The induction of oxidative enzymes in *Streptomyces coelicolor* upon hydrogen peroxide treatment

JONG-SOO LEE, YUNG-CHIL HAH and JUNG-HYE ROE*

Department of Microbiology, College of Natural Sciences, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

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*Streptomyces coelicolor* (Müller) became resistant to killing by hydrogen peroxide (H$_2$O$_2$) when pretreated with non-lethal concentrations of H$_2$O$_2$. When rapidly growing cells were pretreated with 100 μM-H$_2$O$_2$, they became 7–10-fold more resistant to 20 mM-H$_2$O$_2$ than were naive cells. Activities of several oxidative defense enzymes were measured in cells treated with 100 μM-H$_2$O$_2$ in either exponential or stationary phase growth. The specific activity of catalase in crude extracts of cells pretreated in either phase increased about 40%. Peroxidase activity, in cell extracts and culture supernatants, respectively, of cells treated in the stationary growth phase increased two times and four times. Glucose-6-phosphate dehydrogenase increased by 60% at the exponential growth phase. Glutathione reductase increased 80% after treatment in the exponential phase and 4-fold in the stationary growth phase. However, superoxide dismutase activity decreased by 70%. Two mutants resistant to H$_2$O$_2$ were isolated after mutagenesis of spores with N-methyl-N-nitro-N-nitrosoguanidine. In addition to a dramatic increase in the survival rate in 20 mM-H$_2$O$_2$, both mutants exhibited increased activities of all the above enzymes except superoxide dismutase. The pleiotropic phenotype of the mutants suggests that there exists a global regulation of oxidative response in *S. coelicolor*.

Introduction

All aerobic organisms must cope with reactive oxygen species such as the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (HO'), produced as incomplete reduction products of molecular oxygen, and with singlet oxygen formed photochemically (Cadenas, 1989; Morgan *et al.*, 1986; Halliwell, 1990). Bacteria grown aerobically encounter these oxidants not only from inside the cell, but also from outside, as occurs, for example, when pathogenic bacteria are attacked by phagocytic cells (Hassett & Cohen, 1989). The reactive oxygen species can oxidize membrane fatty acids and proteins, and can damage DNA (Morgan *et al.*, 1986; Saran & Bors, 1990). The responses of bacterial cells to these oxidative stresses have been studied mostly in *Escherichia coli* and *Salmonella typhimurium* (Storz *et al.*, 1990; Farr & Kogoma, 1991). Several oxidative defence proteins and their genes have been found in *E. coli* (Storz *et al.*, 1989, 1990; Farr & Kogoma, 1991). These include defence (radical-scavenging) enzymes such as superoxide dismutase (SOD) (*sodA, sodB*), catalase (*katG, katE*), alkylhydroperoxide reductase (*ahpC, ahpF*) and glutathione reductase (*gord*). Several DNA enzymes such as exonuclease III (*xthA*), endonuclease IV (*nfo*) and other endonucleases (*recB, recC*), as well as some metabolic enzymes such as NADH dehydrogenase (*ndh*) and glucose-6-phosphate dehydrogenase (*zwf*) are also involved in oxidative defence. These enzymes might repair DNA damage caused by H$_2$O$_2$ or HO' radicals (Imlay *et al.*, 1988; Imlay & Linn, 1988), protect cells from membrane damage such as lipid peroxidation (Farr *et al.*, 1988), or lower the reducing equivalents to prevent generation of harmful HO' radicals in the presence of metal and reducing equivalents (Imlay *et al.*, 1988). The evolution of such defence mechanisms in order to prevent or repair oxidative damage must have been critical to the survival of aerobic life forms (Halliwell & Gutteridge, 1989).

Induction of the defence proteins by treating cells with low doses of H$_2$O$_2$ or radical-producing oxidants provides the basis for adaptation as well as resistance to the oxidants. Several regulatory proteins mediate expression...
of the genes for these proteins. The genes induced by superoxide-generating compounds such as paraquat, plumbagin or menadione include sodA and zwf, and are all controlled by the regulator SoxR (Tsaneva & Weiss, 1990). The genes induced by H₂O₂ include katG, ahpC, ahpF and gorA, and are all controlled by the positive regulator OxyR (Christman et al., 1985, 1989; Morgan et al., 1986). The so-called soxR and oxyR regulons seem to overlap other stress response regulons such as that for heat-shock, since some proteins are induced in common by different stresses (Greenberg & Demple, 1989; VanBogelen et al., 1987), and some cross-protection has been observed (Christman et al., 1985; Jenkins et al., 1988).

Compared with E. coli and S. typhimurium, little is known about these systems in Bacillus subtilis, and even less in other bacteria. In B. subtilis, several proteins are induced by H₂O₂ (Murphy et al., 1987), and the sensitivity of B. subtilis to H₂O₂ is growth-cycle-dependent. Increased sensitivity in certain sporulation mutants has also been reported (Dowds et al., 1987; Bol & Yasbin, 1990).

We have tried to elucidate the regulation of defences against oxidative stress in Streptomyces coelicolor (Müller). This paper presents a biochemical analysis of the adaptation to H₂O₂ in S. coelicolor, and some evidence for a global genetic system that controls the expression of many defence enzymes in this organism.

**Methods**

**Media and culture conditions.** Growth and maintenance of S. coelicolor (Müller) (ATCC 10147) were essentially as described by Hopwood et al. (1985). S. coelicolor was grown routinely in minimal or YEME media (Hopwood et al., 1985) at 30 °C with shaking. The growth phase of cultures was determined by measuring growth as OD₆₀₀, dried mycelium weight or DNA content (Baserga, 1989).

For adaptation experiments in the early exponential growth phase, germinated spores were used to inoculate fresh minimal media to an OD₆₀₀ of 0.03–0.05. After cultures had been incubated with shaking for 3 h at 30 °C, H₂O₂ was added to a final concentration of 100 μM and the cultures were kept at 30 °C for 1 h. The cultures were then challenged with H₂O₂ at final concentrations up to 20 mM. Samples were withdrawn at 10 min intervals, and diluted immediately in minimal medium; aliquots were plated on nutrient agar and incubated for 2 d at 30 °C to count viable cells.

For adaptation experiments in the mid-exponential growth phase, spores were used to inoculate YEME media. Following growth for 36–40 h, mycelia were collected by centrifugation and resuspended in minimal medium. H₂O₂ was added to a final concentration of 100 μM and the cultures were incubated without shaking at 30 °C for 30 min. The cultures were then challenged with H₂O₂ as above. Protoplasts were obtained as described by Hopwood et al. (1985). Dilutions were made in P buffer (Hopwood et al., 1985), and aliquots of diluted cells were plated on R2YE regeneration medium. They were incubated for 2 d at 30 °C to count viable and regenerated cells.

For enzyme assays, germinated spores were used to inoculate minimal medium and the cultures were grown for 40–48 h, when their OD₆₀₀ was 0.9–1.2 (exponential growth phase) or for 7–10 d (stationary growth phase). The production of both blue and red pigments was regarded as an indication of entry into the stationary growth phase. The mycelium and DNA content of stationary phase cultures decreased by 20–60%.

**Preparation of cell extracts.** Mycelium was harvested by centrifugation at 3000 g for 10 min and was washed twice with 10 mM-potassium phosphate buffer (pH 7.0). The washed mycelium was suspended in 50 mM-potassium phosphate buffer (pH 7.0) containing 1 mM-phenylmethylsulphonyl fluoride and was disrupted by abrasion with glass beads for 5 min in a mini-bead beater (Biospec) at the maximum power setting. The cell debris was pelleted at 12000 r.p.m. for 15 min in a microcentrifuge. Cells and extracts were maintained at 4 °C throughout the procedures described above.

**Enzyme assays.** Catalase activity in extracts was determined as described by Beers & Sizer (1952). The rate of disappearance of H₂O₂ was measured spectrophotometrically at 240 nm. The reaction mixture contained 12 μl 3% (v/v) H₂O₂ and an appropriate amount of enzyme in 50 mM-potassium phosphate buffer (pH 7.0) in a 1 ml total volume. The assay was done at 30 °C in quartz cuvettes of 1 cm path length. One unit of enzyme was defined as the activity that catalysed the degradation of 1 μmol H₂O₂ min⁻¹.

Peroxidase activity in cell extracts and culture supernatants was measured spectrophotometrically by monitoring the oxidation of o-dianisidine at 460 nm in the presence of H₂O₂ (Kapoor & Sreenivasan, 1988). The reaction mixture contained 10 μl 3 M-sodium acetate buffer (pH 4.8), 10 μl H₂O₂ and 10 μl 0.044% o-dianisidine, in a 1 ml total volume. One unit of enzyme was defined as the activity that increased the A₄₆₀ by 0.01 min⁻¹.

The assay for superoxide dismutase (SOD) (Beauchamp & Fridovich, 1971) was based on the transfer of electrons by xanthine oxidase to nitroblue tetrazolium (NBT) via oxygen. This reduction of NBT is inhibited by SOD. The reaction mixture contained 0.1 mM-xanthine, 25 μM-NBT, 0.1 mM-EDTA and 0.01% (w/v) xanthine oxidase, in 50 mM-sodium carbonate buffer (pH 10.2). The initial rate of reduction of NBT in reaction mixtures with and without SOD was followed by measuring the A₄₆₀. The amount of SOD activity causing 50% inhibition was defined as one unit.

Glutathione reductase activity in crude extracts was measured by the modified procedure of Smith et al. (1988). This is based on the increase in A₃₄₀ when 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) is reduced by reduced glutathione (GSH), produced by glutathione reductase. The reaction mixture contained 1 mM-EDTA, 0.3 mM-DTNB, 0.1 mM-NADPH and 1 mM-oxidized glutathione (GSSG), in 200 mM-potassium phosphate buffer (pH 7.5). A unit of activity was defined as the amount that catalysed the production of 1 μmol thiobarbituric acid (TNB) min⁻¹.

Glucose-6-phosphate dehydrogenase was measured using protocols outlined in the Worthington Manual (Decker, 1977), as the rate of reduction of NADP monitored at 340 nm. The assay mixture contained 30 μl 32 mM-glucose-6-phosphate in 100 mM-Tris/HCl (pH 7.5), 10 μl 23 mM-NADP in 1% sodium bicarbonate, and enzyme solution in a 1:10 total reaction volume. One unit of enzyme was defined as the activity that catalysed the reduction of 1 μmol NADP min⁻¹.

**Mutagenesis with N-methyl-N-nitro-N-nitrosoguanidine (NTG) and screening for H₂O₂-resistant mutants.** Spores were treated with NTG (1 mg or 3 mg ml⁻¹) to a survival ratio of 0–10% and then plated on Bennet's medium (Jones, 1949), presoaked with 30 mM-H₂O₂ for 30 min. Spores of surviving colonies were collected to test resistance to H₂O₂. About 10⁴ spores from each colony were spread in parallel rows on Bennet's medium. After 5 h, a strip of filter paper soaked with 3% (880 mM) H₂O₂ was placed at 90° across the centre of the inoculated streaks and incubation was continued for 2 d. Mutants that grew closer to the strip than the wild-type were collected. For disk tests, around 10⁶ spores from each mutant were plated on Bennet's medium; 5 h later 60 μl 30% H₂O₂ absorbed on a filter disk was placed at the centre of a
Results and Discussion

Adaptation to \( H_2O_2 \) stress

We tested the sensitivity of \( S. coelicolor \) to \( H_2O_2 \) as well as its adaptation in early and mid-exponential growth phases. In the adaptation experiments, we pretreated cells with a non-lethal concentration (100 \( \mu \)M) of \( H_2O_2 \) for 1 h before exposure to a lethal concentration (20 mM). To obtain viable cell counts by plating, we grew cultures in minimal medium for 3 h after inoculation with germinated spores. The viability of cells in 20 mM-\( H_2O_2 \) was counted at 10 min intervals after plating. The results (Fig. 1) demonstrate that only 0.1\% of the cells remained viable after 30 min in 20 mM-\( H_2O_2 \). However, when cells were pretreated with \( H_2O_2 \), survival was enhanced almost 10-fold.

The extent of adaptation was similar when cells were grown to mid-exponential phase and counted as regenerated protoplasts after \( H_2O_2 \) treatment. The results suggest that the pretreatment with 100 \( \mu \)M-\( H_2O_2 \) caused mycelial cells to adapt better to a higher dose of \( H_2O_2 \), showing a survival rate seven times higher than naive cells against 20 mM-\( H_2O_2 \) (Fig. 2). Since the colonies went through lysozyme treatment and regeneration in addition to \( H_2O_2 \) treatment, we cannot quantitatively estimate the \( H_2O_2 \) sensitivity or adaptation. However, the results point to qualitatively similar adaptations in early-exponential and mid-exponential growth phases.

The adaptation of \( H_2O_2 \) in \( S. coelicolor \) occurred under conditions slightly harsher than those previously described for \( E. coli \) (Demple et al., 1983), \( S. typhimurium \) (Christman et al., 1985) and \( B. subtilis \) (Dowds et al., 1987). Pretreatment with 60 and 50 \( \mu \)M-\( H_2O_2 \) increased survival against 10 mM-\( H_2O_2 \) by approximately 50- and 100-fold, in \( E. coli \) and in \( B. subtilis \), respectively.

Adaptation is accompanied by induction of several enzyme activities involved in defence against oxidative stress

The specific activities of several oxidoreductases and free-radical scavenging enzymes known to function in protection against oxidative stress were measured (Table 1). Cells in the exponential and stationary growth phases were treated with 100 \( \mu \)M-\( H_2O_2 \) for 30 min. The specific activity of catalase in crude extracts of treated cells increased about 40% above that in untreated cells at both exponential and stationary phases. This is a smaller response than the 4–5-fold induction of catalase activity in \( E. coli \) (Christman et al., 1985) and \( S. typhimurium \) (Finn & Condon, 1975). The constitutive level of catalase activity in \( S. coelicolor \) was about 7-fold and 4-fold higher than that in \( E. coli \) and \( B. subtilis \), respectively (data not shown).

Peroxidase activity was not induced in the exponential and stationary growth phases by treatment with 100 \( \mu \)M-\( H_2O_2 \) (Table 1). However, it increased 2-fold in extracts of treated cells from cultures more than 2 weeks old. Peroxidase activity in cell-free supernatants from cultures treated at this later stationary growth phase was 4-fold higher than in untreated cells. It is not known whether the activity in the culture supernatants originated from active secretion or from passive release due to cell damage.
Reduced glutathione is thought to play a role in preventing excessive damage from toxic oxygen species (Penninckx & Jaspers, 1982). Glutathione can be easily oxidized to the intermolecular disulphide, which subsequently becomes reduced by glutathione reductase (Meister & Anderson, 1983). The activity of glutathione reductase in cell extracts was induced almost 2-fold by following treatment with 100 μM-H₂O₂ in the exponential growth phase (Table 1). Since glutathione was not detected in extracts of stationary phase cells. In contrast, SOD activity in the exponential growth phase was 60% higher in extracts of stationary phase cells. Since glutathione was not detected in extracts of stationary phase cells. Glutathione can be easily oxidized to the intermolecular disulphide, which subsequently becomes reduced by glutathione reductase (Meister & Anderson, 1983). The activity of glutathione reductase in cell extracts was induced almost 2-fold by following treatment with 100 μM-H₂O₂ in the exponential growth phase (Table 1). Since glutathione was not detected in extracts of stationary phase cells.

Table 1. Changes in specific activity (U mg⁻¹) of defence enzymes following treatment with 100 μM-H₂O₂

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>H₂O₂ treatment</th>
<th>Exponential phase</th>
<th>Stationary phase</th>
</tr>
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<tbody>
<tr>
<td>Catalase</td>
<td></td>
<td>634 ± 51</td>
<td>880 ± 114</td>
</tr>
<tr>
<td>Peroxidase</td>
<td></td>
<td>21 ± 0.3</td>
<td>20 ± 0.3</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td></td>
<td>64 ± 3.0</td>
<td>105 ± 5.4</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td></td>
<td>508 ± 233</td>
<td>923 ± 226</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td></td>
<td>285 ± 6.2</td>
<td>83 ± 16</td>
</tr>
</tbody>
</table>

NT, Not tested; ND, not detected.
*Activities from cells grown for 14-21 d in minimal medium.

Reduced glutathione is thought to play a role in preventing excessive damage from toxic oxygen species (Penninckx & Jaspers, 1982). Glutathione can be easily oxidized to the intermolecular disulphide, which subsequently becomes reduced by glutathione reductase (Meister & Anderson, 1983). The activity of glutathione reductase in cell extracts was induced almost 2-fold by following treatment with 100 μM-H₂O₂ in the exponential growth phase and 4-fold in the stationary growth phase (Table 1). Since glutathione was not detected in Streptomyces cacaoiatus, it is possible that the glutathione reductase activity that we measured in S. coelicolor is from another reductase that can use glutathione as a substrate. One plausible candidate is thioredoxin reductase (Y. Aharonowitz, Tel Aviv University, Israel, personal communication).

Reduced NADP, produced by glucose-6-phosphate dehydrogenase can be used as reducing power during protection and repair against oxidative stress. The specific activity of glucose-6-phosphate dehydrogenase in the exponential growth phase was 60% higher in treated than in untreated cells (Table 1). Little activity was detected in extracts of stationary phase cells.

SOD activity in cell extracts decreased by 70% following treatment with 100 μM-H₂O₂ in the exponential growth phase (Table 1). In contrast, SOD activity in E. coli (Gregory & Fridovich, 1973; Hassan & Fridovich, 1977) and S. typhimurium (Christman et al., 1985) was induced following a shift from anaerobic to aerobic conditions or H₂O₂ stress. In E. coli, the activity of manganese SOD increased during oxidative stress, while that of iron SOD decreased (Hassan & Fridovich, 1977). Previous investigations showed that manganese SOD was induced by oxygen, redox-cycling compounds in aerobicosis, and iron chelators in anaerobiosis, suggesting that the regulation of SOD activity might be at the transcriptional and the post-transcriptional level (Touati, 1988). Since the superoxide radical is converted irreversibly to H₂O₂ in vivo, the failure of H₂O₂ to induce SOD activity in S. coelicolor is not surprising. The reduction in activity by H₂O₂ could have resulted from competition for metals among some H₂O₂-induced oxidoreductases, which are metalloproteins.

The difference in the extent of induction depending on the growth phase is consistent with the growth-phase-dependent sensitivity to H₂O₂ observed in B. subtilis (Dowds et al., 1987). This dependence suggests an interaction between the oxidation-inducible and the growth-phase-specific systems.

H₂O₂-resistant mutants overexpress several enzyme activities

As a preliminary to genetic investigation of the regulatory system for the response against oxidation in S. coelicolor, we isolated mutants resistant to up to 880 mm-H₂O₂. The mutants arose during NTG mutagenesis at a frequency of about 1 x 10⁻⁵ per surviving cell. Two mutants showing the highest resistance by disc and strip tests were further characterized. They exhibited marked increases in survival rate against H₂O₂ (Fig. 3). Cross-protection against heat shock (50°C) was also greater in the two mutants compared with the wild-type (data not shown). The specific activity of oxidoreductases in the mutants were measured in the
exponential and stationary growth phases without H$_2$O$_2$ treatment (Table 2). Both mutants contained higher levels than wild-type cells of all the enzymes tested except SOD.

Catalase activity in mutant N7 was 8-fold higher in the exponential growth phase and 4-fold higher in the stationary growth phase than in wild-type cells. In mutant N24, catalase activity was 3-fold higher in the exponential phase and 2-fold higher in the stationary phase. Intracellular peroxidase activity was about 2-fold higher in the later phase of stationary growth, whereas extracellular activity was 3-4-fold higher in the two mutants. Glutathione reductase activity was about 2-fold higher in the two mutants. Glucose-6-phosphate dehydrogenase activity was 4-fold higher in N7 and 50% higher in N24. In both mutants, the increase in catalase activity was much greater than that induced in wild-type cells by 100 μM-H$_2$O$_2$. In the exponential growth phase, the level of catalase in mutants N7 and N24 was 6 times and 2 times greater than the H$_2$O$_2$-induced level in wild-type cells. SOD activity in the mutants was similar to that in naive wild-type cells. The extent of the increase in peroxidase and glutathione reductase activity in the mutants was similar to the extent of induction by H$_2$O$_2$ in wild-type cells. When the mutant cells were treated with 100 μM-H$_2$O$_2$, the levels of all five enzymes did not change significantly (data not shown).

Considering the frequency with which these mutants arose, it is unlikely that separate mutations had occurred in the genes for each enzyme affected. The pleiotropic effect of the mutations is possibly due to a change in a regulatory gene controlling the response to H$_2$O$_2$ stress. It seems plausible to assume that there is global regulation of the oxidative response in S. coelicolor, as in E. coli and S. typhimurium, the oxyR (Christman et al. 1989) and soxR (Tsaneva & Weiss, 1990) genes are known to be required for induction of a response regulon against H$_2$O$_2$ and superoxide, respectively. OxyR is a transcriptional activator for the genes katG (encoding catalase), ahpCF (encoding alkylhydroperoxide reductase) and gorA (encoding glutathione reductase), whereas SoxR is a global regulator governing nfo (encoding endonuclease IV), zwf (encoding glucose-6-phosphate dehydrogenase) and sodA (encoding manganese superoxide dismutase). However, since the enzymes induced in resistant mutants N7 and N24 encompass those from both OxyR and SoxR regulons in E. coli, we suppose that the make-up of the peroxide-inducible regulon in S. coelicolor is different from E. coli.

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### Table 2. Comparison of specific activities (U mg$^{-1}$) of defence enzymes in the wild-type and in resistant mutants at different growth phases

Growth phases were as in Table 1. Values are means ± standard deviations from more than three experiments.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Wild-type</th>
<th>N7</th>
<th>N24</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Exponential</td>
<td>Stationary</td>
<td>Exponential</td>
</tr>
<tr>
<td>Catalase</td>
<td>634 ± 51</td>
<td>690 ± 8</td>
<td>5339 ± 239</td>
</tr>
<tr>
<td>Peroxidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular</td>
<td>2.1 ± 0.3</td>
<td>2.0 ± 0.3</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Extracellular</td>
<td>ND</td>
<td>6.8 ± 0.6*</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>64 ± 30</td>
<td>NT</td>
<td>25.1 ± 0.9</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>508 ± 233</td>
<td>200 ± 46</td>
<td>993 ± 82</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>28 ± 5.62</td>
<td>NT</td>
<td>21.6 ± 4.4</td>
</tr>
</tbody>
</table>

NT, ND, *, as Table 1.

### References


