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1 **Resilience to oxidative and nitrosative stress is mediated by the stressosome,**
2 **RsbP and SigB in *Bacillus subtilis.***
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33 **Running Title:** Bacterial oxidative stress resilience
34 **Keywords:** SigB, oxidative stress, general stress response, stressosome
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38 **Abstract**

39 A bacterium's ability to thrive in the presence of multiple environmental stressors
40 simultaneously determines its resilience. We showed that activation of the SigB-
41 controlled general stress response by mild environmental or nutritional stress provided
42 significant cross-protection to subsequent lethal oxidative, disulfide and nitrosative
43 stress exposure. SigB activation is mediated via the stressosome and RsbP, the main
44 conduits of environmental and nutritional stress, respectively. Cells exposed to mild
45 environmental stress while lacking the major stressosome components RsbT or RsbRA
46 were highly sensitive to subsequent oxidative stress, whereas *rsbRB, rsbRC, rsbRD*
47 and *ytvA* null mutants showed a spectrum of sensitivity, confirming their redundant roles
48 and suggesting they could modulate the signal generated by environmental stress or
49 oxidative stress. Furthermore, from mutant analysis we infer that RsbRA
50 phosphorylation by RsbT was important for this cross-resistance to oxidative stress. By
51 contrast, cells encountering stationary phase stress required RsbP but not RsbT to
52 survive subsequent oxidative stress caused by hydrogen peroxide and diamide.
53 Interestingly, optimum cross-protection against nitrosative stress caused by SNP
54 required SigB but not the known regulators, RsbT and RsbP, suggesting an additional
55 and as yet uncharacterized route of SigB activation independent of the known
56 environmental and energy-stress pathways. Together, these results provide a
57 mechanism for how *Bacillus subtilis* promotes enhanced resistance against lethal
58 oxidative stress during likely physiologically relevant conditions such as mild
59 environmental or nutrient stress.

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60 **Importance**

61 The *Bacillus subtilis* general stress response is a model for gram-positive pathogens
62 because the regulators are conserved, and the Sigma factor, SigB, controls expression
63 of virulence genes in *Listeria monocytogenes*. We showed that *B. subtilis* SigB
64 promotes survival to oxidative, disulfide and nitrosative stress through priming or cross-
65 protection. Moreover, when cells were exposed to nitrosative stress, priming was SigB
66 dependent, yet the known regulators of SigB were not required, suggesting an
67 alternative mode of SigB activation during nitrosative stress. Importantly, we showed the
68 first genetic requirements of stressosome genes, *rsbRB* and *rsbRD,* during oxidative
69 stress cross-protection not explained by environmental stress activation, suggesting a
70 role for stressosome proteins during oxidative stress and advancing the role of SigB
71 during antioxidant protection.
72

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73 **Introduction**

74 The resilience of bacteria to environmental stressors allows them to survive during
75 constantly changing conditions (1). Resilience comes about due to cross-protection or
76 priming, which is when bacteria face mild stress that prepares them for future lethal
77 stress, whether or not the stresses are related. Microbes commonly use this
78 phenomenon in order to survive their dynamic environments (2). Priming is especially
79 beneficial for pathogens because it increases their fitness in the face of the host
80 immune system, which deploys an oxidative burst meant to kill the pathogen (1).
81 Changes to gene expression induced by stress are important for the acquired cross-
82 protection that will let those preprogrammed cells thrive in the presence of a further
83 lethal stressor. Most environmental stresses, such as osmotic shock and temperature
84 shifts, can cause priming and have the highest protective effect against oxidative stress
85 (2). Therefore, evolutionarily conserved mechanisms of bacterial survival, environmental
86 stress induced gene expression and the proteins involved in these processes could
87 have broader biological significance than previously recognized.

88

89 *Bacillus subtilis* uses multiple sigma factors to cope with the changes to its
90 surroundings (3). Some sigma factors are dedicated to specific stressors, but the
91 general stress sigma factor, SigB is responsible for the adaptation to the widest type of
92 environmental conditions and therefore is an important protein during priming (4).
93 Environmental conditions include high and low temperature, alkaline and acidic
94 environments, osmotic stress and changes in carbon sources, ATP levels and oxidative
95 stress (5-8). The activation of SigB leads to the differential expression of 196 genes with

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96 diverse biochemical functions giving cells resistance to multiple stresses, an important
97 aspect of priming (9). Moreover, survival is enhanced in the presence of reactive
98 oxygen species (ROS) when priming is triggered by ethanol stress (10). In this
99 comprehensive analysis of 94 individual SigB-dependent genes, Reder and colleagues
100 showed priming protection to lethal levels of hydrogen peroxide. Cells carrying
101 mutations in individual genes were first given non-lethal ethanol exposure, to trigger
102 priming, followed by lethal levels of hydrogen peroxide and stress-induced tolerance
103 was dependent on many SigB targets (10). It has also been shown that in the presence
104 of oxidative stress alone, caused by hydrogen peroxide or Sodium Nitropruside (SNP),
105 genes belonging to the SigB regulon are induced (11-14), suggesting that SigB is
106 activated by the presence of oxidative stress signals. Furthermore, the need for SigB in
107 resistance against oxidative stress is apparent in stationary phase cells, where
108 exposure to hydrogen peroxide made *sigB* null cells more sensitive than wild type
109 (15).However, the upstream mechanisms controlling SigB-dependent priming during
110 ethanol exposure and in nutritionally stressed cells have not been addressed.
111
112 SigB activity is controlled by two pathways (Figure 1A), which independently sense
113 nutritional and environmental stresses (16). Nutritional stress such as low ATP levels
114 requires the activity of the hydrolase RsbQ and phosphatase RsbP, although the
115 specific nutritional signal is unknown (17-19). Environmental stress uses the
116 stressosome complex consisting of related, putative sensor proteins RsbRA, RsbRB,
117 RsbRC, RsbRD, and YtvA, and the kinase RsbT and antagonist RsbS (20-23). The
118 RsbR paralogs are candidate sensor proteins due to their amino acid sequence

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119 similarity to known sensing domains and their position on the 3D structure of the
120 stressosome. The N' termini, containing a non-heme globin domain are found externally
121 in the structure while the STAS C' terminal domains interact with RsbS and RsbT,
122 potentially transmitting the environmental signal (24-26). Once the stress is sensed,
123 such as ethanol or osmotic stress, RsbT is activated, phosphorylates RsbRA and
124 RsbRB, and leaves the stressosome complex (20, 23, 27). The specific signal that
125 initiates the signaling cascade remains unknown, but could be transmitted from the
126 environment to the stressosome through the N' termini of the RsbR proteins. Once
127 released from the stressosome, RsbT activates the phosphatase RsbU through their
128 direct interaction (28). Active RsbU dephosphorylates RsbV, promoting the partner
129 switching of RsbW bound to SigB to the anti-sigma factor RsbV (29). SigB is normally
130 associated with RsbW, but the dephosphorylation of RsbV causes RsbW to switch
131 partners releasing SigB (30, 31). Nutritional stress works similarly promoting RsbV
132 activation. Upon ATP level depletion the RsbP/RsbQ dimer becomes activated
133 promoting RsbP phosphatase activity towards RsbV resulting in the activation of SigB
134 by releasing RsbW (32). Once SigB is activated, at least 196 genes become
135 differentially expressed leading to the production of important proteins that protect the
136 cell in these stressful circumstances. Although their relative contribution to survival
137 under environmental stresses including oxidative stress has been measured (6, 10), the
138 role of individual stressosome components during extreme oxidative stress, cross-
139 protection has not been determined.
140

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141 Here, measuring the ability of cells mutant in SigB-regulatory proteins to survive
142 exposure to lethal Reactive Oxygen Species (ROS) and Reactive Nitrogen Species
143 (RNS), we probed the role of key SigB regulators in promoting cross-protection during
144 logarithmic and stationary phase. We showed that when priming is prevented by
145 deleting the transducers of environmental and nutritional stress, cells became sensitive
146 when placed in the presence of oxidative and nitrosative stress. In the case of
147 nitrosative stress caused by SNP, *sigB* mutants were the most sensitive followed by
148 individual and double *rsbT*, *rsbP* mutants. This result demonstrated the presence of
149 SigB-dependent pathways responsible for stress protection seen in cells during
150 nitrosative stress that are independent of the stressosome or the nutritional stress
151 sensors. Moreover, we showed for the first time the effect of deleting individual
152 stressosome genes in the physiology of priming suggesting that improper environmental
153 stress signaling is detrimental to cells when dealing with extreme oxidative stress.
154

155 **Materials and Methods**

156 *Bacterial strain construction.* Strains used in these experiments were either made as
157 described in Table1, donated by the Bacillus Genetics Stock Center or courtesy of Dr.
158 Chester Price at the University of California, Davis. Deletions of *rsbRA, rsbT, rsbP* were
159 made by gene replacement with the Chloramphenicol or Kanamycin resistance
160 cassettes from plasmid pGK67. PCR products were made containing 1000 base pairs of
161 homologous regions upstream and downstream of each gene flanking the desired
162 antibiotic resistance gene using NEB Q5 Polymerase. These PCR products were used
163 to transform wild type cells, then antibiotic resistant transformants were confirmed by

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164 PCR of the desired mutation at the endogenous locus. In the case of the *rsbRA*
165 deletion, reverse transcription PCR was performed to confirm that the insertion-deletion
166 was not polar on the operon and that the *rsbT* and *rsbS* transcripts were still expressed.
167 All other deletions were made by chromosomal transformation with DNA from strain
168 PB804 containing the desired mutations of stressosome genes. Strain PB804
169 containing antibiotic marked deletions of *rsbR* genes was used to delete individual
170 stressosome components and selected for single mutations. These strains were also
171 confirmed by PCR of the endogenous locus of each gene. DNA isolation and plasmid
172 preparations were performed using Zymo Reseach kits.
173
174 *Oxidative stress viability and calculations. Bacillus subtilis* strains were grown in Basal
175 Limitation Media (BLM) for experiments in exponential phase, or Glucose Limitation
176 Media (GLM) for stationary phase treatment as previously described (33). BLM consists
177 of 50 mM Tris, 15 mM$(NH_4)_2SO_4$, 8 mM $MgSO_4$, 27 mM KCl, and 7 mM sodium citrate
178 (pH 7.5), 2 mM $CaCl_2$, 1uM $FeSO_4$, 10 uM $MnSO_4$, 4.5 mM Potassium Glutamate, 0.1%
179 glucose (or 0.05% for GLM), 0.6 mM KH_2PO_4 and 160 ug ml^{-1} each of Trp and Phe.
180 Overnight cultures of *B. subtilis* were used to create starting cultures at OD_{600} readings
181 of 0.05, incubated at 37°C while shaking at 300rpm until OD_{600} reached mid-log (~0.4).
182 At mid-log cultures were treated with 2% ethanol for 20 minutes, while shaking. These
183 cultures were split and treated with either 5 mM H_2O_2, 45 mM diamide or 74 mM SNP.
184 After 60 minutes, the cultures were serially diluted and plated on LB agar to recover at
185 37°C for 16 hours before colonies were counted. For oxidative stress induction during
186 nutritional stress, cells were grown in GLM for the entire experiment and growth was

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187 monitored until cells reached transition state. One hour into transition state, 10 mM
188 H_2O_2, 45 mM diamide, or 74 mM SNP was added for an additional 60 minutes while
189 shaking. Both treated and untreated cultures were diluted and plated in LB agar plates
190 and allowed to recover for 16 hours at 37°C. For each individual experiment, treated
191 and untreated bacterial cultures were plated in triplicate, counted and averaged. To
192 calculate percent survival, the number of colonies forming units under stress was
193 divided by the number of colonies forming units without stress. The data shown
194 represent percent viability means of a minimum of three experiments with standard error
195 bars. To calculate the significance of the difference between the percent viability means
196 of different strains, the data were subjected to Ordinary One-Way ANOVAs and all
197 showed P values of 0.005 or lower. Tukey's or Dunnett's multiple comparison tests were
198 performed to compare viabilities between strains. All strains were compared to wild type
199 and to *sigB* nulls when appropriate.
200
201 **Results**
202 **RsbT and RsbP are required for cross-protection to lethal reactive oxygen**
203 **species.** We set out to test the role of key SigB regulators, the stressosome and RsbP,
204 during cross-protection to oxidative stress. Each pathway operates during different
205 growth phases; logarithmic cells are sensitive to environmental stress, transmitted via
206 the stressosome, and early stationary phase cells are nutritionally starved, a condition
207 signaled via RsbP. We hypothesized that each regulator would be required to promote
208 oxidative stress cross-protection in their respective growth phases; therefore we
209 performed experiments in both log phase and early stationary phase using BLM and

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210 GLM respectively (Figure 1B). In order to test priming or cross-protection, in log phase,
211 cells were primed with sub-lethal levels of ethanol and then treated with lethal hydrogen
212 peroxide levels as previously shown (10). Wild type cells preadapted with ethanol were
213 more resistant than cells that received hydrogen peroxide alone and their survival was
214 dependent on SigB since *sigB* deleted cells were extremely sensitive (20 fold, decrease
215 in surival) to the subsequent exposure to hydrogen peroxide (Figure 2A). Since
216 resistance to oxidative stress in nutritionally stress cells was shown to depend on the
217 alternative sigma factor SigB (15), we set out to identify the signaling pathway involved
218 in the cross-protection in this phase. In nutritionally starved cell, RsbP/RsbQ are
219 responsible for SigB activation, therefore we treated *rsbP* deleted cells with lethal
220 amounts of hydrogen peroxide. *rsbP* mutants were highly sensitive to oxidative stress,
221 similarly to *sigB* deleted cells (Figure 2B). In contrast, *rsbT* knock out cells were not
222 sensitive and exhibited survival indistinguishable from wild type cells, demonstrating
223 that the stressosome does not play a role in the stationary phase-induced oxidative
224 stress cross-protection (Figure 2B).
225
226 In order to test the role of environmental stress-activated SigB during priming
227 against oxidative stress, we used mutations in members of the stressosome to assess
228 their role in cross-protection. The kinase RsbT and the co-antagonist RsbRA were
229 deleted individually and logarithmically growing cells were preadapted with mild ethanol
230 stress before being given lethal levels of hydrogen peroxide. *rsbT* and r*sbRA* mutant
231 cells (Figure 2C) were more sensitive than wild type cells and similarly sensitive to *sigB*
232 deleted cells (ANOVA P value 0.0016, Tukey's test showed no significant difference

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233 amongst *rsbT*, *rsbRA* and *sigB* cells) showing that the stressosome is important for the
234 cross-protection that renders the cells resistant to oxidative stress. Deletion of *rsbP* had
235 no effect on survival to hydrogen peroxide exposure (Figure 2C) demonstrating that
236 RsbP is not required for cross-protection to oxidative stress in logarithmically growing
237 cells likely due to not being activated by this stress.
238
239 **Stressosome components play different roles in the resilience to reactive oxygen**
240 **species.** The role of the stressosome in ROS cross-protection has never been tested,
241 so we characterized mutants in individual stressosome components. The stressosome
242 is made up of five paralog proteins RsbRA, RsbRB, RsbRC, RsbRD and YtvA (20).
243 They form a large complex with the kinase RsbT and its antagonist or inhibitor, RsbS
244 (24). We tested individual *rsbR* mutants in the presence of hydrogen peroxide using
245 logarithmic growing pre-adapted cells and saw that *rsbRA* was equally sensitive to
246 hydrogen peroxide as a *sigB* delete as previously shown (Figure 3A). Strains lacking
247 *rsbRB* that were preadapted with ethanol exposure were more sensitive to ROS lethal
248 levels than wild type cells (ANOVA P value <0.0001 and Dunnett's test showed
249 statistical significance). RsbRB is a co-antagonist of RsbT activation, similar to RsbRA,
250 and strains lacking *rsbRB* have elevated SigB-dependent expression in presence of
251 ethanol exposure (20). This suggests that in our experiments, SigB activity is elevated,
252 yet it was not sufficient to protect cells against ROS, therefore the proper modulation
253 that the second co-antagonist, RsbRB, provides is important for surviving lethal
254 oxidative stress. While RsbRB can be a co-antagonist (21, 34), it may need other
255 paralogs for proper regulation as our assay shows that RsbRB function is necessary for

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256 survival even when other co-antagonists are present. Deletion of *rsbRD* also made cells
257 sensitive to lethal ROS even in the presence of ethanol preadaptation (Figure 3A).
258 Interestingly, cells lacking *rsbRD* have no reported defect in SigB activation (20), yet
259 there was a statistically significant difference between *rsbRD* null and wild type cells
260 (ANOVA P value <0.0001, Tukey's and Dunnett's test showed statistical significance).
261 While we do not know how the lack of *rsbRB* affects the stressosome, our results
262 suggest that its presence in the complex plays a role in the regulation of SigB activity
263 during ROS cross-protection.
264
265 In contrast, deletion of *rsbRC* had no effect on the cells' ability to be cross-
266 protected against ROS, showing viability undistinguishable from wild type. (Figure 3A).
267 This is consistent with the absence of a recorded phenotype for cells lacking *rsbRC*
268 (20). Interestingly, cells that contain RsbRC as the only co-antagonist in the
269 stressosome have elevated SigB expression (21, 34) arguing that RsbRC alone is
270 defective at preventing RsbT activation. And in the case of our experiments, removing
271 RsbRC from the stressosome had no effect on the physiological outcome of stress
272 cross-protection. Therefore, RsbRC is not necessary for cross-protection likely due to
273 the redundancy of the paralogs in the complex. Similarly, deletion of *ytvA* had no effect
274 on stress induced, cross-protection against hydrogen peroxide (Figure 3A). YtvA plays a
275 role in the ability of cells to detect light, and cells without *ytvA* have reduced SigB
276 activation under normal laboratory lighting conditions (20). Since our experiments were
277 performed under similar lighting conditions, *ytvA* nulls likely had compromised signaling,
278 yet the predicted lower SigB activity did not prevent cross-protection.

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279

280 **RsbRA phosphorylation is important in the survival to oxidative stress.** Since we
281 saw a defect in *rsbRA*-deleted cells' ability to cross-protect, we tested whether the
282 known phosphorylation steps were involved during ROS exposure. First, we saw that
283 cells where the stressosome consisted of only RsbRA were fully capable of surviving
284 oxidative stress (Figure 3B) suggesting that at least during oxidative stress survival, the
285 other RsbR proteins are not necessary and signaling through RsbRA is sufficient.
286 Mutations in RsbRA phosphorylation site T171, T171A and T171D, made cells deficient
287 at stress induced, ROS protection in our assay (Figure 3B). T171A and T171D mutants
288 are known for having significantly diminished SigB activation measured by *ctc*
289 expression (21). Our sensitivity results are consistent with these mutants having
290 compromised SigB activation when cells were treated with ethanol, which resulted in
291 lower SigB dependent expression of important genes, making cells sensitive to
292 subsequent ROS treatment. Moreover, T171D mutant cells have lower SigB activity in
293 the presence of salt stress compared to wild type, and the T171A mutant RsbRA protein
294 was unable to promote RsbS phosphorylation by RsbT *in vitro* (27, 35), suggesting that
295 the low *ctc-lacZ* expression in these mutants could have been due to lack of RsbS
296 phosphorylation and failure to activate the stressosome or RsbT. These results are
297 consistent with our cross-protection data showing mutations in T171 made cells
298 sensitive to oxidative stress likely due to defects in stressosome priming and eventual
299 cross-protection.

300

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301 Mutations in the phosphorylation site T205 to Alanine or Aspartic acid had
302 different phenotypes likely due to the previously observed effects of each amino acid
303 substitution. First, the T205A mutation had no observable effect in our stress induced,
304 ROS protection survival assay (figure 3B). T205A mutant cells were shown to have wild
305 type levels of SigB dependent expression under 4% ethanol (21), which is higher than
306 the priming stress we used, 2% ethanol. Therefore, SigB activation is likely normal in
307 the T205A mutant and cells had sufficient SigB activity to protect them against
308 subsequent lethal oxidative stress. On the other hand, the T205D mutant was very
309 sensitive to oxidative stress cross-protection (Figure 3B) showing sensitivity similar to
310 *rsbRA* null cells and is consistent with the effect of this mutation on SigB dependent
311 expression since T205D mutant cells have lower SigB dependent expression than wild
312 type cells in presence of salt and ethanol stress (21, 27, 36). It is likely that in our
313 viability assay, 2% ethanol did not cause SigB activation in this mutant therefore, ROS
314 cross-protection could not happen and cells became as sensitive as *rsbRA* as Figure 3B
315 demonstrates.
316
317 **RsbT and RsbP are important during disulfide stress cross-protection.** Disulfide
318 stress happens when thiol groups on proteins are oxidized and non-native covalent
319 bonds form disrupting protein function. Spx and MgsR are disulfide stress regulators
320 responsible for regulation of multiple genes involved in the detoxification of disulfide
321 stress (37, 38). SigB controls their induction during ethanol stress therefore,
322 environmental stress priming could also protect against disulfide stress. Using diamide
323 to induce disulfide stress, *sigB, rsbT* and *rsbP* null cells were tested in cross-protection

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324 during disulfide stress in logarithmically growing cells. We saw that *sigB* deleted cells
325 were defective in survival during diamide exposure compared to wild type cells and
326 preadaptation heightened this difference between wild type and *sigB* null cells (Figure
327 4A). Similarly, *rsbT* mutants showed lower survival than wild type, whereas *rsbP* mutant
328 cells survived to wild type levels (Figure 4B). In stationary phase, which induces
329 nutritional stress, *sigB* and *rsbP* deleted cells were more sensitive to diamide exposure
330 than wild type and *rsbT* deleted cells (Figure 4C). Therefore, nutritional and
331 environmental stress prime cells against disulfide stress.
332
333 **Role of SigB in resilience to nitrosative stress**. We tested how general the oxidative
334 stress cross-protection imparted by SigB was by exposing cells to nitrosative stress.
335 Viability after SigB activation was measured by treating cells with the NO producing
336 compound Sodium Nitropruside (SNP) during log phase or during early stationary state
337 to measure the role of each SigB activating pathway. Wild type and *sigB*-deleted cells
338 were pretreated with ethanol to activate the stressosome and then SNP was added for
339 one hour. As shown in Figure 5A, pre-treatment in log phase made the cells more
340 resistant to lethal levels of SNP, and this resistance was SigB-dependent. SigB-
341 dependent survival to SNP was not observed in previous experiments by Rogstam et al.
342 (12) but the growth medium and stress conditions used in their study and ours were
343 significantly different. We use Basic Limitation Medium and they used Nutrient
344 Sporulation Medium. Additionally, the adaptive response they tested used low level
345 exposure to 0.5 mM SNP followed by lethal SNP levels, whereas our assay uses an
346 unrelated stressor, ethanol, to activate the environmental stress priming effect.

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347 Therefore, under their conditions the general stress response was potentially not
348 activated compared to SigB activation in our system using ethanol.
349
350 To test the upstream activators of the priming pathway, mutants in *rsbT* and *rsbP*
351 were subjected to the cross-protection assay. Wild type and *rsbP*-deleted cells had
352 similar viability rates supporting that RsbP is not activated in log phase by ethanol
353 stress (Figure 5B). Interestingly, cells with deleted *rsbT* showed an intermediate
354 phenotype between wild type and *sigB* mutants (ANOVA P value 0.0001. Tukey's test
355 found no difference between wild type and *rsbT* cells but a significant difference with
356 *sigB* cells). It is possible that under conditions where the stressosome is non-functional,
357 such as in the *rsbT* deleted cells, RsbP becomes required. To test this hypothesis we
358 treated *rsbT, rsbP* double mutant cells with SNP and saw that this strain had similar
359 viability to the *rsbP*-deleted cells, and not like cells lacking *sigB* as was expected if our
360 hypothesis was correct (Figure 5B). Instead, this result suggests a SigB-dependent
361 cross-protection pathway that does not use the known activators RsbT and RsbP. While
362 we have not tested the genetic requirement of downstream regulators such as RsbV in
363 our experiments, the data suggest that SNP causes damage that can be survived if
364 SigB is activated by environmental stress, suggesting an alternative pathway to activate
365 SigB in log phase. RsbV-independent activation was observed during chill (15°C) and
366 high temperature (51°C) exposure (39, 40). In these temperatures, *rsbV*-deleted cells
367 had higher than usual SigB protein levels as if deleting these regulators causes hyper-
368 activation of SigB, which would also explain our viability results in the double mutant

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369 strain. Whether there is another branch of the general stress signaling network is worthy
370 of further investigation.
371
372 In stationary phase, cells were treated with lethal levels of SNP and viability was
373 measured. Wild type and *rsbT*-deleted cells showed the same resistance as with other
374 types of oxidative stress arguing that in stationary phase the stressosome is not
375 required (Figure 5C). The single *rsbP* mutant and the double *rsbP, rsbT* mutant were
376 less sensitive than *sigB* deleted strains when exposed to lethal SNP concentrations
377 (Figure 5C). Both results suggest that SNP resistance may require SigB activation that
378 happens through a pathway other than the known RsbV anti, anti-sigma factor, since so
379 far only the phosphatase activity of RsbP and the stressosome-activated RsbU are
380 required for RsbV activation. Alternatively, SNP may cause RsbV activation through a
381 yet uncharacterized mechanism, which works in both logarithmic and transition state.
382 We have shown that SNP causes stress that requires SigB activity for optimal survival
383 but the mechanism of SigB activation under nitrosative stress remains unknown.
384

385 **Discussion**

386 The general stress response activated by SigB gives cells an advantage to
387 uncertain, future environmental conditions. We characterized the SigB regulatory
388 pathways required for enhanced survival during oxidative stress due to environmental
389 and nutritional stress priming. We showed that upstream regulators of SigB are involved
390 in *B. subtilis* stress priming against oxidative stress, disulfide stress and reactive
391 nitrogen species and provide evidence that stressosome components, RsbRB and

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392 RsbRD, may play a role in ROS signaling outside of environmental stress SigB
393 activation. Bacteria have multiple strategies to deal with their natural ecosystems, these
394 include slowing down metabolism during transition state, inducing competence, biofilm
395 formation, sporulation and virulence in pathogenic bacteria. Since SigB affects some of
396 these processes (41, 42) it is possible that priming is also involved in these distinct
397 states. If low-level SigB activity gives cells an advantage, then normal environmental
398 fluctuations in temperature, osmotic pressure and carbon limitation might help cells
399 more successfully transition between developmental and life style states. Moreover,
400 endogenously produced radicals through metabolic reactions and aerobic respiration
401 must be detoxified (43) and SigB could play a more important role in ROS and RNS
402 detoxification than previously thought. In pathogens redox sensing of the extracellular
403 environment is essential to survival, and for those species that express SigB, it appears
404 to be important in the initial steps that lead to successful colonization (1, 44). In their
405 natural environments, populations may experience sporadic SigB activation due to small
406 changes in temperature or pH and these changes may prepare the cells for extreme
407 oxidative conditions such as the ones imposed by the immune system.
408

409 **Role of the stressosome in modulating SigB activity during oxidative stress.**

410 We saw that deregulated SigB-dependent transcription was counter-productive to
411 the benefits of priming. Using viability as a measure for proper SigB function, we were
412 able to separate mutations in stressosome genes into three categories. Mutations
413 compromised at the priming step were most sensitive, *rsbRA* null, *rsbRA* T171A, T171D
414 and T205A, and had viability similar to *sigB* nulls, as expected if their only role was in

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415 priming. Mutations that were priming-proficient but oxidative stress sensitive, such as
416 *rsbRB* and *rsbRD* suggest a priming-independent role in ROS sensing or signaling for
417 the stressosome that has never been observed. Finally, mutations in *rsbRC* that
418 retained the ability of cells to be primed even to a lower degree, as in *ytvA* nulls,
419 survived oxidative stress like wild type cells. The redundancy of stressosome proteins
420 could be at play during priming so that *rsbRC* and *ytvA* null cells activated SigB to
421 sufficient levels.
422
423 Using an assay that measures the physiological effects of oxidative stress
424 exposure, we were able to show a novel phenotype for two stressosome genes, *rsbRB*
425 and *rsbRD* that cannot be explained by a lack of priming. Cells with mutations in *rsbRA*
426 that reduced SigB activity, were less efficient at oxidative stress cross-protection (Figure
427 3B) as expected if priming is the only role the stressosome plays. Yet, mutations that
428 induce SigB activity such as deletion of the stressosome antagonist protein RsbRB
429 lowered the cell's resilience or ability to meet subsequent oxidative stress. We propose
430 two alternative explanations for this observation. First, hyperactive SigB signaling could
431 be detrimental to the expression patterns required for cross-protection by some general
432 disruptive mechanism of imbalanced gene products. Alternatively, RsbRB and/or
433 RsbRD proteins could have a direct or indirect role in sensing oxidative stress, which
434 contributes to the cross-protection we observed. While, no sensing mechanism has
435 been described for the *B. subtilis* stressosome, both direct and indirect sensing
436 functions have been reported in *Vibrio brasiliensis* (45) and *Listeria monocytogenes* (46)
437 stressosomes. In the *Vibrio* system, the RsbR co-antagonist bound oxygen, which could

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438 make this species stressosome an oxidative stress sensing complex (45). *L.*
439 *monocytogenes* stressosomes did not directly bind a ligand, but a transmembrane
440 protein, Prli42, directly interacted with RsbRA and was required for SigB dependent
441 expression during hydrogen peroxide exposure (46). This mechanism could be
442 conserved in *B. subtilis*, making RsbRB and RsbRD interesting candidates for oxidative
443 stress signal transducers.
444

445 **Cross-protection and SigB regulatory pathways**

446 SigB's importance in oxidative stress cross-protection was first appreciated for its
447 contribution to transition state (15) and later for its role in logarithmic growth (10). While
448 oxidative stress resistance is known to be SigB dependent, we provide evidence that in
449 stationary phase RsbP is the most important SigB regulator for priming and RsbT plays
450 a more significant role in logarithmic phase. During nutritional stress the potential redox
451 imbalance caused by depletion of ATP could be sensed and processed by the two
452 functions in the RsbP- RsbQ complex. The PAS domain on RsbP could bind the signal
453 molecule (47) and RsbQ's hydrolase domain could process it; yet imbalanced redox
454 state was not involved in the activation of RsbP arguing against the redox sensing
455 model (19). However, our cross-protection experiments revealed a potentially
456 uncharacterized SigB activating pathway involved in oxidative stress caused by reactive
457 nitrogen species (Figure 5). Nitrosative stress is an inducer of SigB-dependent gene
458 expression (12, 48, 49). In aerobic conditions, *rsbT* and *rsbP* were each required
459 depending on mode of NO production (49) so how the stress signal(s) activates SigB
460 remains unknown. Our results are consistent with this observation because we saw a

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461 decrease in survival in *rsbT*-deleted cells, although not to the extent of *sigB* deleted
462 cells. Moore and colleagues measured SigB-dependent transcription, so a direct
463 comparison is difficult given that our assay measures the physiological effect of SigB
464 activation. Importantly, we saw that nitrosative stress cross-protection required SigB but
465 not necessarily RsbT or RsbP (Figure 5) arguing for an RsbV-independent pathway or
466 regulation of RsbV independent of the known phosphatases. It is known, however that
467 chill and high temperature induce SigB in an RsbV-independent way (39, 40). While we
468 do not know whether nitrosative stress activates SigB through the same pathway used
469 by extreme temperatures, these results together raise the possibilities that SigB can be
470 activated by more uncharacterized mechanisms.
471

472 **General Stress Response and Antioxidant Activity**

473 Disulfide stress sensing is conserved in many bacterial species through the
474 disulfide sensing, transcription factor Spx. It is responsible for regulating genes such as
475 thioredoxins that reduce inappropriate disulfide bonds between proteins (37). Since Spx
476 is under the regulation of SigB during ethanol stress (50), its activation could explain the
477 cross-protection, i. e. resilience, observed when cells were treated with lethal amounts
478 of diamide (Figure 4). Likewise, the Spx homolog, MgsR is regulated transcriptionally by
479 SigB (38). The sensitivity of *sigB*-deleted cells to diamide exposure could be explained if
480 transcription factors, Spx and MgsR, were not induced. Additionally, some MgsR
481 regulated genes have SigB dependent promoters (38), making their transcription both
482 directly and indirectly sensitive to SigB activity. Appropriate Spx and MgsR activity
483 levels could be required for the concerted transcription of SigB-dependent genes with

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484 potential detoxification properties such as predicted dehydrogenases and reductases
485 regulated by Spx and MgsR (38).
486
487 Nitric oxide production by SNP and diamide stress cause disulfide bond
488 intermediates (51) that result in non-native disulfide bonds requiring detoxification and
489 antioxidant activity for survival. *B. subtilis* produces bacillithiol, the low molecular-weight
490 thiol, involved in redox chemistry. It is synthesized by acillithiol biosynthethic enzymes
491 and transferred to toxic substrates for detoxification by Bacillithiol-S-Transfereses (52,
492 53). Two bacillithiol transferase genes, *bstB* and *bstD* show mRNA expression patterns
493 similar to SigB-dependent genes, high in ethanol, heat, hydrogen peroxide and diamide
494 exposure (54) yet they are not known SigB-targets. If *bstB* and *bstD* expression is
495 induced by environmental stress conditions, they could be indirect targets of SigB
496 through MgsR activity, contributing to the SigB dependent survival we observed during
497 priming. Consistent with a detoxifying role of bacillithiol in disulfide stress, the promoters
498 of bacilithiol biosynthetic genes, *bshA, bshB1/2, BshC*, are upregulated by Spx during
499 difulside stress (53). Ultimately, stress priming triggered through ethanol exposure could
500 induce bacillithiol synthesis and utilization promoting the enhanced resistance of cells
501 subsequently exposed to toxic diamide and nitrosative stress. Potentially, SigB
502 regulatory proteins such as the stressosome and the RsbP/RsbQ complex could
503 function in the cross-protection to all types of oxidative stress conditions, providing
504 primed antioxidant capabilities to the cell.
505

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514

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515 References
516

517 1. Guldimann C, Boor KJ, Wiedmann M, Guariglia-Oropeza V. 2016. Resilience in
518 the Face of Uncertainty: Sigma Factor B Fine-Tunes Gene Expression To
519 Support Homeostasis in Gram-Positive Bacteria. Appl Environ Microbiol 82:4456-
520 4469.
521 2. Andrade-Linares DR, Lehmann A, Rillig MC. 2016. Microbial stress priming: a
522 meta-analysis. Environ Microbiol 18:1277-88.
523 3. Haldenwang WG. 1995. The sigma factors of Bacillus subtilis. Microbiol Rev
524 59:1-30.
525 4. Hecker M, Pané-Farré J, Völker U. 2007. SigB-dependent general stress
526 response in Bacillus subtilis and related gram-positive bacteria. Annu Rev
527 Microbiol 61:215-36.
528 5. Völker U, Maul B, Hecker M. 1999. Expression of the sigmaB-dependent general
529 stress regulon confers multiple stress resistance in Bacillus subtilis. J Bacteriol
530 181:3942-8.
531 6. Höper D, Völker U, Hecker M. 2005. Comprehensive characterization of the
532 contribution of individual SigB-dependent general stress genes to stress
533 resistance of Bacillus subtilis. J Bacteriol 187:2810-26.
534 7. Boylan SA, Redfield AR, Brody MS, Price CW. 1993. Stress-induced activation of
535 the sigma B transcription factor of Bacillus subtilis. J Bacteriol 175:7931-7.
536 8. Boylan SA, Redfield AR, Price CW. 1993. Transcription factor sigma B of Bacillus
537 subtilis controls a large stationary-phase regulon. J Bacteriol 175:3957-63.

bioRxiv preprint doi: https://doi.org/10.1101/460303; this version posted November 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

538 9. Nannapaneni P, Hertwig F, Depke M, Hecker M, Mader U, Volker U, Steil L, van
539 Hijum SA. 2012. Defining the structure of the general stress regulon of Bacillus
540 subtilis using targeted microarray analysis and random forest classification.
541 Microbiology 158:696-707.
542 10. Reder A, Hoper D, Gerth U, Hecker M. 2012. Contributions of individual sigmaB-
543 dependent general stress genes to oxidative stress resistance of Bacillus subtilis.
544 J Bacteriol 194:3601-10.
545 11. Helmann JD, Wu MF, Gaballa A, Kobel PA, Morshedi MM, Fawcett P, Paddon C.
546 2003. The global transcriptional response of Bacillus subtilis to peroxide stress is
547 coordinated by three transcription factors. J Bacteriol 185:243-53.
548 12. Rogstam A, Larsson JT, Kjelgaard P, von Wachenfeldt C. 2007. Mechanisms of
549 adaptation to nitrosative stress in Bacillus subtilis. J Bacteriol 189:3063-71.
550 13. Völker U, Engelmann S, Maul B, Riethdorf S, Völker A, Schmid R, Mach H,
551 Hecker M. 1994. Analysis of the induction of general stress proteins of Bacillus
552 subtilis. Microbiology 140 (Pt 4):741-52.
553 14. Mostertz J, Scharf C, Hecker M, Homuth G. 2004. Transcriptome and proteome
554 analysis of Bacillus subtilis gene expression in response to superoxide and
555 peroxide stress. Microbiology 150:497-512.
556 15. Engelmann S, Hecker M. 1996. Impaired oxidative stress resistance of Bacillus
557 subtilis sigB mutants and the role of katA and katE. FEMS Microbiol Lett 145:63-
558 9.

bioRxiv preprint doi: https://doi.org/10.1101/460303; this version posted November 2, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

559 16. Voelker U, Voelker A, Maul B, Hecker M, Dufour A, Haldenwang WG. 1995.
560 Separate mechanisms activate sigma B of Bacillus subtilis in response to
561 environmental and metabolic stresses. J Bacteriol 177:3771-80.
562 17. Brody MS, Vijay K, Price CW. 2001. Catalytic function of an alpha/beta hydrolase
563 is required for energy stress activation of the sigma(B) transcription factor in
564 Bacillus subtilis. J Bacteriol 183:6422-8.
565 18. Vijay K, Brody MS, Fredlund E, Price CW. 2000. A PP2C phosphatase
566 containing a PAS domain is required to convey signals of energy stress to the
567 sigmaB transcription factor of Bacillus subtilis. Mol Microbiol 35:180-8.
568 19. Zhang S, Haldenwang WG. 2005. Contributions of ATP, GTP, and redox state to
569 nutritional stress activation of the Bacillus subtilis sigmaB transcription factor. J
570 Bacteriol 187:7554-60.
571 20. Akbar S, Gaidenko TA, Kang CM, O'Reilly M, Devine KM, Price CW. 2001. New
572 family of regulators in the environmental signaling pathway which activates the
573 general stress transcription factor sigma(B) of Bacillus subtilis. J Bacteriol
574 183:1329-38.
575 21. Kim TJ, Gaidenko TA, Price CW. 2004. A multicomponent protein complex
576 mediates environmental stress signaling in Bacillus subtilis. J Mol Biol 341:135-
577 50.
578 22. Delumeau O, Chen CC, Murray JW, Yudkin MD, Lewis RJ. 2006. High-
579 molecular-weight complexes of RsbR and paralogues in the environmental
580 signaling pathway of Bacillus subtilis. J Bacteriol 188:7885-92.

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581 23. Chen CC, Lewis RJ, Harris R, Yudkin MD, Delumeau O. 2003. A supramolecular
582 complex in the environmental stress signalling pathway of Bacillus subtilis. Mol
583 Microbiol 49:1657-69.
584 24. Marles-Wright J, Grant T, Delumeau O, van Duinen G, Firbank SJ, Lewis PJ,
585 Murray JW, Newman JA, Quin MB, Race PR, Rohou A, Tichelaar W, van Heel
586 M, Lewis RJ. 2008. Molecular architecture of the "stressosome," a signal
587 integration and transduction hub. Science 322:92-6.
588 25. Marles-Wright J, Lewis RJ. 2010. The stressosome: molecular architecture of a
589 signalling hub. Biochem Soc Trans 38:928-33.
590 26. Murray JW, Delumeau O, Lewis RJ. 2005. Structure of a nonheme globin in
591 environmental stress signaling. Proc Natl Acad Sci U S A 102:17320-5.
592 27. Gaidenko TA, Yang X, Lee YM, Price CW. 1999. Threonine phosphorylation of
593 modulator protein RsbR governs its ability to regulate a serine kinase in the
594 environmental stress signaling pathway of Bacillus subtilis. J Mol Biol 288:29-39.
595 28. Delumeau O, Dutta S, Brigulla M, Kuhnke G, Hardwick SW, Völker U, Yudkin
596 MD, Lewis RJ. 2004. Functional and structural characterization of RsbU, a stress
597 signaling protein phosphatase 2C. J Biol Chem 279:40927-37.
598 29. Yang X, Kang CM, Brody MS, Price CW. 1996. Opposing pairs of serine protein
599 kinases and phosphatases transmit signals of environmental stress to activate a
600 bacterial transcription factor. Genes Dev 10:2265-75.
601 30. Benson AK, Haldenwang WG. 1993. Bacillus subtilis sigma B is regulated by a
602 binding protein (RsbW) that blocks its association with core RNA polymerase.
603 Proc Natl Acad Sci U S A 90:2330-4.

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604 31. Dufour A, Haldenwang WG. 1994. Interactions between a Bacillus subtilis anti-
605 sigma factor (RsbW) and its antagonist (RsbV). J Bacteriol 176:1813-20.
606 32. Delumeau O, Lewis RJ, Yudkin MD. 2002. Protein-protein interactions that
607 regulate the energy stress activation of sigma(B) in Bacillus subtilis. J Bacteriol
608 184:5583-9.
609 33. Stülke J, Hanschke R, Hecker M. 1993. Temporal activation of beta-glucanase
610 synthesis in Bacillus subtilis is mediated by the GTP pool. J Gen Microbiol
611 139:2041-5.
612 34. Cabeen MT, Russell JR, Paulsson J, Losick R. 2017. Use of a microfluidic
613 platform to uncover basic features of energy and environmental stress responses
614 in individual cells of Bacillus subtilis. PLoS Genet 13:e1006901.
615 35. Kim TJ, Gaidenko TA, Price CW. 2004. In vivo phosphorylation of partner
616 switching regulators correlates with stress transmission in the environmental
617 signaling pathway of Bacillus subtilis. J Bacteriol 186:6124-32.
618 36. Akbar S, Kang CM, Gaidenko TA, Price CW. 1997. Modulator protein RsbR
619 regulates environmental signalling in the general stress pathway of Bacillus
620 subtilis. Mol Microbiol 24:567-78.
621 37. Nakano S, Küster-Schöck E, Grossman AD, Zuber P. 2003. Spx-dependent
622 global transcriptional control is induced by thiol-specific oxidative stress in
623 Bacillus subtilis. Proc Natl Acad Sci U S A 100:13603-8.
624 38. Reder A, Höper D, Weinberg C, Gerth U, Fraunholz M, Hecker M. 2008. The Spx
625 paralogue MgsR (YqgZ) controls a subregulon within the general stress response
626 of Bacillus subtilis. Mol Microbiol 69:1104-20.

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627 39. Brigulla M, Hoffmann T, Krisp A, Volker A, Bremer E, Volker U. 2003. Chill
628 induction of the SigB-dependent general stress response in Bacillus subtilis and
629 its contribution to low-temperature adaptation. J Bacteriol 185:4305-14.
630 40. Holtmann G, Brigulla M, Steil L, Schutz A, Barnekow K, Volker U, Bremer E.
631 2004. RsbV-independent induction of the SigB-dependent general stress regulon
632 of Bacillus subtilis during growth at high temperature. J Bacteriol 186:6150-8.
633 41. Knobloch JK, Jager S, Horstkotte MA, Rohde H, Mack D. 2004. RsbU-dependent
634 regulation of Staphylococcus epidermidis biofilm formation is mediated via the
635 alternative sigma factor sigmaB by repression of the negative regulator gene
636 icaR. Infect Immun 72:3838-48.
637 42. Reder A, Gerth U, Hecker M. 2012. Integration of σB activity into the decision-
638 making process of sporulation initiation in Bacillus subtilis. J Bacteriol 194:1065-
639 74.
640 43. Imlay JA. 2013. The molecular mechanisms and physiological consequences of
641 oxidative stress: lessons from a model bacterium. Nat Rev Microbiol 11:443-54.
642 44. Reniere ML. 2018. Reduce, Induce, Thrive: Bacterial redox sensing during
643 pathogenesis. J Bacteriol doi:10.1128/JB.00128-18.
644 45. Jia X, Wang JB, Rivera S, Duong D, Weinert EE. 2016. An O2-sensing
645 stressosome from a Gram-negative bacterium. Nat Commun 7:12381.
646 46. Impens F, Rolhion N, Radoshevich L, Becavin C, Duval M, Mellin J, Garcia Del
647 Portillo F, Pucciarelli MG, Williams AH, Cossart P. 2017. N-terminomics identifies
648 Prli42 as a membrane miniprotein conserved in Firmicutes and critical for
649 stressosome activation in Listeria monocytogenes. Nat Microbiol 2:17005.

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650 47. Taylor BL, Zhulin IB. 1999. PAS domains: internal sensors of oxygen, redox
651 potential, and light. Microbiol Mol Biol Rev 63:479-506.
652 48. Hochgrafe F, Wolf C, Fuchs S, Liebeke M, Lalk M, Engelmann S, Hecker M.
653 2008. Nitric oxide stress induces different responses but mediates comparable
654 protein thiol protection in Bacillus subtilis and Staphylococcus aureus. J Bacteriol
655 190:4997-5008.
656 49. Moore CM, Nakano MM, Wang T, Ye RW, Helmann JD. 2004. Response of
657 Bacillus subtilis to nitric oxide and the nitrosating agent sodium nitroprusside. J
658 Bacteriol 186:4655-64.
659 50. Petersohn A, Bernhardt J, Gerth U, Hoper D, Koburger T, Volker U, Hecker M.
660 1999. Identification of sigma(B)-dependent genes in Bacillus subtilis using a
661 promoter consensus-directed search and oligonucleotide hybridization. J
662 Bacteriol 181:5718-24.
663 51. Grossi L, D'Angelo S. 2005. Sodium nitroprusside: mechanism of NO release
664 mediated by sulfhydryl-containing molecules. J Med Chem 48:2622-6.
665 52. Perera VR, Lapek JD, Jr., Newton GL, Gonzalez DJ, Pogliano K. 2018.
666 Identification of the S-transferase like superfamily bacillithiol transferases
667 encoded by Bacillus subtilis. PLoS One 13:e0192977.
668 53. Gaballa A, Antelmann H, Hamilton CJ, Helmann JD. 2013. Regulation of Bacillus
669 subtilis bacillithiol biosynthesis operons by Spx. Microbiology 159:2025-35.
670 54. Nicolas P, Mäder U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, Bidnenko E,
671 Marchadier E, Hoebeke M, Aymerich S, Becher D, Bisicchia P, Botella E,
672 Delumeau O, Doherty G, Denham EL, Fogg MJ, Fromion V, Goelzer A, Hansen

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673 A, Härtig E, Harwood CR, Homuth G, Jarmer H, Jules M, Klipp E, Le Chat L,
674 Lecointe F, Lewis P, Liebermeister W, March A, Mars RA, Nannapaneni P,
675 Noone D, Pohl S, Rinn B, Rügheimer F, Sappa PK, Samson F, Schaffer M,
676 Schwikowski B, Steil L, Stülke J, Wiegert T, Devine KM, Wilkinson AJ, van Dijl
677 JM, Hecker M, Völker U, Bessières P, et al. 2012. Condition-dependent
678 transcriptome reveals high-level regulatory architecture in Bacillus subtilis.
679 Science 335:1103-6.

680

681

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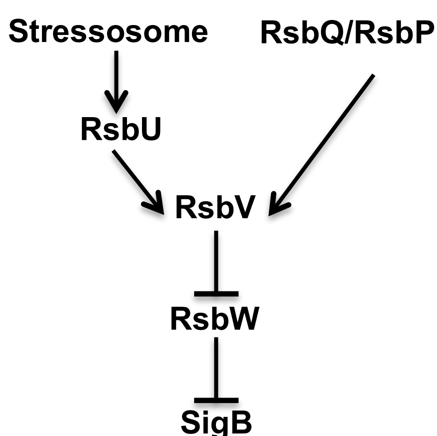
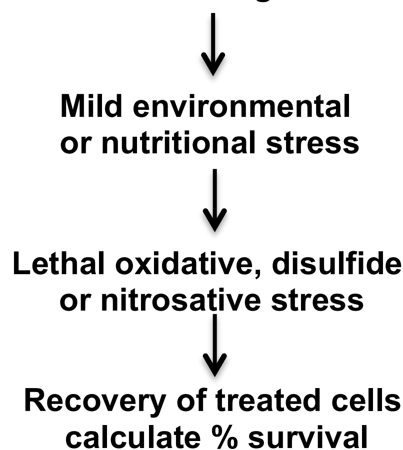
682 **Figure 1. SigB activation pathway.**

683 Environmental stress and nutritional stress activate the stressosome and RsbP
684 respectively. The phosphatase RsbU and RsbP activate the anti-anti sigma factor RsbV
685 which then inhibits the anti-sigma factor RsbW, releasing SigB. (B) Experimental
686 approach to test bacterial cross-protection to oxidative stress in log or transition state.

687
688 **A.** **B.**





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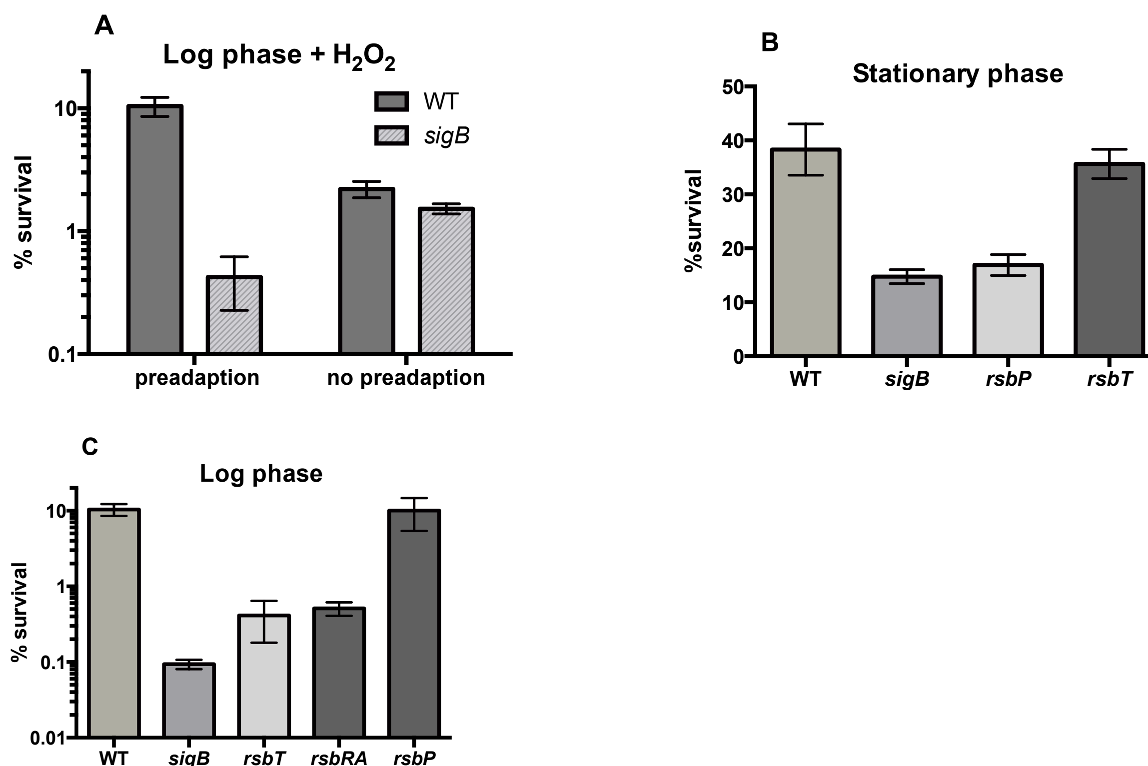
692 **Figure 2. Environmental and nutritional stress protects cells from oxidative**
693 **stress.** (A) Cells were grown in BLM until OD_{600nm} 0.4 and were either preadapted with
694 2% ethanol for 20 minutes or received no preadaptation. Hydrogen peroxide was added
695 at 5 mM final concentration for 1 hour before cells were allowed to recover overnight on
696 LB plates. (B) Cells were grown in Glucose Limitation Media and monitored until
697 transition state. One hour into transition state, cell were given 10 mM hydrogen peroxide
698 for one hour and then plated. (C) Cells were grown to midlog in BLM, treated with 2%
699 ethanol for 20 minutes and then given 5 mM hydrogen peroxide for one hour. Every
700 experiment was done a minimum of three times and averaged; standard error bars are
701 shown for all experiments. One-way ANOVAs were performed, followed by Tukey's
702 multiple comparison tests to determine statistical significant differences between
703 means.



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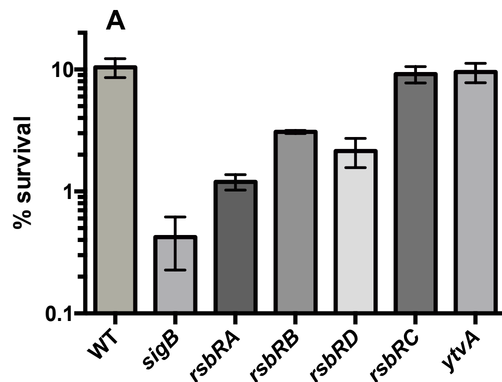
707
708 **Figure 3. RsbRA phosphorylation is required for protection against oxidative**
709 **stress.** (A) Bacterial survival was measured in BLM, after 2% ethanol for 20 minutes
710 and subsequent 5 mM hydrogen peroxide for another hour. Cells were allowed to
711 recover overnight on LB plates. All mutants were tested and compared to wild type and
712 *sigB*. The percent survival for each mutant was calculated compared to wild type
713 survival (100%): *sigB* 5%, *rsbRA* 9%, *rsbRB* 52%, *rsbRC* 75%, *rsbRD* 43% and *ytvA*
714 82%. (B) Position of single amino acid mutations are labeled, RsbRA only strain has
715 deletions in *rsbRB*, *rsbRC* and *rsbRD*. Every experiment was done a minimum of three
716 times and averaged; standard error bars are shown for all experiments. One-way
717 ANOVAs were performed, followed by Tukey's and Dunnett's multiple comparison tests
718 to determine statistical significances between means.



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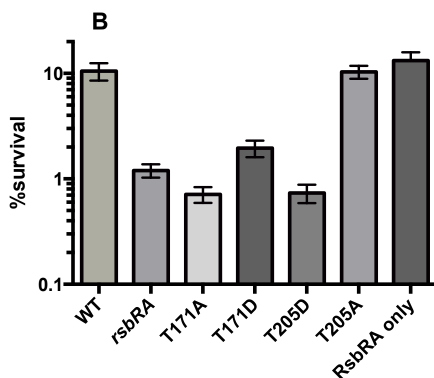

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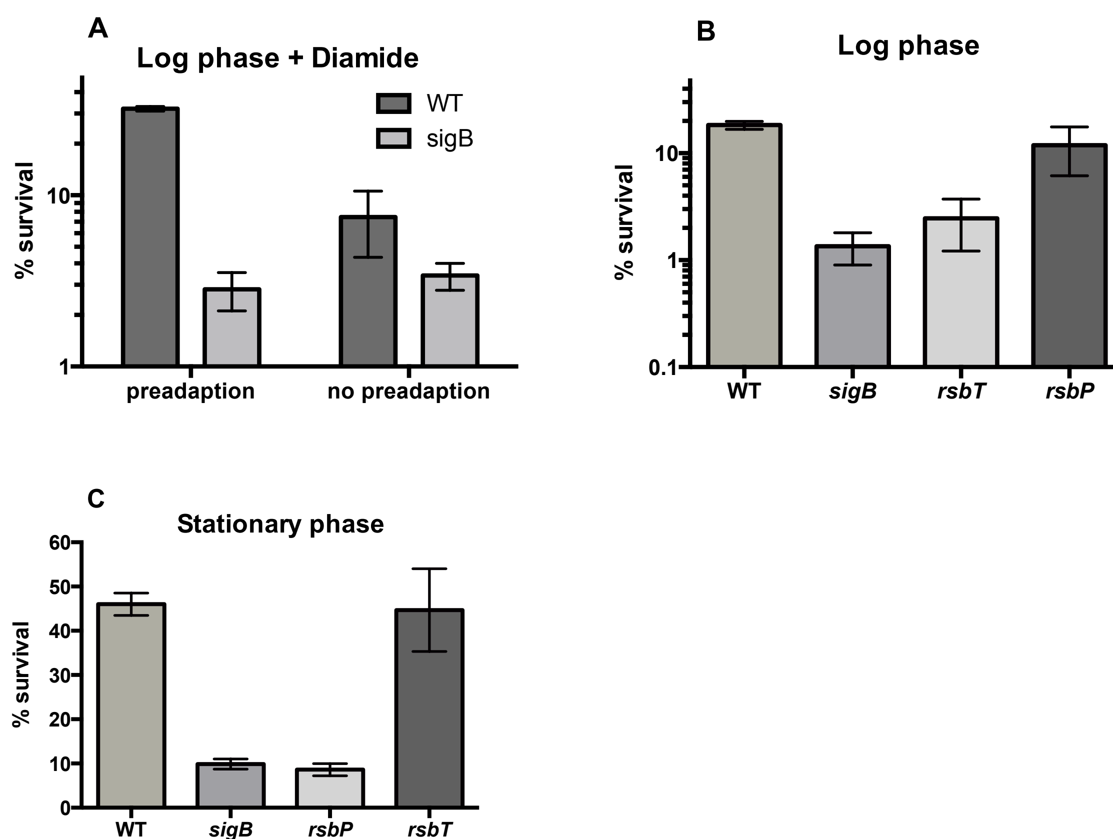
721 **Figure 4. Environmental and nutritional stress protects against disulfide stress.**
722 (A) Cells were grown in BLM until OD600nm 0.4 and were either preadapted with 2%
723 ethanol for 20 minutes or received no preadaptation. Diamide was added at 45 mM final
724 concentration for 1 hour before cells were allowed to recover overnight on LB plates. (B)
725 Cells were grown to mid-log in BLM, treated with 2% ethanol for 20 minutes and then
726 given 45 mM diamide for one hour. (C) Cells were grown in Glucose Limitation Media
727 and monitored until transition state. One hour into transition state, cell were given 45
728 mM diamide for one hour. Every experiment was done a minimum of three times and
729 averaged; standard error bars are shown for all experiments. One-way ANOVAs were
730 performed, followed by Tukey's and Dunnett's multiple comparison tests to determine
731 statistical significant differences between means.
732



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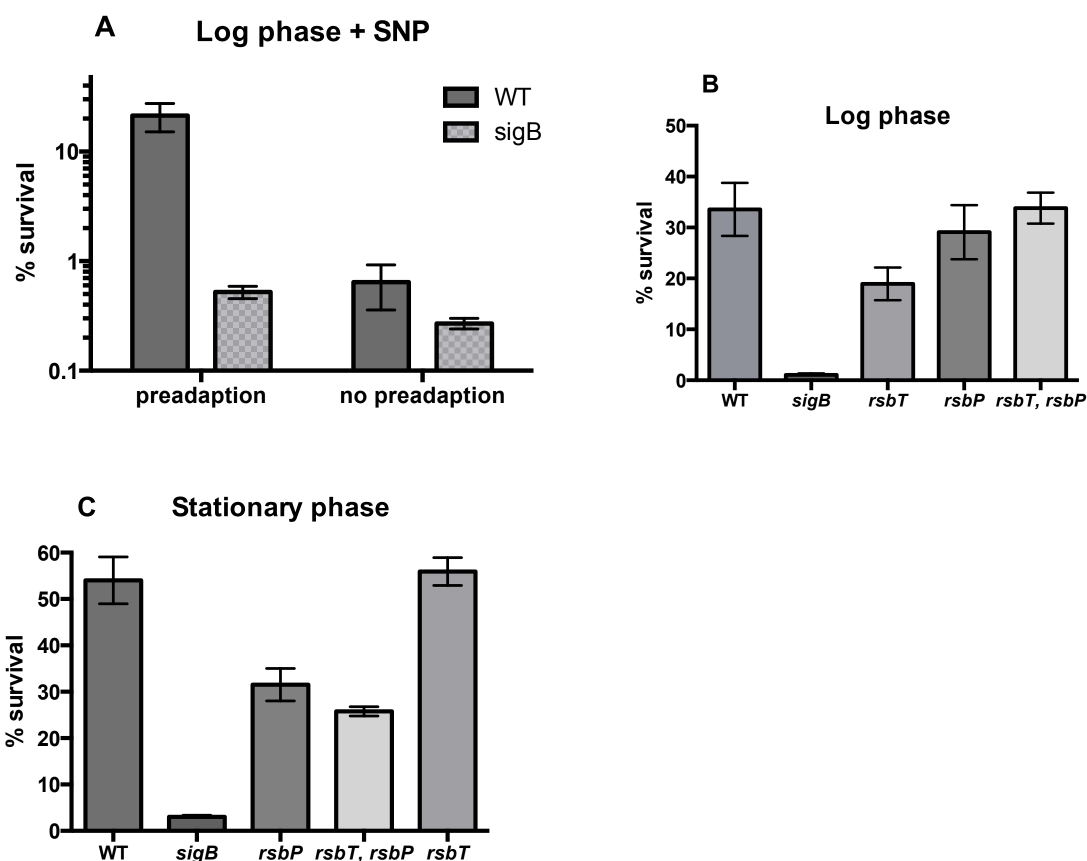
736 **Figure 5. Environmental and nutritional stress protects against nitrosative stress.**
737 (A) Cells were grown in BLM until midlog and were either preadapted with 2% ethanol
738 for 20 minutes or received no preadaptation. Sodium nitropruside was added at 74 mM
739 final concentration for one hour before being plated on LB overnight. (B) Cells were
740 grown to midlog in BLM, treated with 2% ethanol for 20 minutes and then given 74 mM
741 sodium nitropruside for one hour. (C) Cells were grown in Glucose Limitation Media and
742 monitored until transition state. One hour into transition state, cell were given 74 mM
743 sodium nitropruside for one hour and then plated. Every experiment was done a
744 minimum of three times and averaged; standard error bars are shown. One-way
745 ANOVAs were performed, followed by Tukey's multiple comparison tests to determine
746 statistical significant differences between means.



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749 **Table 1.** Strains used in experiments.

Strain	Genotype	Reference or Construction steps
PB502	*rsbRAT205D trpC*	Kim et al, 2004
PB505	*rsbRAT205A trpC*	Kim et al, 2004
PB557	*rsbRAT171D trpC*	Kim et al, 2004
PB804	*rsbRBΔ1::kan rsbRCΔ1::ery, rsbRDΔ1::spc amyE::ctc-lacZ trpC2*	Kim et al, 2004
PB829	*rsbRAT171A trpC*	Kim et al, 2004
CYB1	*trpC2 pheA1*	JH642 from Grossman lab
CYB34	*sigB::catR, trp, phe*	CYB1 transformed *sigB::catR* DNA (PB2)
CYB40	*rsbT::catR, trp, phe*	CYB1, *rsbT* gene deletion
CYB42	*rsbRA::catR, trp, phe*	CYB1, *rsbRA* gene deletion
CYB46	*rsbP::catR, trp, phe*	CYB1, *rsbP* gene deletion
CYB47	*rsbRB::kan, trp, phe*	CYB1 transformed with PB804
CYB48	*rsbRC::erm, trp, phe*	CYB1 transformed with PB804
CYB49	*rsbRD::spc, trp, phe*	CYB1 transformed with PB804
CYB57	*rsbT:catR, rsbP::kan*	CYB40 transformed with *rsbP::kan* DNA

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