MiniReview

The oxidative stress response in *Bacillus subtilis*

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Abstract: *Bacillus subtilis* undergoes a typical bacterial stress response when exposed to low concentrations (0.1 mM) of hydrogen peroxide. Protection is thereby induced against otherwise lethal, challenge concentrations (10 mM) of this oxidant and a number of proteins are induced including the scavenging enzymes, catalase and alkyl hydroperoxide reductase, and a putative DNA binding and protecting protein. Induced protection against higher concentrations (10–30 mM) of hydrogen peroxide is eliminated in a catalase-deficient mutant. Both RecA and Spo0A influence the basal but not the induced resistance to hydrogen peroxide. A regulatory mutation has been characterized that affects the inducible phenotype and is constitutively resistant to high concentrations of hydrogen peroxide. This mutant constitutively overexpresses the proteins induced by hydrogen peroxide in the wild-type. The resistance of spores to hydrogen peroxide is partly attributable to binding of small acid soluble proteins by the spore DNA and partly to a second step which coincides with the depletion of the NADH pool, which may inhibit the generation of hydroxyl radicals from hydrogen peroxide.

Key words: Stress; Oxidative stress; Hydrogen peroxide; *Bacillus subtilis*

Introduction

The oxidative stress response of bacteria may be categorized according to the type of oxidant involved. For example, hydrogen peroxide differs from superoxide radical generating agents in its effect. This minireview focuses on the response of *Bacillus subtilis* to hydrogen peroxide in the context of the more thoroughly studied responses of *Escherichia coli* and *Salmonella typhimurium* to hydrogen peroxide and superoxide radicals. Bacteria react in different ways to hydrogen peroxide depending on their stage in the growth cycle, so the minireview is separated into the effects at exponential phase, stationary phase and sporulation. Evidence is discussed to support the contention that bacterial responses to hydrogen peroxide during stationary phase and sporulation may be part of a general resistance to stress.

Biochemistry of oxygen toxicity

The biochemical effects of active oxygen species have been reviewed elsewhere [1] and will only be repeated here very briefly. The reactions
of molecular oxygen are constrained to one-electron transfers. Molecular oxygen is reduced to water by the addition of four electrons, thus generating the following intermediates after each subsequent electron addition: superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (·OH). Other active oxygen species include singlet oxygen, ozone and iron-oxygen complexes. Reactive oxygen species can be generated by aerobic metabolism, environmental sources such as UV radiation, or redox cycling agents which divert electrons from NAD(P)H to molecular oxygen thereby generating $O_2^-$.

Active oxygen species are highly reactive and cause damage to DNA, RNA, protein and lipids. In *E. coli* there are two modes of killing by $H_2O_2$. Mode one killing appears to be due to DNA damage and is caused by 1–3 mM concentrations of $H_2O_2$. Mode two killing requires a > 20 mM dose of $H_2O_2$ and the sites of the toxic injury have not been established. Much of the DNA damage in mode one is caused by hydroxyl radicals generated from $H_2O_2$ via the Fenton reaction which requires DNA-bound iron and a source of reducing equivalents (possibly NADH) to regenerate Fe(II).

Bacterial defences against reactive oxygen species include scavenging enzymes such as superoxide dismutase (SOD, converts $O_2^-$ into $H_2O_2$), catalase (converts $H_2O_2$ into $H_2O$ and $O_2$), glutathione peroxidase and DT-diaphorase. In addition to the enzymes, small molecules such as ascorbic acid, $\alpha$-tocopherol, glutathione, $\beta$-carotene and uric acid also have antioxidant activity. Secondary defences include DNA-repair systems (reviewed in [2]) and proteolytic and lipolytic enzymes. Oxidative stress arises when the concentration of active oxygen increases to a level over the basal level of the cell’s defence capacity.

**Response of exponential phase bacteria to oxidative stress**

*E. coli* and *S. typhimurium* are the bacteria most studied in terms of their response to oxidative stress, and the subject has been comprehensively reviewed by Farr and Kogoma [1]. Exponential phase phenomena include separate responses to peroxide and superoxide stresses. In response to low concentrations of $H_2O_2$ or organic peroxides, *E. coli* increases the levels of at least 30 proteins, including HP1 catalase, alkyl hydroperoxide reductase, DnaK, RecA [1] and Dps [3]. Dps (also called PexB) is a histone-like protein which forms highly structured complexes with DNA in vitro and probably protects DNA against attack by $H_2O_2$. Pretreatment with a low concentration of $H_2O_2$, which induces the peroxide stimulon, leads to resistance to a subsequent challenge dose. Eight of the induced proteins in *E. coli* are regulated by the OxyR protein which is bifunctional, acting as both a sensor of oxidative stress and a transcriptional activator of genes of the regulon. The reduced form of OxyR is oxidized upon exposure of the cells to oxidative stress. Both forms of the protein can bind to specific sites on target genes but only the oxidized form activates gene expression.

Similarly, cells pretreated with a non-lethal dose of a superoxide generator, which induces a superoxide stimulon, display enhanced survival upon exposure to a challenge dose. $O_2^-$ induces more than 40 proteins, most of which are different from those of the peroxide stress stimulon. At least ten of these genes are regulated by a two gene locus, *soxRS*. A redox signal produced by $O_2^-$ generating agents activates pre-existing SoxR protein, which then triggers expression of *soxS* whose gene product activates members of the *soxRS* regulon. Other oxidant responsive regulatory genes are anticipated since at least 60 of approximately 80 proteins induced by oxidative stress in *E. coli* are not under the control of oxyR or *soxRS*.

$H_2O_2$ stress in *B. subtilis* during exponential phase

The study of oxidative stress in *B. subtilis* to date has been mainly confined to examining the response to $H_2O_2$. A superoxide stimulon has not yet been described.
Oxidants and other agents which induce an adaptive response

Exponentially growing cells of *B. subtilis* exhibit approximately 0.01–0.1% survival after treatment with a killing concentration (challenge dose) of H$_2$O$_2$ (10 mM) [4,5]. A pretreatment (protective, inducing) concentration (0.05–0.1 mM) was defined that is sublethal but protects the cells 100-fold against the otherwise lethal effects of 10 mM H$_2$O$_2$. This is effective whether the pretreatment and treatment last for 1 h [4,6] or for 10–15 min [5,7–10]. Several proteins are rapidly induced after the pretreatment suggesting that they play a role in the induced protection [4,6,7].

Cross-protection has been observed for many stress responses, i.e. pretreatment with a low (inducing or protective) level of one stress can protect cells against lethal levels of a second stress. Four of the proteins (including the two subunits of alkyl hydroperoxide reductase and a DNA binding protein) induced by H$_2$O$_2$ in *B. subtilis* JH642 are also induced to a minor extent by heat shock [4,7]. However they constitute a small part of the heat shock response since a number of other proteins are induced to a much greater extent [4]. Thus it is not surprising that heat shock (48°C) does not protect against H$_2$O$_2$ (10 mM), nor does H$_2$O$_2$ (0.05 mM) protect against a lethal heat treatment (56°C) [8]. However, pretreatment with ethanol (4%) affords 100-fold protection against H$_2$O$_2$ and the effect depends on protein synthesis, suggesting that ethanol induces all necessary proteins to protect the cells against H$_2$O$_2$ [8].

A H$_2$O$_2$-resistant mutant of *B. subtilis* (MA991) which is more resistant than the wild-type to cumene hydroperoxide and t-butyl hydroperoxide [7], led to the expectation that there might be cross-reaction in the response of the cells to different peroxides. However low concentrations of these organic peroxides did not protect the wild-type against 10 mM H$_2$O$_2$ [8]. Indeed, it seems likely that *B. subtilis* does not have an adaptive response to organic peroxides since attempts to determine a low pretreatment concentration which would induce protection against a lethal concentration of the same agent were also unsuccessful [8].

The cross-protective effects of three superoxide generating agents have been examined since it was expected that they would increase the levels of H$_2$O$_2$ by dismutation of the O$_2^-$ which they generate [8,9]. Riboflavin (RF) generates superoxide anion in the presence of light and is toxic to *B. subtilis*. Its toxicity was enhanced by the addition of SOD, indicating that extracellularly generated H$_2$O$_2$ is partially responsible for the effect; the membrane being impermeable to O$_2^-$. RF (10 μM) + light ± SOD protected *B. subtilis* against 10 mM H$_2$O$_2$, but SOD was not needed for the protective effect, implying that O$_2^-$ itself can facilitate protection [9]. Two agents which generate increased intracellular levels of O$_2^-$ (and presumably also of H$_2$O$_2$) were also assessed. Plumbagin protected *B. subtilis* against H$_2$O$_2$ but a second redox cycling agent, paraquat did not, indicating a difference in the uptake or effect of the two chemicals [8]. The effect of plumbagin suggested that the H$_2$O$_2$-resistant mutant, MA991 might also be resistant to plumbagin but this was found not to be the case [7]. There was also no difference between the wild-type and strain MA991 in their sensitivity to a number of other stresses including cadmium chloride, mitomycin C, N-ethylmaleimide and paraquat [7].

*Rose bengal* produces singlet oxygen in the presence of light and is highly phototoxic to *B. subtilis*. However, attempts to define adaptive conditions for rose bengal were unsuccessful suggesting the lack of an inducible response associated with this compound [9].

Induced genes and proteins

Some of the genes and proteins induced by the low concentration of H$_2$O$_2$ and which protect against challenge doses, have been identified and are thought to play a role in the protection. One-dimensional gel analysis of extracts from exponential phase cultures of *B. subtilis* strain JH642 indicated that at least eight proteins are induced by 0.05 mM H$_2$O$_2$. Initial attempts to characterize the genes induced by H$_2$O$_2$, relied on the generation of transcriptional fusions to the
lacZ gene and screening for induction by $H_2O_2$ [10]. Two fusions were isolated which were induced late following treatment with sub-lethal concentrations of $H_2O_2$. Partial sequences of the two promoter regions failed to identify them. One is induced 4–5 fold and maps at 11° while the second is induced 20-fold and maps close to the right of the defective prophage PBSX at about 120°. The latter (oxy-2) is induced by mitomycin C as well as $H_2O_2$ (0.1 mM) which correlates with the induction of PBSX by these agents. It seems likely that the induction of oxy-2 by $H_2O_2$ does not occur at the transcriptional level, but rather as a consequence of the induction of PBSX replication extending into adjacent regions of the chromosome. In view of this result, subsequent work in this laboratory used the YB886 strain of *B. subtilis* which has an uninducible PBSX and lacks phage SPβ. Attempts to isolate genes induced rapidly (within 30 min) by $H_2O_2$ were unsuccessful using gene fusion methodology. A second operon fusion method [11] which relied on the regulation of some of the $H_2O_2$-inducible genes by Spo0A [6] also failed (O. Hartford, unpublished data).

Several enzymes, including SOD, haem oxygenase, glucose-6-phosphate dehydrogenase, glutathione reductase and DT-diaphorase which might be expected to be induced by $H_2O_2$, are unaffected (J. O’Rourke, unpublished data). However, the specific activity of catalase is increased several fold by the addition of $H_2O_2$ [7,12, J. O’Rourke, unpublished data]. Moreover, induced protection against very high concentrations (10–30 mM) of $H_2O_2$ is completely dependent on the presence of a functional catalase gene whereas protection can be induced more than 10-fold against 2 and 5 mM $H_2O_2$ even in a kat− mutant [13]. Thus, proteins other than catalase that are induced by protective concentrations of $H_2O_2$ only protect against relatively low levels of $H_2O_2$. At higher concentrations, they are unable to protect in the absence of catalase.

The more abundant $H_2O_2$-induced proteins were identified in the $H_2O_2$-resistant mutant, MA991 which constitutively over-expresses all of the $H_2O_2$-induced proteins of the parental strain (YB886) as well as three other proteins [7]. N-terminal sequencing revealed that three of the proteins induced in the wild-type and over-expressed in the mutant are catalase and the two subunits of alkyl hydroperoxide reductase. These degrade $H_2O_2$ and organic hydroperoxides (including cumene hydroperoxide and t-butyl hydroperoxide) respectively. The specific activity of superoxide dismutase was the same in the two strains, suggesting that a separate, non-overlapping response to superoxide may exist in *B. subtilis* as is the case for *E. coli*. A 16 kDa protein is constitutively over-expressed in the mutant [7] and is induced by $H_2O_2$ [4,7] or by entry into stationary phase [6] in the wild-type. The 16 kDa protein has the same N-terminal sequence (H. Cameron, unpublished data) as a 113 kDa protein [7] which accumulates to higher levels in the mutant. The latter is probably an exceptionally stable oligomer of the former. They also share an N-terminal sequence with the MrgA protein [14] of *B. subtilis* (J. Helmann, personal communication). It seems likely that there is an error in the published [7] sequence of the 113 kDa protein from the 16th amino acid onwards since its sequence diverges at this point from the MrgA and 16 kDa proteins which are identical to each other. The MrgA protein is encoded by a gene which is induced under conditions of metal ion limitation [14], and is homologous to the Dps/PexB DNA binding and protecting protein of *E. coli* (J. Helmann, personal communication).

Three proteins accumulate to a much smaller extent in MA991 than in the wild-type and one of these was identified as flagellin [7]. The mutant also has a different pattern of $t_7$ spore coat proteins than the wild-type and a 41 kDa band that is intense in the wild-type but absent from the mutant was identified as flagellin (O. Hartford, unpublished results). Flagellar genes are subject to metalloregulation in *E. coli* and *B. subtilis* [15]. The control of both mrgA and flagellar genes by metals as well as $H_2O_2$ suggests a possible link between the responses to these two agents. MA991 is filamentous [7], again reflecting the induced characteristics of the wild-type where filamentation is induced by both $H_2O_2$ (1 mM) and mitomycin C in a RecA independent manner [13].
Catalases in B. subtilis

Loewen and Switala [12] noted an increase in the specific activity of catalase as exponential growth proceeded with a maximal level occurring at stationary phase followed by a decrease as spores appeared in the medium. Mid-exponential phase cells contain a single catalase, named catalase 1 which appears to be a hexamer with a subunit molecular weight of 65 kDa. This corresponds in terms of subunit Mr and pattern of expression to the catalase characterized in other laboratories [7,16, J. O'Rourke, unpublished data]. Levels of catalase 1 increase 15-fold during growth into stationary phase. This enzyme is induced by 0.25 mM H₂O₂ during exponential phase [12] and mutants lacking the enzyme are not only highly sensitive to H₂O₂, but also lose inducible protection against concentrations of H₂O₂ of 10 mM or greater [13,16]. Loewen and Switala [17] mapped a locus, katA which affects catalase 1 activity, to 70° on the B. subtilis chromosome and Bol and Yasbin [16] cloned and sequenced the structural gene (kat-19) for catalase 1. The sequence revealed greater homology to the catalases of various eukaryotes than to those of other prokaryotes. Loewen and Switala [12] noticed a faint activity, labelled catalase 3, which appears between 5 and 10 h post-inoculation. A much more prominent band, catalase 2, emerges 20 h after inoculation [12]. The latter species is not induced by H₂O₂ and is the only catalase present in spores. Its synthesis was blocked in mutants defective in stage 0, but not in a mutant with an undefined (presumably later) block in sporulation. Catalases 1 and 2 have very similar properties and catalase 1 is present in 40-fold greater amounts during spore formation. However, catalase 2 is the only isozyme incorporated into spores [12]. The constitutive and inducible catalase activities present during exponential phase are located largely (about 90% of the total) in the membrane (J. O'Rourke, unpublished data). Thus, B. subtilis may require decomposition of extracellular as well as intracellular H₂O₂.

Since catalase is a haem-containing enzyme, we investigated the induction of haem and its porphyrinogen precursors by H₂O₂ (J. O'Rourke, unpublished data). Porphyrin synthesis is induced by H₂O₂ and both haem and porphyrin levels are higher in the H₂O₂ resistant mutant MA991 than in the wild-type parent strain. However, 5-aminolevulinic synthetase, the first rate-limiting enzyme in the classical pathway of haem synthesis, and also other enzymes in the pathway are not induced by H₂O₂. Therefore the second B. subtilis pathway for aminolevulinic synthesis, via glutamyl-tRNA, may be responsible for the H₂O₂-induced synthesis of porphyrin and haem.

Regulation of the response to H₂O₂

At least three regulatory mutations alter the response of B. subtilis to H₂O₂ stress.

The mutant strain, MA991 [7], is highly resistant to short- and long-term exposure to H₂O₂ and is less sensitive than the wild-type to cumene hydroperoxide and t-butyl hydroperoxide. The resistance to H₂O₂ cannot be further induced by pretreatment with low concentrations of H₂O₂ and mutant cultures display a similar degree of survival (approximately 10%) after a short-term H₂O₂ (10 mM) treatment to that of induced (pretreated) wild-type cultures. Proteins induced in the wild-type are present constitutively in the mutant at a higher level than in the induced wild-type and the mutant is filamentous [7] like the induced wild-type [13]. Thus, MA991 has the characteristics of an induced wild-type strain. These attributes of MA991 are consistent with it bearing a mutation in a gene controlling a peroxide-response regulon. Attempts have been made to isolate this regulatory gene by trying to transform the wild-type to H₂O₂-resistance with a bank of MA991 DNA on an integrating plasmid. The lack of success of these experiments suggests that MA991 contains more than one mutation or alternately that the selection conditions used were inadequate. Regardless of whether the pleiotropic phenotype of MA991 derives from a single or multiple mutations, it is probable that MA991 has been affected in at least one regulatory gene.

B. subtilis strains with null mutations in the spoOA gene are approximately 100-fold less sensitive to 10 mM H₂O₂ than their isogenic wild-type strain during exponential phase [5,6]. However,
some further resistance of spoOA mutants can be induced by low concentrations of \( \text{H}_2\text{O}_2 \) [5]. In addition, while only two of the proteins induced in the wild-type are also induced in the spoOA strain, most of the remaining proteins are not synthesized at a high level in the mutant [6]. Thus it is clear that MA991 and the spoOA mutants contain different regulatory mutations, the former affecting the inducible phenotype and the latter affecting the basal, uninduced sensitivity to \( \text{H}_2\text{O}_2 \). Inability to sporulate per se does not lead to \( \text{H}_2\text{O}_2 \) resistance, whereas lack of a functional Sp00A protein does cause this phenotype [5,6]. Sp00A is phosphorylated in response to nutrient deprivation and initiates the cascade of events that lead to sporulation. In addition, phosphorylated Sp00A regulates many late log/early stationary phase events via its negative control of the \( \text{abrB} \) gene. The AbrB protein in turn negatively or positively regulates the expression of a number of genes including a positive effect on \( \text{hpr} \) gene expression. The Hpr protein negatively regulates protease production and at least one gene required for sporulation. The \( \text{H}_2\text{O}_2 \) resistance phenotype of a spoOA strain is suppressed by mutations in the \( \text{abrB} \) gene or in the \( \text{hpr} \) gene, suggesting that the resistance of spoOA strains to \( \text{H}_2\text{O}_2 \) is due to the lack of a protein directly controlled by the \( \text{hpr} \) negative regulator [5]. This work was all performed on exponential phase cultures of \( \text{B. subtilis} \), thus suggesting a role for these genes during growth arrest (e.g. by 10 mM \( \text{H}_2\text{O}_2 \)) and not just stationary phase. Yasbin et al. [18] report the dependence on spoOA of the temporal expression of \( \text{kat-19} \) and the abolition of this dependence in the absence of a functional \( \text{abrB} \) gene. This suggests that spoOA has the opposite effect on catalase [18] from that expected based on \( \text{H}_2\text{O}_2 \) resistance [5,6]. It is not yet clear whether this discrepancy arises from the use of different spoOA strains or from the physiological state of the cultures during these experiments.

\( \text{B. subtilis} \) responds to \( \text{H}_2\text{O}_2 \) by the induction of at least one other regulon in addition to that specific for oxidative stress [13]. This second regulon is the SOS DNA repair system which is controlled by the \( \text{rec}A \) gene (previously called \( \text{recE} \)). The basal level of sensitivity to \( \text{H}_2\text{O}_2 \) is approximately the same in a \( \text{rec}A \) mutant as in the wild-type for concentrations of \( \text{H}_2\text{O}_2 \) of 10–30 mM. However the \( \text{rec}A^- \) strain is more sensitive than the wild-type to concentrations of \( \text{H}_2\text{O}_2 \) up to 10 mM though the mutant and wild-type cultures display similar degrees of survival in high concentrations of \( \text{H}_2\text{O}_2 \) after pretreatment with 0.05 mM \( \text{H}_2\text{O}_2 \). Thus \( \text{rec}A \) appears to affect the basal resistance to \( \text{H}_2\text{O}_2 \) concentrations up to 10 mM, but not the induced resistance to \( \text{H}_2\text{O}_2 \). Three DNA-damage-inducible (\( \text{din} \)) loci which are induced in a \( \text{rec}A \)-dependent fashion by mitomycin C are also induced by \( \text{H}_2\text{O}_2 \). At least one of these \( \text{H}_2\text{O}_2 \)-inductions is \( \text{rec}A \)-dependent. A \( \text{dinB} \) gene fusion is induced maximally by \( \text{H}_2\text{O}_2 \) at a concentration (3 mM) that slows growth and the degree of induction is comparable to that obtained with mitomycin C. Another SOB characteristic, that of prophage induction, is also affected by \( \text{H}_2\text{O}_2 \) since \( \phi 105 \) is induced by the oxidant. Thus the SOB system is clearly induced by \( \text{H}_2\text{O}_2 \), though its full induction (by mitomycin C) is not sufficient to render cells resistant to a range of \( \text{H}_2\text{O}_2 \) concentrations.

**General stress responses**

Stationary phase cultures of \( \text{B. subtilis} \) display complete viability after treatment with 10 mM \( \text{H}_2\text{O}_2 \), a concentration that reduces the viability of exponential phase cells to approximately 0.01% [6]. The same phenomenon has been observed in \( \text{E. coli} \) where the response has been partly characterized and found to be quite separate from that occurring during exponential phase [19,20]. The inhibition of growth by a variety of means including nutrient starvation and application of growth inhibiting concentrations of \( \text{H}_2\text{O}_2 \) causes the induction of general stress proteins (Gsps) in \( \text{B. subtilis} \) [21,22] and PEX proteins in \( \text{E. coli} \) [19,20].

**\( \text{E. coli} \)**

There is little apparent structural or regulatory overlap between stationary phase protection against \( \text{H}_2\text{O}_2 \) and the adaptive oxidative stress responses controlled by OxyR and SoxS/S which
involve catalase HP1 and different DNA repair enzymes. Many of the stationary phase characteristics of *E. coli*, including resistance to oxidative and other stresses, are dependent on an intact rpoS allele which codes for σ^+ (also called σ^38 or KatF) [19,20]. Several of the genes comprising the σ^+ regulon are involved in the development of increased resistance to environmental stresses, e.g. *katE* encoding the stationary phase catalase HP11, *xth* encoding the DNA repair enzyme, exonuclease 111 and *dps* are all important in protecting against H_2O_2 [19,20]. The *dps* gene is also involved in protection against H_2O_2 during growth but is regulated differently in this case, being activated by σ^+ in stationary phase but by OxyR during exponential growth [3]. Mutant cells lacking Dps fail to develop starvation-induced resistance to H_2O_2 (45 mM), despite having normal levels of catalase HP11 and also show changes in the pattern of proteins synthesized during starvation though not during log phase [23]. It has been suggested that starved cells must prepare in advance for the possibility of environmental stress, as their capacity for rapid response is severely compromised and that Dps carries out two of these important functions, i.e. it physically protects the DNA and maintains a low level of gene expression.

A universal stress protein (UspA) gene has been characterized from *E. coli* [24]. This differs from the genes that respond to many forms of growth inhibition in that it is transcribed by σ^70 and is not inversely dependent on growth rate. However, it is induced by growth arrest or by perturbations in unrestricted balanced growth. Fifteen different starvation and stress conditions (including H_2O_2), causing growth inhibition induce the expression of uspA but mutations in various global regulatory networks (including an oxyR mutation) do not affect induction of UspA. The properties of a *uspA* mutant suggest that UspA may modulate and reorganize the flow of carbon in the central metabolic pathways during growth arrest.

**B. subtilis**

The proteins induced in *B. subtilis* by growth limiting levels of a variety of stresses have been investigated using 2-dimensional gels [21,22]. Some proteins are stress-specific e.g. the DnaK and GroEL chaperones are induced by heat shock but not by oxidative stress or a number of other stresses. Stress proteins specific for H_2O_2 have also been detected but not investigated further. However, numerous proteins, termed general stress proteins (Gsps), are induced by a variety of stresses including H_2O_2 (0.005% or 1.5 mM), heat shock, salt stress, glucose limitation and oxygen limitation [21,22]. Most of the Gsp genes have SigB-dependent promoters though a few are SigB-independent. Volker et al. [22] speculate that Gsps may constitute the equivalent of the PEX proteins of *E. coli* [19,20] and may provide an unspecific protection of the cell under non-growing or starving conditions. N-terminal sequencing of these Gsps identified four as ClpP, GsiB, Ctc and RsbW [22].

The ClpP protein is induced by H_2O_2, heat shock, salt stress, glucose limitation and oxygen limitation [21,22] and its induction is not influenced by mutations of sigB [22]. It has strong homology with the ClpP proteolytic subunit of *E. coli*. The *gsiB, ctc* and *rsbW* genes are induced by H_2O_2, heat shock, salt stress, glucose limitation and oxygen limitation in a SigB-dependent fashion [22]. The functions of the *ctc* and *gsiB* genes are not known but deletion of the latter has no obvious effect on bacterial growth or viability or catabolite repression of sporulation. RsbW is an anti-sigma factor which inhibits SigB activity. SigB is activated after stress by the activation of RsbV, an anti-anti-sigma factor, which binds to RsbW, thereby releasing it and activating SigB [25]. Another SigB dependent gene codes for UDP-glucose pyrophosphorylase, which, in *E. coli*, forms part of the biosynthetic pathway for trehalose, a protective compound required for osmotolerance and thermotolerance in the stationary growth phase.

Boylan et al. [26] propose that SigB may control a general stress regulon that is induced when cells encounter a variety of growth limiting conditions. SigB is activated early in stationary phase growth but is not required for sporulation and the *sigB* operon which includes the *sigB* and *rsbW*
genes is induced by heat shock and by salt during log phase when SigB is normally inactive [22,26]. Salt, heat shock and ethanol all increase the amount of SigB protein within the cell but \( \text{H}_2\text{O}_2 \) (0.005%) has no effect. It is clear that SigB controls the expression of several general stress genes, but Boylan et al. [26] note that the fact that \( \text{H}_2\text{O}_2 \) does not induce SigB activity but does induce the general stress regulon indicates that there is additional control on this system. Furthermore, a \( \text{sigB} \) null mutant is not at a survival disadvantage compared to the wild-type when exposed to osmotic or heat shocks of exponential or stationary phase cells or to extreme dessication [26]. Similarly, a \( \text{sigB} \) mutant and the wild-type parent strain were equally sensitive to 10 mM \( \text{H}_2\text{O}_2 \) during exponential phase and both completely insensitive during stationary phase (B. Dowds, unpublished data). Boylan et al. [26] suggest the possibility that Gsps controlled by SigB might compose part of a redundant system whereby the cells respond to a variety of stresses, but it is clear that SigB does not play a role in the response of \( \text{B. subtilis} \) to oxidative stress.

**Resistance of spores to \( \text{H}_2\text{O}_2 \)**

The spore of \( \text{B. subtilis} \) is metabolically inactive in contrast to starved cells of \( \text{E. coli} \). Its DNA is protected by being covered with small acid soluble proteins (SASP) of the \( \alpha/\beta \) type that protect the DNA from a variety of damaging agents including heat and UV light. Spore DNA is also protected by the decreased water content in the spore retarding chemical reactions with DNA, and by DNA repair being initiated minutes after the onset of spore germination. Dormant spores of \( \text{B. subtilis} \) are quite resistant to \( \text{H}_2\text{O}_2 \). Setlow and Setlow [27] showed that \( \text{H}_2\text{O}_2 \) (4 M) kills 99% of mid-log phase cells in less than 1 min compared with more than 1 h for 99% of spores. Mutant spores lacking \( \alpha/\beta \)-SASP are intermediate in displaying 1% survival after about 15 min and the mutant spores which survive the treatment have a significant number of mutations including single strand breaks. Generation of a large number of abasic sites did not accompany \( \text{H}_2\text{O}_2 \)-killing, unlike heat killing, of SASP-deficient spores [28]. Removal of spore coats from \( \text{B. subtilis} \) has no effect on \( \text{H}_2\text{O}_2 \)-resistance [27], as found previously for \( \text{B. cereus} \), but unlike the case for \( \text{Clostridium bifermentans} \). \( \text{H}_2\text{O}_2 \)-resistance was acquired in two steps during spore formation; during \( \alpha/\beta \)-type SASP synthesis and during dipicolinic acid accumulation [27]. Mutants blocked in progressing from this first stage to the second failed to acquire the second increment of resistance. The author notes that the NADH pool of the developing forespore is lost completely at about the time of dipicolinic acid accumulation and that this loss of reducing equivalents would slow down the generation of hydroxyl radicals from \( \text{H}_2\text{O}_2 \) via the Fenton reaction. Another contribution to the \( \text{H}_2\text{O}_2 \)-resistance of spores might be expected to come from the spore-specific catalase. However, this is first synthesized during stationary phase [12] before the acquisition of the first significant increment of resistance [27].

**Conclusions**

Much work remains to be done to elucidate the responses of \( \text{Bacillus subtilis} \) to \( \text{H}_2\text{O}_2 \) in growing and stationary phase cells and in spores. There is as yet a sketchy picture of the proteins or other protective molecules induced during these stages and little mutagenesis work has been done which would allow us to assess the importance of these molecules. The connection between the response at stationary phase and following growth inhibition caused by relatively high concentrations of \( \text{H}_2\text{O}_2 \) needs to be further clarified. Knowledge about regulatory genes controlling basal and induced resistance to \( \text{H}_2\text{O}_2 \) is not far advanced and virtually nothing at all is known about the response of \( \text{B. subtilis} \) to other oxidants besides \( \text{H}_2\text{O}_2 \).

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