Relationship among Oxidative Stress, Growth Cycle, and Sporulation in *Bacillus subtilis*

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The sensitivity of *Bacillus subtilis* to hydrogen peroxide (oxidative stress) was found to vary with the position of the culture in the growth cycle. The most dramatic change occurred at the stationary phase, when the cells became totally resistant to 10 mM H$_2$O$_2$, in contrast to the loss of 3 to 4 log units of viability when treated at the early log phase. Two of the eight proteins induced by a protective concentration of H$_2$O$_2$ (50 μM) were also induced (in the absence of oxidative stress) on entry into the late log phase of growth. The response of five isogenic spo0 mutants (spoOB, spoOE, spoOF, spoOH, and spo0U) to oxidative stress was identical to that of the wild-type parental strain. In an isogenic spo0A strain, mid-log-phase cells were 100-fold less sensitive to 10 mM H$_2$O$_2$ than was the wild type. Pretreatment with 50 μM H$_2$O$_2$ induced little further protection, suggesting that the response is constitutive in this strain. By comparison of proteins induced by 50 μM H$_2$O$_2$ in the wild-type, spo0A, spo0H, and spo0J strains, four proteins were identified that may be essential for protection against lethal concentrations of H$_2$O$_2$. The presence of multiple copies of the spo0H gene in a spo0A background converted the stress phenotype of the spo0A mutant to that of the wild type but left the sporulation phenotype unaltered.

When exposed to stress, organisms respond rapidly and adapt their metabolism to the altered environmental condition. Analysis of stress responses has revealed a network of interlocking regulatory systems. Each regulatory system controls the expression of a group of operons (termed a regulon). An important feature is that some genes are induced by more than one stress. For example, UV light and nalidixic acid induce repair functions through a *recA* lexA-dependent mechanism, but can also induce the heat shock genes *groEL* and *dnaK* in a *htpR*-dependent fashion in *Escherichia coli* (8). The *oxyR* locus of *Salmonella typhimurium* controls the expression of nine proteins after oxidative stress, and also controls the expression of three proteins of the heat shock regulon in response to oxidative stress (3). The response of one of these three proteins to heat shock is also dependent on *oxyR*. Analysis of the *fitE* gene cluster, which is involved in cell division, reveals that it may be linked to the heat shock, DNA repair, and stringent response regulons (7). The emerging picture is of interlocking regulatory systems which fine-tune the response of organisms to changing environmental conditions.

The sporulation response of *Bacillus subtilis* to starvation has been characterized extensively, and mutants blocked at all eight morphological stages have been identified (9). Of particular interest are the spo0 mutants which are blocked at the earliest stage of sporulation. It is possible that such mutants are unable to detect the environmental stimulus which initiates the sporulation pathway (11). Exposure of *B. subtilis* to low concentrations of hydrogen peroxide induces protection against higher concentrations. It also induces a characteristic set of eight proteins, some of which are induced by elevated temperatures (10). It was of interest to study the oxidative stress response in mutants blocked early in sporulation. In addition, the oxidative stress response was studied in cells at different stages of the growth cycle. Our results indicate that cells with a spo0A background display a constitutive protective response to oxidative stress, whereas strains carrying other spo0 mutations display a wild-type response. In addition, cells at different stages of the growth cycle display dramatic differences in their sensitivity to hydrogen peroxide.

**MATERIALS AND METHODS**

**Strains and plasmids.** An isogenic set of *B. subtilis* stage 0 mutants and their parent strain were obtained from the Bacillus Genetic Stock Center, Ohio State University (Columbus, Ohio). These were JH642 (pheA1 trpC2 spo*), JH646 (pheA1 trpC2 spo0A12), JH648 (pheA1 trpC2 spo0B136), JH647 (pheA1 trpC2 spoOE11), JH649 (pheA1 trpC2 spo0F221), JH651 (pheA1 trpC2 spo0H81), and JH696 (pheA1 trpC2 spo0J87).

The genotype of the strains was checked for phenylalanine and tryptophan auxotrophy. They were then streaked onto sporulation medium (12). JH642 grew as brown colonies after 3 days of incubation at 37°C, whereas the other six strains all yielded the transparent colonies expected of spo mutants. JH651 reverted to a Spo* phenotype when transformed with pL1, a plasmid carrying the spo0H gene. JH646 was noncompetent, as is expected of a spo0A strain.

The IS1 plasmid, kindly provided by Issar Smith (Public Health Research Institute of the City of New York), was constructed by insertion of the spo0H gene of *Bacillus licheniformis* into the plasmid pBD64 (5). The chemicals used were as described in the accompanying paper (10).

**Protection experiments.** The experiment for which the results are reported in Table 1 was performed under exactly the same conditions as the equivalent experiment on the wild-type strain (10). Protection was initiated when the cultures reached an optical density at 550 nm of 0.15.

**Growth cycle experiments.** Single colonies from fresh plates of *B. subtilis* were inoculated into 1 ml of Luria broth (LB) and shaken at 37°C for 3 h. Minimal medium (50 ml in a 250-ml flask) was inoculated with 0.3 ml of the LB culture and shaken at 200 rpm at 37°C in a Gallenkamp orbital.
incubator. Samples were taken at regular intervals starting 30 min after inoculation. At each time point, three fractions (1 ml each) were removed into prewarmed 10-ml culture tubes. Hydrogen peroxide was present in two of the tubes so that the concentration came to 10 mM immediately after addition of the culture; the third tube was the untreated control. After shaking for 10 min (200 rpm, 37°C), the samples were appropriately diluted in LB and 0.1 ml of three dilutions was plated onto LB agar. In the case of the strains that carried the plSi plasmid, chloramphenicol (5 µg/ml) was included in all media.

After overnight growth of the plates at 37°C, the viable titer was assayed from the plates of the untreated sample, and the growth curves were drawn from this set of data. The titer obtained from the hydrogen peroxide-treated sample was divided by that from the untreated sample and was plotted as the percent survival of the treated sample relative to that of the untreated sample on the same time scale as the growth curve.

The cells were grown as described above throughout the growth cycle for labeling with [35S]methionine. Approximately the same number of cells was removed at each time point so that the volume sampled was 4 ml initially and decreased with time. The amount of [35S]methionine was appropriately increased with the larger volumes so that the activity was always 30 µCi/ml. Samples were diluted with cold methionine and spun in an Eppendorf minifuge. The cell pellets were suspended, lysed, and electrophoresed as described previously (10).

Metabolic labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (10). Strains JH642 and JH651 were made competent and transformed with plSi by the method described by Dubnau and Davidoff-Abelson (4). Protoplasts of JH646 were made and transformed with plSi, as described by Bourne and Dancer (1).

RESULTS

Oxidative stress and the growth cycle. Wild-type *B. subtilis* JH642 was grown in minimal medium, and samples were taken at different times throughout the growth cycle to be tested for their sensitivity to 10 mM H2O2. The results (Fig. 1) demonstrate that during growth at the early to mid-log phase, survival increased from 0.4 to 0.01% relative to that of untreated samples at the same phase of growth. During the mid- to late log phase, survival increased from 0.01 to approximately 100%, while at the stationary phase, cells were totally resistant to H2O2. This experiment was performed on three occasions with identical results. Duplicate samples at each time point did not vary by more than 0.13 log units.

The complete resistance of stationary-phase cells to killing by H2O2 could be explained by increased accumulation of catalase, which would effectively decrease the concentration of H2O2 to which the cells were exposed. To test this possibility, cell extracts of mid-log- and stationary-phase cells were assayed for catalase activity. No difference in levels was observed, indicating that the resistance to H2O2 at the stationary phase is not mediated by catalase (see the accompanying paper [10] for activity levels).

Protein synthesis throughout the growth cycle. It was hypothesized that, in the stationary phase, cells are constitutively resistant to hydrogen peroxide stress and that in the log phase they are resistant only after induction with sublethal doses of peroxide (10). To test this hypothesis, JH642 was pulse-labeled with [35S]methionine at different stages of the growth cycle. Results demonstrated that the 16-kilodalton (kDa) protein was induced at the late log phase and that the 59.5-kDa protein was induced at the stationary phase. Induction of these proteins, however, was less than that observed during exposure to protective concentrations of H2O2. The total synthetic profile varied very little during the growth cycle, with changes in synthesis of the 16- and 59.5-kDa proteins and a 45-kDa protein (not induced during oxidative stress) being the only reproducible differences observed. Increased synthesis of the 45-kDa protein was observed during entry into the stationary phase.

Effect of hydrogen peroxide on spo0 mutant strains. Strains carrying spo0 mutations are blocked in the sporulation process before morphological differentiation occurs. It is possible that some of these mutants fail to sporulate since they cannot detect the signal to initiate sporulation. It was thus of interest to examine the response of these mutants to oxidative stress. Six isogenic spo0 mutants (spo0A, spo0B, spo0E, spo0F, spo0H, and spo0J) were grown to the early log phase and tested for survival on exposure to 10 mM H2O2 with and without pretreatment with the protective level of 50 µM H2O2. The experiments were repeated four or more times. The response of spo0B, spo0E, spo0F, and spo0J to killing and protection with H2O2 was identical to that of the wild-type strain. The response of the spo0A strain differed, however (Table 1). Exposure to 10 mM H2O2 resulted in only one to two log units of killing, in contrast to 4 log units of killing for the wild type. Pretreatment of spo0A with 50 µM H2O2 prior to the administration of 10 mM H2O2 did not induce significant and reproducible added protection. Thus,
the \textit{spoOA} strain may be regarded as having a constitutive response to oxidative stress.

\textbf{Effect of the growth cycle on the resistance of the \textit{spoOA} strain JH646 to hydrogen peroxide.} In view of the increased resistance of early-log-phase \textit{spoOA} cells to 10 mM H$_2$O$_2$, it was of interest to determine the kinetics of resistance of this strain throughout the growth cycle. Resistance was determined as described in Materials and Methods. The experiment was performed three times with insignificant variation. Results (Fig. 2) demonstrated that resistance fluctuated throughout the growth cycle in a manner distinct from that of the wild type (Fig. 1). The maximal level of killing of 2 log units was observed in the mid- to late log phase of growth. Entry of the cells into the stationary phase resulted in 100% resistance to killing. It is concluded that the \textit{spoOA} strain is more resistant to killing than is the wild type and that the kinetics of resistance during the growth cycle is also different from that of the wild type. The kinetics of resistance of a \textit{spoOH} strain to 10 mM H$_2$O$_2$ throughout the growth cycle was determined as a control. Results (data not shown) demonstrated that the response was identical to that observed with the wild-type strain (Fig. 1). It is thus concluded that (i) the increased resistance of the wild-type strain at the stationary phase is not due to the entry of cells into the sporulation pathway, and (ii) the decreased sensitivity of the \textit{spoOA} strain to 10 mM H$_2$O$_2$ is a feature of the \textit{spoOA} mutation and not a generalized effect of a sporulation-defective phenotype.

\textbf{Proteins induced by hydrogen peroxide in the \textit{spoOA} strain JH646.} The \textit{spoOA} strain JH646 was pulse-labeled with [35S]methionine at various times after 50 \textmu M H$_2$O$_2$ treatment. The labeling was repeated three times, and each set of samples was run on both 8 and 12.5% gels. Two different exposures of each autoradiograph were taken. A representative autoradiograph is shown in Fig. 3, and composite results for the wild type (four repeats) and the \textit{spoOA} strain (three repeats), along with those for \textit{spoOH} (five repeats) and \textit{spoOH} (three repeats) controls, are shown in Fig. 4 and 5. All inductions listed were observed in all labeling repeats, although not necessarily on the particular percentage of gel or exposure shown (Fig. 3, and Fig. 1 in the accompanying paper [10]).

Of the eight proteins induced in the wild type, only two (49 and 16 kDa) were induced in the \textit{spoOA} strain; the 49-kDa protein was induced only slightly but reproducibly. The other six proteins were synthesized at a constant rate following oxidative stress of the \textit{spoOA} strain, but in most cases it was not clear whether they were synthesized at levels similar to those in induced or noninduced wild-type cells. The proteins required for protection are expected to be induced in the wild-type strain but to be synthesized at a constitutively high level in the \textit{spoOA} strain. This was

![Fig. 2](image)

**Fig. 2.** Growth cycle and sensitivity to oxidative stress of the \textit{spoOA} strain JH646. Symbols: \(\Delta\), culture titer; \(\bullet\), percent survival of cells after a 10-min treatment with 10 mM H$_2$O$_2$ relative to that of an untreated control.

![Fig. 3](image)

**Fig. 3.** One-dimensional polyacrylamide gel of \textit{B. subtilis} JH646 (\textit{spoOA} mutant) proteins synthesized after treatment with 50 \textmu M H$_2$O$_2$. The proteins were radioactively labeled with [35S]methionine during 5 min of growth. Lanes 1 and 2, Wild-type (WT) protein profiles, without and with treatment, respectively (0 to 5 min after treatment); lane 3, molecular weight markers, from top to bottom, as follows: 200,000, 92,000, 69,000, 46,000, 30,000, 14,300. Lanes 4 and 5 were not treated; lane 4 was labeled at 0 to 5 min, and lane 5 was labeled at 60 to 65 min. Lanes 6 to 12 were labeled at intervals during 1 h of growth following H$_2$O$_2$ treatment: lane 6, 0 to 5 min; lane 7, 5 to 10 min; lane 8, 10 to 15 min; lane 9, 15 to 20 min; lane 10, 20 to 30 min; lane 11, 40 to 50 min; lane 12, 60 to 65 min. Only two inductions were observed in \textit{spoOA}; these are indicated by the arrows on the right side of the gel. \(M_r\) at left and right are given in thousands.
FIG. 4. A diagrammatic representation of the protein inductions observed in *B. subtilis* JH646 (spo0A) and JH642 (wild type) following treatment with 50 μM H₂O₂. Of the eight proteins induced in the wild type, only two are seen in spo0A. MₙS are given in thousands.

particularly clear for the 20-kDa protein, which was constitutively synthesized in JH646 at a level comparable to that in the induced wild type.

By comparing the induction profiles for the spo0H and spo0J strains with that for the wild type (Fig. 5), we could rule out the possibility that certain proteins are essential for the observed protection against killing by H₂O₂, since these two mutants had a wild-type phenotype with respect to oxidative stress. Neither the 20- nor the 40-kDa proteins were induced in the spo0H strain. Therefore, an essential role for either of these proteins in protection is unlikely. It should be emphasized, however, that these proteins are synthesized in the two mutants; there is simply no increase in their rate of synthesis following oxidative stress. The remaining four proteins might play a role in the induced protection of the wild type (and spo0H and spo0J mutants) and the constitutive protection of the spo0A strain against high concentrations of hydrogen peroxide. However, the 88-kDa (and the 49-kDa) protein was induced later than the time required for maximal protection.

**Complementation of the spo0A mutation with multiple copies of the spo0H gene.** The plasmid pIS1, which carries the *B. licheniformis* spo0H gene on the multicopy plasmid pBD64, was transformed into wild-type, spo0H, and spo0A strains. Transformants were checked for the plasmid of the correct size and restriction pattern. The spo0H (pIS1) and wild-type (pIS1) strains had a Spo⁺ phenotype, whereas the spo0A (pIS1) strain had a Spo⁻ phenotype. Early-log-phase cells of the wild-type and spo0A (pIS1) strains were tested for sensitivity to 10 mM H₂O₂. The survival of the wild type containing the plasmid was identical to that of the strain without the plasmid. In the case of the spo0A (pIS1) strain, survival frequencies of 0.07 and 0.2% were observed in separate experiments. Pretreatment with 50 μM H₂O₂ followed by 10 mM H₂O₂ resulted in an increase in survival to 22 and 23%, respectively (cf. Table 1 and Table 1 in the accompanying paper [10]). The sensitivity of the spo0A (pIS1) strain to 10 mM H₂O₂ was assayed throughout the growth cycle. Results demonstrated a response that was identical to that of the wild type at all stages of growth. It is concluded that the presence of multiple copies of the spo0H gene in a spo0A background reverts the response to protective and killing levels of H₂O₂ to that of the wild type.

**DISCUSSION**

In this study we analyzed the relationship among oxidative stress, growth cycle, and sporulation in *B. subtilis*. We found that *B. subtilis* became resistant to high concentrations of hydrogen peroxide during the stationary phase. This did not depend on the ability of cells to sporulate since spo0A and spo0H survived such treatment with unimpaired viability. Neither was the resistance of stationary-phase cells to killing by H₂O₂ due to increased levels of catalase.

Five of the six spo0 mutants displayed a normal response to hydrogen peroxide, whereas the sixth mutant, spo0A,
showed decreased sensitivity during the log phase of growth. The spoOA mutation may be somewhat analogous to the oxyR mutation studied by Christman et al. (3), in that both are pleiotropic, regulatory mutations which render the cells resistant to hydrogen peroxide.

Results obtained on protein inductions for oxidative stress in spo0 mutants allow speculation on the proteins that are essential to the oxidative stress response and to stress responses in general. Synthesis of eight proteins is induced on exposure of cells to 50 μM H2O2 (10). Induction of two of these proteins (16 and 49 kDa) in spoOA (which has a partially constitutive oxidative stress response) and noninduction of two proteins (20 and 40 kDa) in the spo0H strain (which has a wild-type oxidative stress response) indicates that the observed induction of these proteins on pretreatment with H2O2 is not essential to the observed protective response. In addition, the 88-kDa protein appears to be induced too late to play a role in the protection response. Thus, the 64-, 59.5-, and 54-kDa proteins may play essential roles in protecting the cells against the effects of H2O2. Of particular interest, however, is the 16-kDa protein, which was induced on pretreatment of the wild type and spo0H and spo0J mutants with 50 μM H2O2 and which was also induced on entry of wild-type cells into the stationary phase and on temperature upshift. Thus, the 16-kDa protein may play a more general role in detecting or responding to changing environmental conditions. This protein is also induced in the spoOA strain in response to oxidative stress. The induction was less than that observed in the wild type, however, which is consistent with the partially constitutive protection displayed by the spoOA strain.

We suggest that there are a number of stress genes which are expressed when induced by protective concentrations of hydrogen peroxide. The products of these genes protect the cells against the lethal effects of high concentrations of hydrogen peroxide. Furthermore, we suggest that the SpoOA protein (from the wild-type allele) directly or indirectly inhibits expression of the stress genes. Low concentrations of hydrogen peroxide partially reverse this process so that the genes are switched on. The spoOA gene is thought to be responsible for the initial detection of starvation which signals sporulation (11). On contact with a starvation signal, the putative inhibitor is inactivated or removed and stress genes are switched on.

The ability of multiple copies of a spo0H gene to complement a spoOA mutant, giving it a wild-type stress phenotype, suggests that multiple copies of the spo0H gene play a similar role to that of a single copy of the spoOA gene. The sequence of the spo0H gene indicates that its product is a sigma factor, probably sigma30 (Issar Smith, personal communication). The spoOA gene does not code for a sigma factor but, rather, displays homology with genes that code for proteins which regulate membrane components (13). However, the SpoOA protein may modulate RNA polymerase activity by sigma28 (2) and, possibly, by sigma26 (6). In conclusion, therefore, the SpoOA protein may block expression of stress genes by competing out binding of stress-specific sigma factors by promoting binding of others such as sigma30. The SpoOH sigma factor may function in the same way, albeit with a lower efficiency.

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LITERATURE CITED