA]	0.00055	3
NWB21	I149F [ATT→TTT]	0.00067	4

Suppressor strains that partially overcome the strong sporulation defect exhibited by the *B. subtilis* wild-type strain JH642 carrying mutations in the coding region of *sigH*, the structural gene for the σ^H sporulation-specific sigma factor. The two mutations resulting in single amino acid substitutions in σ^H are indicated. The spore titers and fold increases in sporulation efficiency of the suppressor strains are indicated.

of these two suppressors only a single nucleotide polymorphism was observed in comparison to the JH642 reference genome (Smith *et al*., 2014). In strain NWB6, we identified a point mutation in the promoter region of *kinA* (Table 1), and in strain NWB7 a point mutation in the coding region of the *sigH* gene was present that resulted in an amino acid substitution (E146A) in the σ^H protein (Table 2). With this information in hand, we PCR-amplified and sequenced the *kinA*, *sigH* and *spo0A* genes from the remaining nine suppressor strains. This analysis identified eight additional mutant alleles of the *kinA* promoter (Table 1) and one further allele of the *sigH* coding region (Table 2). None of the 11 suppressor strains harboured a mutation in *spo0A*. We also sequenced *kinA* and *sigH* (and their promoters) from *B. subtilis* 168, PY79, 3610 and DK1042 to investigate whether the strain-to-strain variation in sporulation efficiency at high salinity (Fig. 1C) could be explained by polymorphisms in these loci. Both genes and their promoter regions were identical to those in JH642, indicating that other differences among these strains account for the observed variability.

Overexpression of kinA upon entry into sporulation bypasses the early salt-stress imposed block

The identification of suppressor mutations in the promoter of *kinA* that modestly increased sporulation under high salinity (1.2 M NaCl) conditions, prompted us to investigate whether higher levels of KinA could overcome the salt-sensitive block. σ^H is required for the expression of both *spo0A* and *kinA* (see Fig. 2B) and thus it seemed possible that the overexpression of *kinA* could trigger enhanced sporulation in hypertonic medium. Accordingly, we used a strain in which *kinA* is expressed under the control of a strong IPTG-inducible promoter ($P_{hyperspank}$). Previous work from Fujita and coworkers has shown that increased expression of *kinA* using this expression system can bypass the need for starvation signals and induce sporulation during growth in rich medium

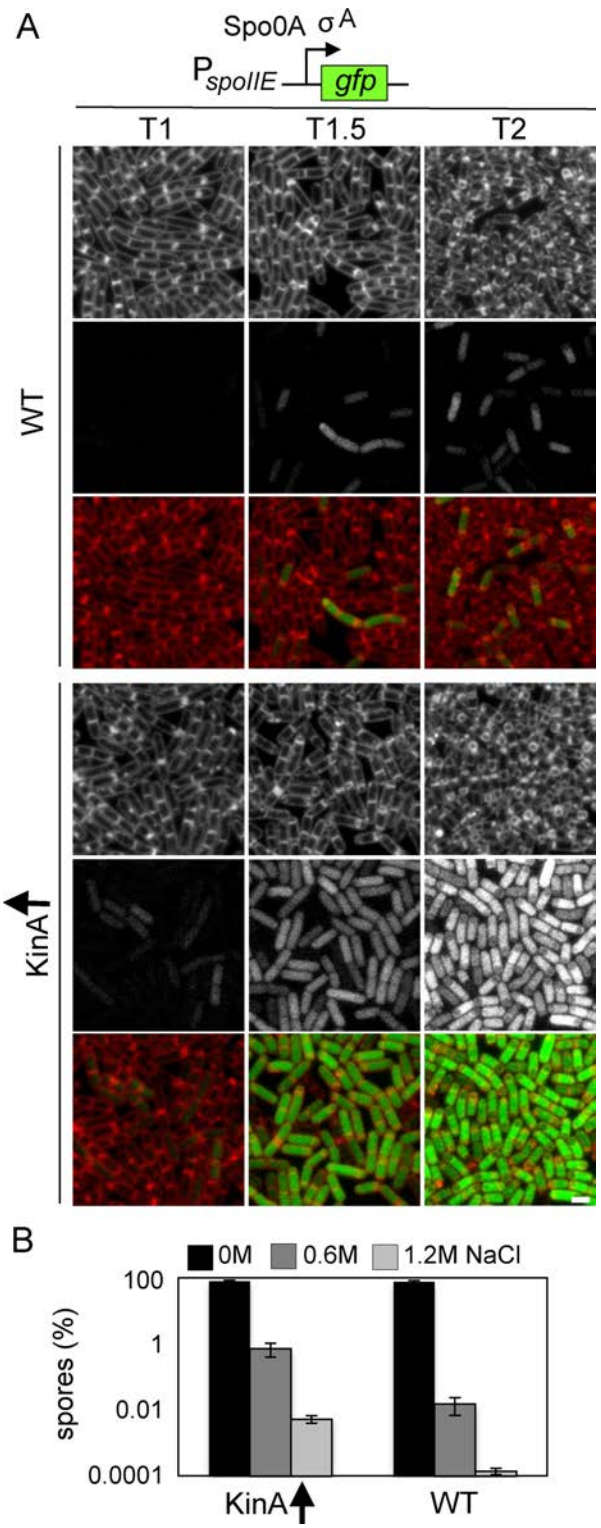

Fig. 6. Overexpression of *kinA* bypasses the early salt-sensitive block in sporulation.
A. Representative images of cells harbouring a Spo0A activity reporter (P_{spoIIE}-*gfp*) induced to sporulate in the presence of 0.6 M NaCl. Wild-type (WT; strain BDR2128) (Supporting Information Table S1) and a strain (BDR3087; Supporting Information Table S1) harbouring an IPTG-inducible allele of *kinA* were imaged at the indicated times after the initiation of sporulation. A 5 h sporulation time course of cells overexpressing *kinA* (BDR3087) comparing 0 M, 0.6 M and 1.2 M NaCl can be found in Supporting Information Fig. S9. Scale bar indicates 2 μm.
B. Bar graphs of sporulation efficiency in the wild-type (WT) and the *kinA* overexpression strain induced to sporulate in the presence of the indicated concentration of NaCl.

(Fujita and Losick, 2005; Eswaramoorthy *et al.*, 2009; Eswaramoorthy *et al.*, 2010). We induced sporulation in the presence of 0, 0.6 and 1.2 M NaCl and monitored Spo0A activity using the P_{spoIIE}-*gfp* reporter and followed sporulation with the fluorescent membrane dye TMA-DPH (Fig. 6A and Supporting Information Fig. S9A). Overexpression of *kinA* in all three conditions resulted in similar levels of KinA protein (Supporting Information Fig. S9B). Strikingly, KinA overproduction at 0.6 M NaCl resulted in high Spo0A activity and a statistically significant increase ($>$79%, $n > 430$) in the number of cells with polar divisions and engulfing forespores at hour 2 (T2) (Fig. 6A and Supporting Information Fig. S9). Even in the presence of 1.2 M NaCl, asymmetric septa and engulfing forespores were readily detectable, albeit with some delay compared to 0.6 M NaCl and the no-salt condition (Supporting Information Fig. S9). Thus, these results indicate that increasing the level of phosphorylated Spo0A can bypass the salt-sensitivity of σ^H and any other possible early blocks to sporulation.

Importantly, over-expressing *kinA* from the $P_{hyperspank}$ promoter significantly increased the number of heat-resistant spores in the presence of 0.6 M and 1.2 M NaCl (Fig. 6B). However, despite the near complete bypass of the early morphological events in 0.6 M NaCl (Fig. 6A and Supporting Information Fig. S9), the sporulation efficiency was still considerably reduced (by ~100-fold) compared to the no salt condition in which about 90% of the cells formed heat resistant spores (Fig. 6B). These data suggest that high salinity also impairs additional, later steps in the sporulation pathway.

High salinity impairs engulfment, σ^G activity and causes lysis

To define additional steps in the sporulation program that are sensitive to high salinity, we took advantage of a strain that harbours fluorescent reporters for all of the sporulation-specific sigma factors (Meeske *et al.*, 2016). In this strain, the reporter for the first compartment-specific transcription factor σ^F was a promoter (P_{spoIIQ}) fused to *yfp* (P_{spoIIQ}-*yfp*) (Londono-Vallejo *et al.*, 1997). The P_{spoIID} promoter that is recognized by the first

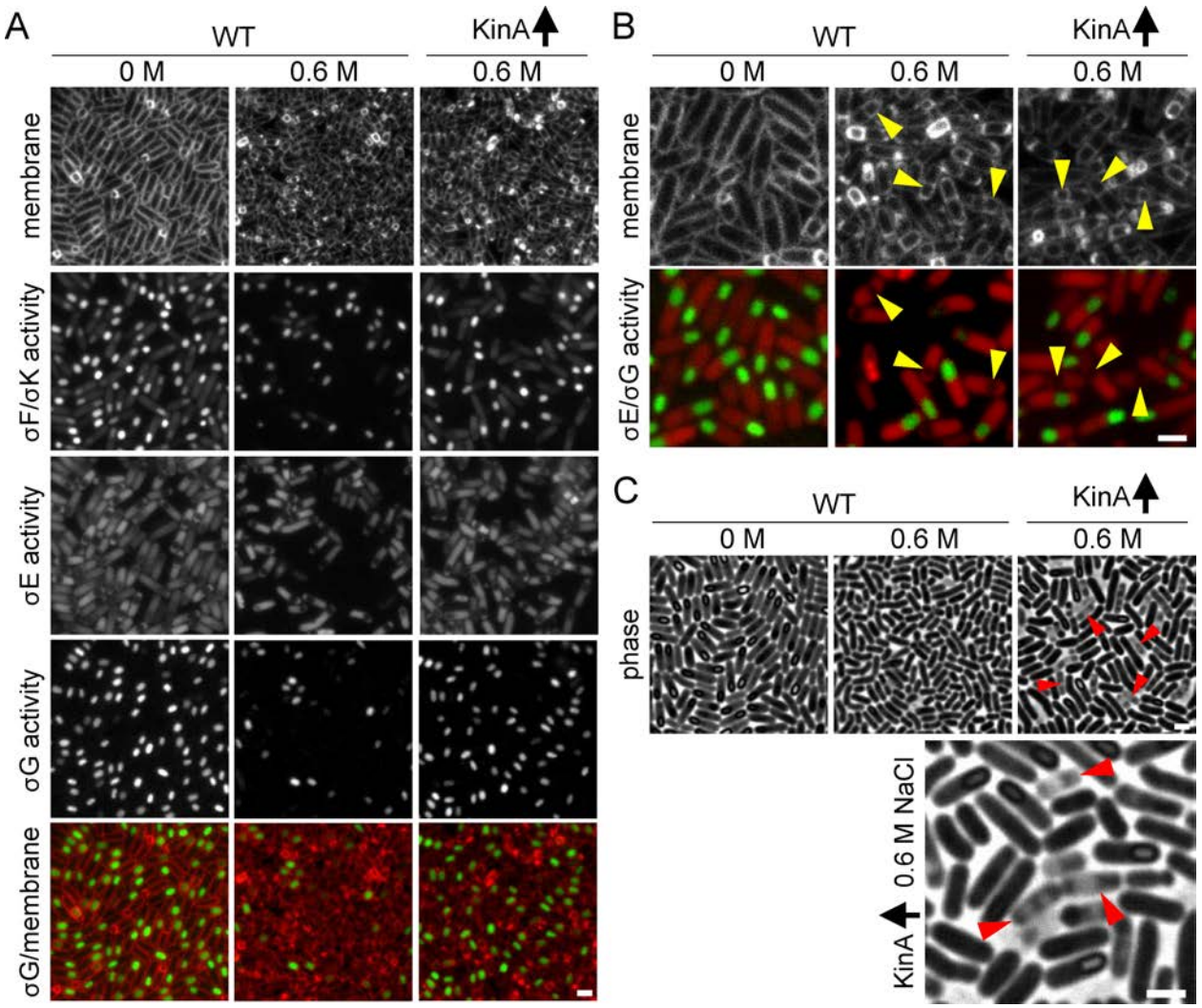

Fig. 7. High salinity impairs engulfment and late forespore gene expression and causes cell lysis.
A. Representative images of cells harbouring transcriptional reporters for all four stage- and compartment-specific sigma factors induced to sporulate in the presence of the indicated concentrations of NaCl. The membranes and reporters for σ^F (P_{spoIIQ}-*yfp*), σ^E (P_{spoIID}-mCherry), σ^G (P_{sspB}-*cfp*) and σ^K (P_{gerE}-*yfp*) are shown in black/white or as a merged image with membranes (red) and σ^G activity (green). Wild-type (WT) sporulating cells (strain BCR1071; Supporting Information Table S1) and a strain overexpressing *kinA* (BCR1274; Supporting Information Table S1) were imaged at hour 4 of sporulation. Additional time points can be found in Supporting Information Fig. S10A.
B. Representative images highlighting impaired engulfment and the defect in σ^G activation in a subset of sporulating cells grown in the presence of 0.6 M NaCl. Forespores with reduced σ^G activity are indicated (yellow arrow heads). Images show membranes (top) and an overlay of the σ^G activity reporter (green) and the σ^E activity reporter (red).
C. Representative phase contrast images from hour 5 of sporulation highlighting the increased frequency of cell lysis and reduction in phase-grey and phase-bright spores when cells bypass the early salt-sensitive block to sporulation. Additional time points can be found in Supporting Information Fig. S10B. Lysed cells are highlighted by red arrow heads. Scale bar indicates 2 μm.

mother-cell-specific transcription factor σ^E (Rong *et al.*, 1986) was fused to *mCherry* (P_{spoIID}-*mCherry*), and the reporter for the late forespore transcription factor σ^G was a promoter (P_{sspB}) fused to *cfp* (Nicholson *et al.*, 1989). Finally, the P_{gerE} promoter, recognized by the late mother-cell transcription factor σ^K (Cutting *et al.*, 1989), was fused to *yfp* (P_{gerE}-*yfp*). σ^F-dependent YFP fluorescence is first observed in the forespore at hour 2. By the time σ^K is activated in the mother cell (hour 4), σ^F activity is largely absent and YFP fluorescence is significantly reduced. Accordingly, we could use this fluorescent protein twice.

We introduced the $P_{hyperspank}$-*kinA* construct into this multireporter strain and induced sporulation in the presence of 0.6 M NaCl and IPTG. Over-expression of *kinA* resulted in activation of σ^F in the forespore and σ^E in the mother cell in almost all the sporulating cells (Fig. 7A and Supporting Information Fig. S10A). Interestingly,

many cells exhibited defects in the morphological process of engulfment: the migration of the mother-cell membranes around the forespore was uncoordinated and the engulfed forespores were smaller and contained membrane aberrations (Figs 6A, 7A and B, Supporting Information Figs S9A and S10A). Furthermore, although many sporulating cells displayed forespore-specific σ^G activity after the completion of engulfment, a subset of cells appeared to have reduced or undetectable levels (Fig. 7A and B and Supporting Information Fig. S10A). These phenotypes were also apparent in the 0.6 M NaCl condition where *kinA* was not overexpressed (Fig. 7A and B). Phase contrast images at later time points revealed a second and more striking phenotype: a reduction in phase grey spores and a concomitant increase in lysed cells (Fig. 7C and Supporting Information Fig. S10). Altogether, our results indicate that membrane remodeling and σ^G activity in the forespore display salt-sensitivity but the principal defect in spore formation in the presence of high salt is lysis of the sporulating cell prior to the maturation of the dormant spore.

Discussion

Building on previous observations (Kunst and Rapoport, 1995; Ruzal *et al*., 1998), we report here that spore formation in *B. subtilis* is highly sensitive to even modest increases in the external salinity, an environmental challenge that leads to an almost exponential drop in sporulation efficiency once a threshold of 0.6 M NaCl is achieved (Fig. 1B). Interestingly, at this level of external salinity, growth of *B. subtilis* begins to be impaired (Boch *et al*., 1994) and the cellular osmostress protection mechanisms of vegetative cells (e.g. the import of osmostress protectants and the synthesis of the compatible solutes) are already active (Brill *et al*., 2011; Nau-Wagner *et al*., 2012; Hoffmann *et al*., 2013). Under extreme saline conditions (1.2 M NaCl) where growth of *B. subtilis* is more significantly retarded (Boch *et al*., 1994), only one out of a million vegetative cells can form a mature, heat-resistant endospore (Fig. 1B). The goal of our study was to define the molecular basis for the salt-stress-imposed block in sporulation. Data reported by Sánchez-Rivas and coworkers (Ruzal and Sanchez-Rivas, 1998; Ruzal *et al*., 1998) had already indicated that this happened early in the sporulation process but the underlying molecular mechanism(s) and the precise stage at which the salt-stress-imposed block occurred remained ill defined. Our data clarify these issues.

Our study shows that entry into sporulation by the *B. subtilis* cell is already impaired prior to polar division and the activation of the forespore-specific transcription factor σ^F (Fig. 2A, Supporting Information Figs S2 and S4). Since cells that have formed the asymmetric septum and activated σ^F are fully committed to the sporulation process (Errington, 2003; Dworkin and Losick, 2005; Higgins and Dworkin, 2012), the high-salinity-imposed block occurs prior to the 'point of no return'. Consistent with these findings, we further show that gene expression under the control of the master regulator of sporulation, Spo0A, and of the earliest acting sporulation-specific sigma factor σ^H, was strongly affected by high salinity (Fig. 3). Our data suggest that the molecular underpinnings for these defects rest on an insufficient cellular level of active Spo0A (Fig. 4), and the inability of σ^H to associate effectively with core RNA-polymerase (Fig. 5).

Previous work from Hecker and coworkers (Reder *et al*., 2012a,b) demonstrated that the sigma factor (σ^B) that controls the general stress regulon (Hecker *et al*., 2007; Price, 2011) activates *spo0E* encoding a phosphatase that attenuates flux through the phosphorelay. Although salt-shocks are among the strongest inducers of the general stress response in *B. subtilis* (Nannapaneni *et al*., 2012), induction of σ^B-controlled genes by this environmental cue is only short-lived (Young *et al*., 2013). Importantly, in cells experiencing chronic exposure to high salinity, as was used in our experiments, the general stress regulon is not induced (Spiegelhalter and Bremer, 1998). Accordingly, it is unlikely that the σ^B-dependent down-regulation of Spo0A$\sim$P makes a significant contribution to the early block in sporulation observed here. In support of this idea, the reduction in sporulation efficiency resulting from high salinity was indistinguishable between wild-type and an isogenic Δ*sigB* mutant (Supporting Information Fig. S11).

The genetic control of *spo0A* transcription is complex and involves just-in-time regulatory events (Chastanet *et al*., 2010; Chastanet and Losick, 2011) that ensure that the cellular level of Spo0A$\sim$P increases in such a fashion that promoters with different affinities for this transcription factor become active at defined time points during starvation (Fujita and Losick, 2005; Fujita *et al*., 2005). To initiate spore-formation, high threshold levels of Spo0A$\sim$P and proper activation dynamics are required (Grossman, 1995; Molle *et al*., 2003; Fujita *et al*., 2005; Vishnoi *et al*., 2013). Our immunoblot analysis assessing Spo0A levels indicate that reduced amounts of Spo0A are present in cells cultured in the presence of 0.6 M NaCl and they are even further decreased when the salinity is increased to 1.2 M NaCl (Fig. 4). Hence, the inability of *B. subtilis* cells to sporulate efficiently at high salinity is likely explained by the insufficient accumulation of the master regulator of sporulation, Spo0A. Our data suggest that Spo0A protein is itself sensitive to high salinity (Figs 3B and 4) although

the molecular mechanism underlying this instability remains unknown. However, a second salt-sensitive event that contributes to the drop in Spo0A levels is the reduced association of σ^H with core RNA-polymerase (Fig. 5). Since the σ^H-dependent promoter of *spo0A* is a key genetic control element in setting cellular levels of Spo0A (Predich *et al*., 1992; Chibazakura *et al*., 1995; Eymann *et al*., 2001), insufficient amounts of the σ^H-RNA-polymerase holoenzyme will contribute to a failure to achieve sporulation-promoting levels of Spo0A (Hoch, 1991; Predich *et al*., 1992; Britton *et al*., 2002).

We do not know the reasons why σ^H associates ineffectively with core RNA-polymerase (Fig. 5). One possibility is that an increase in ECF sigma factors in response to salt stress competes for core. However, we favour the idea that an altered composition of the cytoplasmic ion and solute pools is the culprit. Indeed, a *B. subtilis* mutant defective in the major Na^+-sodium extrusion system Mrp (Ito *et al*., 1999; Swartz *et al*., 2005) not only becomes highly sensitive to Na^+ ions but also exhibits a severe sporulation defect (Kosono *et al*., 2000). *B. subtilis* typically maintains a very low cytoplasmic Na^+ level. As assessed by ^{23}Na-nuclear magnetic resonance spectroscopy, the intracellular Na^+ pool in an *mrp* mutant increases from practically nonmeasurable levels in the wild-type strain to approximately 12 mM under growth conditions where the external NaCl concentration was only 80 mM (Gorecki *et al*., 2014). Since the Mrp system functions as a Na^+/H^+ antiporter, its operation is also connected to pH homeostasis (Swartz *et al*., 2005; Krulwich *et al*., 2011). It is, therefore, of interest to consider data reported by Zuber and coworkers who found, as reported here for salt-stressed cells, a defect in the association of σ^H with core RNA-polymerase in *B. subtilis* cells that were subjected to low pH stress (Liu *et al*., 1999). The ion pool, in particular that of potassium, is certainly different in osmotically stressed cells from those that are not subjected to this challenge (Whatmore *et al*., 1990). Furthermore, transient changes in the intracellular Na^+ content of high-salinity-exposed *B. subtilis* cells might occur as a consequence of the operation of osmotically induced and Na^+-driven osmostress protectant importers for glycine betaine (OpuD) and proline (OpuE) (Kappes *et al*., 1996; von Blohn *et al*., 1997).

Suppressor mutants with single nucleotide polymorphisms that map either in *sigH* or in the promoter region of *kinA* partially bypassed the salt-stress-imposed block in sporulation (Tables 1 and 2). Although these mutations only modestly enhanced sporulation under high salinity, they provided additional support for the idea that Spo0A and σ^H activities are impaired under these conditions. Consistent with our findings that the interaction between σ^H and core RNA polymerase is reduced in high salt, the two amino acid substitutions in σ^H (E146A and I149F) both reside in the putative helix-turn-helix located within subregion 3.1, which is associated with sigma binding to core RNA polymerase (Murakami and Darst, 2003). All the other suppressor mutations map in the regulatory region of *kinA* (Table 1), a gene that is dependent on σ^H and Spo0A for its expression (Predich *et al*., 1992; Fujita and Sadaie, 1998a). Since these mutations likely increase *kinA* transcription, we tested a strain in which *kinA* could be overexpressed. Strikingly, KinA overproduction almost completely bypassed the block to entering the sporulation pathway in 0.6 M NaCl (Fig. 6A and Supporting Information Fig. S9). It also significantly increased the frequency of mature, heat-resistant spores; however, sporulation efficiency was still 100-fold lower than that observed in the absence of salt stress (Fig. 6B). This difference can be explained, in part, by impaired engulfment and reduced σ^G activity but is principally due to cell lysis prior to maturation of the spore (Fig. 7, Supporting Information Figs S9 and S10).

At first glance, the presence of a salt-stress imposed block to sporulation runs contrary to the idea that highly desiccation-resistant endospores (Setlow, 1995; Nicholson *et al*., 2000; Setlow, 2014) might serve as an effective osmostress escape route when vegetative growth is no longer possible (Boch *et al*., 1994). One possible explanation for this block is that *B. subtilis* has evolved an alternative strategy to survive starvation at high salinity that is more compatible with lower activities of σ^H and Spo0A. Indeed, we find that wild-type cells retain nearly complete viability in 1.2 M NaCl for at least 24 h after they have exhausted their nutrients (Supporting Information Fig. S12). After 24 h, the viability of these starved and salt-stressed cells steadily declines.

An alternative view places the strong and early block to sporulation by high salinity in a wider cellular context that takes the physiological status of starving (Errington, 2003; Eichenberger *et al*., 2004; Higgins and Dworkin, 2012) and salt-stressed cells (Bremer and Krämer, 2000; Bremer, 2002) into account. The commitment to sporulate is a measure of last resort after the cell has run out of other options (Lopez *et al*., 2009; Vlamakis *et al*., 2013). It is a time-consuming and energy demanding process and involves the coordinated transcription of almost a quarter of the genome (Fawcett *et al*., 2000; Molle *et al*., 2003; Eichenberger *et al*., 2004; Steil *et al*., 2005; Nicolas *et al*., 2012). Even under favourable laboratory conditions, it takes about 7 h to complete (Eichenberger *et al*., 2004), and after polar division and the first compartment-specific transcription factors are activated, a point of no return in this developmental program is reached (Errington, 2003; Higgins and Dworkin, 2012). Hence, the *B. subtilis* cell

must assure that the dwindling nutritional and energetic resources available to it, and the environmental circumstances, will allow completion of the sporulation process. Otherwise, all things are lost: the mother cell will lyse and no spore will be formed.

High salinity environments impose considerable constraints on the physiology and growth of the *B. subtilis* cell (Boch *et al*., 1994; Bremer and Krämer, 2000; Bremer, 2002). A key event in its defense against osmotic stress is the *de novo* synthesis and high-level accumulation of the compatible solute proline to maintain physiologically adequate levels of cellular hydration and turgor (Whatmore *et al*., 1990; Brill *et al*., 2011). The size of the proline pool is linearly dependent on the degree of the environmentally imposed osmotic stress and reaches about 500 mM in *B. subtilis* cells exposed to very high (1.2 M NaCl) saline growth conditions (Brill *et al*., 2011; Hoffmann *et al*., 2013; Zaprasis *et al*., 2013). Genetic disruption of the osmostress-adaptive proline synthesis route makes *B. subtilis* highly salt sensitive, highlighting the important role of the pool of this compatible solute for growth under osmotically unfavourable circumstances (Brill *et al*., 2011). The synthesis of a single proline molecule requires the expenditure of ~20 high-energy phosphate bonds (Akashi and Gojobori, 2002). Hence, the maintenance of proline up to levels of 500 mM (Hoffmann *et al*., 2013; Zaprasis *et al*., 2013) is an enormous physiological task for starving *B. subtilis* cells that are faced with entering the sporulation pathway while simultaneously experiencing severe osmotic stress. The data presented here suggest that the block to sporulation under salt stress is not an accident; rather we propose it serves as a safeguard to avert osmotically stressed *B. subtilis* cells from committing to a developmental program they cannot complete and would die trying.

Experimental procedures

General methods

B. subtilis strains were derived from 168, JH642 or PY79. Unless otherwise indicated, cells were grown in LB (Lennox) or CH (Sterlini–Mandelstam) media at 37°C. Sporulation was induced by resuspension at 37°C according to the method of Sterlini–Mandelstam or by nutrient exhaustion in supplemented DS medium (DSM complete) (Harwood and Cutting, 1990). Sporulation efficiency was determined in cultures grown for 24–36 h as the total number of heat-resistant (80°C for 20 min) colony-forming units (CFUs) compared with the total CFUs or heat-resistant CFUs of cells sporulated without NaCl. Tables of strains, plasmids and oligonucleotide primers and a description of strain and plasmid constructions can be found online as supplementary data (Supporting Information Tables S1, S2 and S3).

Fluorescence microscopy

Sporulating cells were concentrated by centrifugation at 8000 rpm for 1 min and immobilized on 2% agarose pads. Fluorescence microscopy was performed using an Olympus BX61 microscope equipped with a UplanF1 100X phase contrast objective lens and a CoolSnapHQ digital camera (Photometrics) or a Nikon TE2000 inverted microscope with a Nikon CFI Plan Apo VC 100X objective lens. Images were acquired using Metamorph software (Molecular DEVICES, Sunnyvale, CA, USA). Membranes were stained with TMA-DPH (50 μM) (Molecular Probes; ThermoFisher Scientific) and fission of mother cell membranes was assessed as previously described (Doan *et al*., 2013). Image analysis and processing was performed in Metamorph.

Immunoblot analysis

Whole-cell lysates from sporulating cells were prepared as previously described (Doan and Rudner, 2007). Samples were heated for 5 min at 65°C prior to loading. Equivalent loading of proteins was based on the OD_{600} of the cell cultures at the time of harvest. Samples were separated on a 12.5% SDS-polyacrylamide gel and transferred to a methanol-activated PVDF membrane. Membranes were blocked in 5% nonfat milk with 0.5% Tween-20 for 1 h. Blocked membranes were probed with anti-σ^H (diluted 1:2500), anti-Spo0A (diluted 1:5000) or anti-SpoIIQ (diluted 1:10,000) (Doan *et al*., 2009), anti-σ^A (diluted 1:10,000), anti-KinA (diluted 1:5000) and anti-core RNA-polymerase (diluted 1:5000). These primary antibodies were diluted into PBS with 0.05% Tween-20 and incubations were carried out at 4°C overnight. Primary antibodies were detected with horseradish-peroxidase conjugated anti-mouse or anti-rabbit antibodies and detected with Western Lightning ECL reagent as described by the manufacturer (PerkinElmer, Waltham, MA, USA).

Protein pull-down assay

25-ml cultures of strain BDR485 (Supporting Information Table S1) harbouring a functional *rpoC-his6* fusion were grown in CH medium in the presence and absence of 0.6 M NaCl. At an OD_{600} of ~0.5, sporulation was induced by resuspension in the presence and absence of 0.6 M NaCl. 1.5 hours later, 20 ml from each culture were harvested by centrifugation (5000 rpm for 10 min at room temperature), washed two times in 10 ml cold lysis buffer (20 mM Tris pH7.5, 300 mM NaCl, 20 mM imidazole, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μg ml^{-1} leupeptin and 1 μg ml^{-1} pepstatin) and the pellets were flash frozen and stored at −80°C. The cell pellets were thawed and resuspended in 1 ml cold lysis buffer with 2 mg/ml lysozyme and incubated at 4°C for 20 min. The cell suspension was lysed by sonication followed by the addition of 10 μl of 1 mg/ml DNase (Worthington, Lakewood, NJ, USA), 10 μl of 10 mg/ml RNaseA and 20 μl of 100 mM PMSF. The lysate was clarified by centrifugation at 13,000 rpm at 4°C for 5 min and the supernatant was

incubated with 50 μl of Ni^{2+}-agarose (Qiagen Hilden, Germany) for 1 h at 4°C. The resin was washed 5 times with 1.5 ml wash buffer (20 mM Tris pH 7.5, 300 mM NaCl, 20 mM imidazole and 5 mM β-mercaptoethanol) and then resuspended in 100 μl SDS sample buffer and heated at 80°C for 10 min. Protein fractions were separated on a 12.5% SDS-polyacrylamide gel and visualized with Coomassie brilliant blue or by immunoblot.

Suppressor enrichment

To enrich for mutants that could form heat-resistant spores in the presence of high salinity, cells of the wild-type *B. subtilis* strain JH642 (Smith *et al.*, 2014) were sporulated (30 h after reaching stationary phase) in DS medium containing 1.2 M NaCl. After eliminating vegetative cells by pasteurization (80°C, 20 min), serial dilutions were plated onto LB agar plates and the spore titer was determined. Colonies arising from spores were then reinoculated in fresh DS medium containing 1.2 M NaCl. After 30 h of growth after reaching stationary phase, the spore titer of the cultures was again determined. Cultures that showed an increased spore titer in comparison to that of the JH642 wild-type strain were analyzed for mutations either by whole genome resequencing, or by targeted sequencing of PCR products derived from the *spo0A*, *kinA* and *sigH* genes. The DNA primers used to amplify these genes and their flanking regions can be found in Supporting Information Table S3.

Mapping suppressors by whole genome sequencing and PCR analysis

Chromosomal DNA from *B. subtilis* was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). To identify the mutation(s) in the suppressor mutant strains NWB6 and NWB7 (Table 1), the genomic DNA was subjected to whole-genome sequencing. Library preparation and sequencing analysis were performed by the Göttingen Genomics Laboratory (Göttingen, Germany). The reads were mapped on the reference genome of the *B. subtilis* JH642 strain (GenBank accession number: CP_007800) (Smith *et al.*, 2014). Mapping of the reads was performed as previously described (Zaprasis *et al.*, 2014) using the Geneious software package (Biomatters Ltd., New Zealand). Single nucleotide polymorphisms (SNPs) were considered as significant when the total coverage depth exceeded 25 reads with a frequency variance of >90%. For the molecular analysis of all other suppressor strains, the promoter and coding regions of *kinA*, *sigH* and *spo0A* were PCR-amplified and Sanger sequenced (MWG Eurofins, Ebersberg, Germany).

Acknowledgements

This work was supported by contributions of the Fonds der Chemischen Industrie (to F.M.C and E.B.), the Max-Buchner-Forschungsstiftung (MBFSt-Kennziffer 3381) (to F.M.C) and the National Institute of Health grant GM086466 to D.Z.R. We thank Masaya Fujita (University of Houston, TX, USA) for generously providing antibodies and an anonymous reviewer for noting the location of the amino acid substitutions in σ^H. Jörg Schuldes and Rolf Daniel from the Göttingen Genomics Laboratory (G2L) (Göttingen, Germany) are acknowledged for kindly performing genome re-sequencing. We appreciate the expert help of Vickie Koogle in the editing of the manuscript.

N.W. and K.E.F. are recipients of Ph.D. fellowships from the International Max-Planck Research School for Environmental, Cellular and Molecular Microbiology (IMPRS-Mic, Marburg) and gratefully acknowledge its generous support. N.W. thankfully acknowledges the receipt of an EMBO short-term fellowship that funded his stay in the laboratory of D.Z.R at the Harvard Medical School (Boston, MA, USA). F.G. R-G. is a recipient of a Conacyt postdoctoral fellowship (Mexico).

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