Regulation of the oxidative stress response by the *hpr* gene in *Bacillus subtilis*

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Bacillus subtilis mutants with null mutations in the *spo0A* gene are resistant to oxidative stress during the exponential phase of growth. This resistance phenotype can be suppressed by mutations in the *abrB* gene, or in the *hpr* gene. Both of these gene products are negative regulatory proteins which are over-produced in a *spo0A* strain, and the over-production of the *hpr* gene product results from over-production of the *abrB* gene product. The results suggested that the resistance to oxidative stress in a *spo0A* strain is due to the lack of a protein directly controlled by the *hpr* negative regulator. Other mutations in the *spo0A* gene conferring resistance to ethanol stress (*eth*) or suppressors of sporulation phenotypes (*sof*) had no effect on the sensitivity to oxidative stress of strains bearing them.

Introduction

Bacteria have evolved mechanisms for allowing them to cope with a variety of stresses including the presence of oxidants in their environment. Sub-lethal levels of stress-inducing agents evoke a protective response which allows the cells to survive more extreme levels of the same agent (or, in some cases, different agents) (Lindquist, 1986). The protective response to a given type of stress involves the induction of several proteins. There is overlap in the proteins induced in response to a diverse range of stresses, while some remain unique to the inducing agent (Morgan *et al.*, 1986; Van Bogelen *et al.*, 1987).

In one example of this phenomenon, sub-lethal levels (50 μ M) of hydrogen peroxide can induce a protective response in the obligate aerobe *Bacillus subtilis* so that the cells survive otherwise lethal concentrations (10 mM) of this agent. Treatment with 50 μ M-H₂O₂ induces the synthesis of at least eight proteins, some of which may be important in the protective response (Murphy *et al.*, 1987). Paradoxically, strains with mutations in the *spo0A* gene were constitutively resistant to 10 mM-H₂O₂, and some of the proteins induced (by 50 μ M-H₂O₂) in the wild-type strains were synthesized constitutively in a *spo0A* mutant in the absence of stress (Dowds *et al.*, 1987). Surprisingly, wild-type *B. subtilis* cultures

become resistant to the effects of 10 mM-H₂O₂ upon reaching stationary phase (Dowds *et al.*, 1987). Mutations in the *spo0* genes which disrupt the expression of early stationary-phase and sporulation genes did not prevent the stationary phase resistance phenomenon.

The *spo0A* gene product is thought to be required for the initial stage of sporulation in which the cells detect changes in their environment which induce sporulation. *spo0A* mutants have extremely pleiotropic phenotypes which may result from the Spo0A protein controlling sporulation as well as the expression of a number of genes expressed at stationary phase (Hoch, 1976). Many of the stationary-phase genes are regulated by the *spo0A* gene via the *abrB* gene (Trowsdale *et al.*, 1979). *spo0A* mutants over-express the AbrB protein, which is a transcription regulator acting negatively on the expression of many genes (Perego *et al.*, 1988; Strauch *et al.*, 1989). The AbrB protein also acts in a positive manner to induce the synthesis of the Hpr protein, which negatively regulates protease production and at least one gene required for sporulation (Perego & Hoch, 1988).

In this work we investigated the pathway whereby *spo0A* regulates the oxidative stress response and found that over-expression of the *hpr* gene leads to resistance of *B. subtilis* to oxidative stress during the exponential phase.

Table 1. *B. subtilis* strains

Strain	Genotype	Reference or source
W168	Wild-type	Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio, USA
JH642	<i>trpC2 phe-1</i>	
JH646	<i>trpC2 phe-1 spo0A12</i>	
JH703	<i>trpC2 phe-1 spo0AΔ204</i>	
Eth7	<i>trpC2 ssa-7</i>	
Eth415	<i>trpC2 ssa-15</i>	Bohin & Lubochinsky (1982)
Sof105	<i>trpC2Δspo0F sof-105</i>	Bohin & Lubochinsky (1982)
JH12588	<i>trpC2 phe-1 spo0A12 abrBΔCAT</i>	Hoch <i>et al.</i> (1985)
JH12607	<i>trpC2 phe-1 abrBΔCAT</i>	M. Perego & J. A. Hoch (unpublished)
Hpr2	<i>trpC2 hpr-2</i>	
JH12769	<i>trpC2 phe-1 spo0A12 hprΔCAT</i>	Perego & Hoch (1988)
JH12611	<i>trpC2 phe-1 abrBΔCAT amyE::[abrB'-lacZ pC194CAT]</i>	This study
JH12527	<i>trpC2::pJM2488 [hpr'-lacZ]</i>	M. Perego & J. A. Hoch (unpublished)
JH12464	<i>trpC2 phe-1 amyE::[aprE'-lacZ pC194CAT]</i>	Perego & Hoch (1988)
		Ferrari <i>et al.</i> (1988)

Methods

Strains. The *Bacillus subtilis* strains used in this study are listed in Table 1.

Growth conditions. *B. subtilis* was grown for all experiments in minimal medium as described by Murphy *et al.* (1987). Chloramphenicol (5 µg ml⁻¹) was added where appropriate.

Induction of protection against oxidative stress. The method of Murphy *et al.* (1987) was used but was varied in the following details. Cultures were grown to an OD₅₅₀ of 0.1 and treated with H₂O₂ at a final concentration of 100 µM (inducing concentrations) for 15 min or 10 mM (killing concentration) for 10 min.

Induction of *lacZ* fusions with H₂O₂. *B. subtilis* strains containing *lacZ* fusions were grown to an OD₅₅₀ of 0.1. Fractions of 1 ml were removed into prewarmed 10 ml culture tubes. Half of the tubes contained H₂O₂ at a final concentration of 100 µM and half were untreated. The tubes were vigorously shaken at 37 °C for various lengths of time (15 min and 40 min in the case of the *abrB-lacZ* fusion and 10 min, 25 min and 45 min in the case of the *hpr-lacZ* fusion and the *apr-lacZ* fusion). The entire contents of each tube were then spun in an Eppendorf centrifuge for 5 min at 4 °C, the supernatants were discarded and the pellets were frozen at -70 °C. The thawed pellets were each resuspended in 1 ml Z buffer (Ferrari *et al.*, 1986). An appropriate volume of the suspension was then lysed and assayed for β-galactosidase (Ferrari *et al.*, 1986) so that the A₄₂₀ (of the *o*-nitrophenol reaction product) fell between 0.15 and 0.6.

Results

spo0A mutants and oxidative stress

We have previously shown the effects of 1 h treatments with H₂O₂ on the subsequent survival of wild-type and *spo0A* strains of *B. subtilis* (Dowds *et al.*, 1987). We found considerable day-to-day variation in the effects of H₂O₂ and concluded that some or all of this variability could be attributed to the growth phase of the cells. In addition we found that a 10 min H₂O₂ treatment was sufficient to give the maximum. However, even with a short exposure

Table 2. Percentage survival of JH642 (*spo0A*⁺) after H₂O₂ treatment

The numbers in this and subsequent tables are the percentage survival of the treated cultures compared to the same culture untreated.

Expt	H ₂ O ₂ treatment		
	10 mM	100 µM, then 10 mM	100 µM
1	0.1	6.7	97
2	0.23	48	100
3	0.17	8.7	87
4	0.003	9.0	73
5	0.03	1.2	90

time and treatment of the cells at exactly the same culture density, some day-to-day variation was still observed (e.g. Tables 2 and 3).

Table 2 shows the effect of induction by 100 µM-H₂O₂ on the subsequent challenge by 10 mM-H₂O₂ in a sporulation-proficient strain. Typically about 0.1% of the uninduced cells of strain JH642 survived a challenge with 10 mM-H₂O₂. Preinduction with 100 µM-H₂O₂ resulted in an increase in survival of 40 to several hundred fold. Similar experiments with the isogenic *spo0A* mutant strain JH646 showed that the mutant strain was constitutively five- to 6000-fold more resistant to challenge with 10 mM-H₂O₂ (Table 3). Preinduction of the *spo0A* mutant strain still resulted in a 2–50-fold increase in survival over the uninduced culture and the strain was resistant to H₂O₂ in the stationary phase. The classical wild-type strain, W168, gave the same results as JH642 (data not shown).

Treatment of other strains carrying mutations in the *spo0A* locus (Table 4) revealed that their oxidative stress

Table 3. Percentage survival of JH646 (*spo0A12*) after H_2O_2 treatment

Expt	H_2O_2 treatment		
	10 mM	100 μ M, then 10 mM	100 μ M
1	5.8	19	78
2	19	33	113
3	1.2	67	100
4	1.1	24	93
5	10	24	114

phenotype correlated with the functionality of the *spo0A* gene in terms of sporulation. Thus *spo0A* strains JH646 (nonsense mutation, *spo0A12*) (Kudoh *et al.*, 1985) and JH703 (deletion mutation) (Ferrari *et al.*, 1985) are both unable to sporulate because of loss of the Spo0A protein and both are resistant to oxidative stress. On the other hand two mutations in the *spo0A* locus which have a Spo⁺ phenotype cause a wild-type inducible oxidative stress phenotype. These include the *eth-7* and *eth-415* mutations, which make strains resistant to inhibition of sporulation by ethanol (Bohin & Lubochinsky, 1982). The *sof-105* bearing strain, which has a mutation in the *spo0A* gene resulting in sporulation becoming independent of the *spo0B*, *spo0E* and *spo0F* genes (Hoch *et al.*, 1985) has a phenotype intermediate between those of the wild-type and the *spo0A* null mutants.

Resistance to oxidative stress of *spo0A abrB* mutants

The Spo0A protein is a negative regulator of expression of the *abrB* gene (Perego *et al.*, 1988). Null mutations in the *abrB* gene suppress most of the stationary-phase defects but not the sporulation defects of *spo0A* mutants. We found that an *abrB* mutation suppressed the exponential-phase resistant oxidative stress phenotype of a *spo0A* mutant (Table 5). The *spo0A abrB* double

Table 4. Percentage survival of *spo0A* mutants after H_2O_2 treatment

Strain	H_2O_2 treatment		
	10 mM	100 μ M, then 10 mM	100 μ M
JH703	9	20	95
Eth7	0.009	1.7	89
Eth415	0.11	3.8	54
Sof105	0.35	24	100

Table 5. Percentage survival of *B. subtilis* strains after treatment with H_2O_2 (10 mM or 100 μ M)

Strain	Relevant genotype	Exponential phase		Stationary phase 10 mM
		10 mM	100 μ M, then 10 mM	
JH12588	<i>spo0A abrB</i>	0.017 0.018 0.015 0.010	0.42 0.75	44
JH12607	<i>abrB</i>	0.019	2.8	ND
Hpr2	<i>hpr</i>	0.09	2	80
JH12769	<i>spo0A hpr</i>	0.006 0.001 0.0008	41 0.89 0.017	100

ND, Not determined.

mutant remained inducible by 100 μ M- H_2O_2 and showed the typical stationary-phase resistance. A strain bearing the *abrB* mutation in the absence of the *spo0A* mutation was indistinguishable from the wild-type in stress phenotype (Table 5).

Effect of the *hpr* mutation on resistance to oxidative stress

The *abrB* mutation may suppress *spo0A* mutations directly or indirectly via its control of expression of other genes. *spo0A* mutations lead to over-expression of the *abrB* gene, which is in turn responsible for overproduction of the *hpr* gene product (Perego & Hoch, 1988; Strauch *et al.*, 1989). It was of interest to determine if the stress phenotype of *spo0A* mutants was due to a direct effect of *abrB* or to one of the gene products controlled by the *abrB* gene since an *abrB* mutation suppressed the *spo0A* phenotype. In order to test whether the *hpr* gene was involved in the stress resistance phenomenon, we constructed a *spo0A hpr* strain by transforming a *spo0A* mutant with plasmid pJM2479 (Perego & Hoch, 1988), which contains an internal fragment of the *hpr* gene. Integration of this plasmid by Campbell recombination inactivates the *hpr* gene. When this mutant was tested in our usual protocol, it was found that the H_2O_2 resistance of the strain conferred by the *spo0A* mutation was suppressed by the *hpr* mutation (Table 5). Thus, the *spo0A* effect is an indirect consequence of the *spo0A* mutation in that the *hpr* gene is over-expressed in the *spo0A* strains and it is this over-expression that presumably results in constitutive expression of the stress resistance.

We found that *hpr* deletion mutants and *abrB* mutants showed a wild-type inducible stress response (Table 5). Thus, while *hpr* over-expression leads to protection

against oxidative stress, it is not required for the protection induced by pre-treatment with 100 μM - H_2O_2 . Therefore protection can be induced in two completely independent ways.

The hpr gene is not required for the resistance of stationary-phase cells to oxidative stress

Wild-type *B. subtilis* has an inducible stress response during the exponential phase but a constitutive response (i.e. resistance to 10 mM- H_2O_2) at stationary phase (Dowds *et al.*, 1987). We hypothesized that the constitutive stationary-phase resistance might be caused by the increase in expression of the *hpr* gene at stationary phase (Perego & Hoch, 1988). However, we found (Table 5) that this is not the case and that *hpr* deletion mutants are as resistant as wild-type cells to H_2O_2 at stationary phase.

Effect of oxidative stress on the expression of abrB- and hpr-controlled genes

We considered the possibility that while the *hpr* gene product was not necessary for the induction of protection with 100 μM - H_2O_2 , it might nevertheless constitute part of one route whereby H_2O_2 could induce protection. Thus we asked whether the *hpr* gene product was induced by 100 μM - H_2O_2 . Using fusions of the regulatory region of *hpr* to a promoterless *lacZ* gene (strain JH12527; Perego & Hoch, 1988) we found that β -galactosidase was not induced by the addition of 100 μM - H_2O_2 to the medium. The specific activity in the absence (–) and presence (+) of H_2O_2 , was 13 (–) and 13 (+) U mg^{-1} (10 min treatment), 33 (–) and 30 (+) U mg^{-1} (25 min treatment) and 37 (–) and 43 (+) U mg^{-1} (45 min treatment). Similarly, we found that β -galactosidase was not induced by 100 μM - H_2O_2 in an *abrB-lacZ* transcriptional fusion (strain JH12611; Perego *et al.*, 1988). Here the specific activity was 1800 (–) and 1700 (+) U mg^{-1} (15 min treatment) and 1400 (–) and 1200 (+) U mg^{-1} (40 min treatment). Thus, H_2O_2 is unable to induce in exponential growth either the *abrB* gene or the *hpr* gene.

Since the *hpr* gene product is known to directly control the synthesis of subtilisin in *B. subtilis* (Ferrari *et al.*, 1988) we asked whether the subtilisin gene was controlled by conditions of oxidative stress. Thus, a strain carrying the promoter region of the alkaline protease gene, *aprE*, connected to the *lacZ* gene (JH12464) was treated with 100 μM - H_2O_2 and the production of β -galactosidase determined. The specific activity was 26 (–) and 28 (+) U mg^{-1} (10 min treatment), 31 (–) and 25 (+) U mg^{-1} (25 min treatment) and 19 (–) and 18 (+) U mg^{-1} (45 min treatment). In this case, as above,

no induction of β -galactosidase was found due to the oxidative stress conditions.

Discussion

There appear to be at least two different mechanisms by which *B. subtilis* cells respond to oxidative stress, an *hpr*-dependent and an *hpr*-independent mechanism. None of the mutations tested affect the inducible phenotype although the uninduced level of resistance to 10 mM- H_2O_2 may be different depending upon the mutant strain. Furthermore all of the mutant strains are resistant to 10 mM- H_2O_2 in the stationary phase of growth. Thus inducibility and resistance in stationary phase are unimpaired in all of these mutants and it is impossible to determine from these data whether induction and stationary-phase resistance are brought about by the same or different mechanisms.

The phenomenon addressed by the results presented here is the observed constitutive resistance of *spo0A* strains to high levels of H_2O_2 (Dowds *et al.*, 1987). It was found in the previous work that other strains with mutations in *spo0* genes (*spo0B*, *spo0E*, *spo0F*, *spo0H* and *spo0J*) were identical to the wild-type parental strain in their response to oxidative stress. However, in a *spo0A* strain, mid-exponential-phase cells are 100-fold more resistant to 10 mM- H_2O_2 than the wild-type or the other *spo0* strains. The focus of the experiments described here was to determine if the oxidative stress phenomenon is a direct consequence of *spo0A* activity or if it results from the other regulatory proteins controlled by *spo0A*.

In a *spo0A* mutant, negative regulation of the *abrB* locus is lost, with the consequence that the *abrB* gene product is overproduced (Perego *et al.*, 1988; Strauch *et al.*, 1989). Overproduction of the AbrB protein is responsible for overproduction of the *hpr* gene product, which is itself a negative regulatory protein controlling the transcription of several genes (Perego & Hoch, 1988). The introduction of an *abrB* mutation into a *spo0A* strain results in the return of *hpr* expression to normal levels. Thus, one consequence of mutation in the *spo0A* gene is to overproduce the Hpr negative regulator and therefore repress those genes controlled by the *hpr* gene product. It was found in this study that a mutation in the *hpr* gene could suppress the oxidative resistance phenotype of the *spo0A* strain, suggesting that Hpr can behave as both a positive and a negative regulator and that Hpr is a positive regulator for a resistance determinant. Alternatively Hpr may behave as a negative regulator only but might negatively regulate a repressor of a proposed resistance gene.

Two other types of mutations in the *spo0A* gene had no effect on the H_2O_2 sensitivity phenotype of strains

bearing them. Resistance to ethanol stress can be caused by *eth* mutations in the *spo0A* gene (Bohin & Lubochinsky, 1982). Such mutations did not confer resistance to oxidative stress. Thus, the role of the Spo0A protein in ethanol stress must be different from its role in oxidative stress sensitivity. Similarly, the *sof* mutations are alterations in the *spo0A* gene that compensate for the lack of all of the other *spo0* genes except for *spo0H* (Hoch *et al.*, 1985). These proteins must have some unique regulatory properties but the one allele tested, *sof-105*, did not change the H₂O₂ sensitivity phenotype of the strain.

Regulation of the bacterial oxidative stress response has been most studied in enteric bacteria. The *oxyR* locus regulates expression of genes which protect *Escherichia coli* and *Salmonella typhimurium* against oxidative stress (Christman *et al.*, 1985; Goerlich *et al.*, 1989; Storz *et al.*, 1990). We here discuss another regulatory gene, *hpr*, which controls the oxidative stress response in *B. subtilis*. However the *hpr* gene seems to play a different role from the *oxyR* gene. The *hpr* gene regulates the resistance of *B. subtilis* to high concentrations of H₂O₂ but unlike *oxyR* it is not involved in the induction of protection by low concentrations of H₂O₂.

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