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Characterization of an Inducible Oxidative Stress System in *Bacillus subtilis*

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Exponentially growing cells of *Bacillus subtilis* demonstrated inducible protection against killing by hydrogen peroxide when prechallenged with a nonlethal dose of this oxidative agent. Cells deficient in a functional $recE^+$ gene product were as much as 100 times more sensitive to the H_2O_2 but still exhibited an inducible protective response. Exposure to hydrogen peroxide also induced the $recE^+$-dependent DNA damage-inducible (*din*) genes, the resident prophage, and the product of the $recE^+$ gene itself. Thus hydrogen peroxide is capable of inducing the SOS-like or SOB system of *B. subtilis*. However, the induction of this DNA repair system by other DNA-damaging agents is not sufficient to activate the protective response to hydrogen peroxide. Therefore, at least one more regulatory network (besides the SOB system) that responds to oxidative stress must exist. Furthermore, the data presented indicate that a functional catalase gene is necessary for this protective response.

Following exposure of many bacteria to nonlethal doses of a variety of environmental stresses, the cells demonstrate an enhanced resistance to the noxious compounds. These responses are generally defined by the induction of a unique set of genes. An example of such a system is the regulon in *Bacillus subtilis* that is induced following exposure of the bacteria to UV light and other DNA-damaging agents (5, 16, 23). This system, termed the SOS-like or SOB response (23), is analogous to the SOS regulon of *Escherichia coli* (12). The SOB system is a set of coordinately controlled genes which regulate responses such as prophage induction, Weigle reactivation, and error-prone repair (15). In *Salmonella typhimurium* and *E. coli* there exists a separate regulon, OxyR, that responds to oxidative stress (1, 4). Similarly, the heat shock regulon in *E. coli* is a set of operons that are transcribed following a temperature upshift (21). Interactions between stimulated regulons (stimulons) can often be demonstrated by the induction of one response rendering a cell population more tolerant to another stimulon-inducing agent (20). For instance, starved *E. coli* are more resistant to oxidative stress and heat (11). Additionally, some of the proteins produced during the heat shock response are considered oxidative stress proteins because they are under OxyR control (19). Furthermore, the competence regulon of *B. subtilis* (3), which consists of those genes required for the binding, uptake, and incorporation of exogenous DNA, has been shown to overlap with the SOB regulon (14). This interaction was demonstrated by the observation that genes controlled by the SOB system are induced in the absence of DNA-damaging agents when the organism reaches its natural competent state (14). Finally, the competence-controlled induction of the SOB genes is under the control of the regulatory genes $spo0A^+$ and $spo0H^+$, the products of which are also essential for sporulation to proceed correctly (22, 24).

When *B. subtilis* is grown to late stationary phase or exposed to hydrogen peroxide, a specific set of genes is induced (2). The transcription of some of these genes is also dependent on the products of $spo0A^+$ and $spo0H^+$ genes (2). Because competence, a growth-phase dependent event, also induces the SOB system and requires the products of $spo0A^+$ and $spo0H^+$, it is considered possible that there exists an interaction between the oxidative stress-inducible system and the other stress-related regulons. Accordingly, we began to elucidate the molecular and genetic mechanisms that control the inducible oxidative stress system and to define the interactions between this system and the SOB response. Results presented in this report demonstrate that inducible resistance to oxidative stress appears to be independent of $recE^+$ but dependent on a functional catalase gene.

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Cell survival and induced protection. Resistance to H_2O_2 in three *B. subtilis* strains was measured by exposing mid-exponentially growing cultures of each strain to various concentrations of peroxide for 15 min before the survivors were plated. Of the strains tested, repair-proficient strain YB886 showed the greatest resistance to H_2O_2 (Fig. 1). Recombination- and repair-deficient strain YB1015 (*recE4*) was 100-fold more sensitive to low concentrations of H_2O_2 than was wild-type strain YB886. However, both strains were killed with equal efficiency at higher concentrations of the oxidative agent.

Two catalases, one of which has been shown to be inducible by hydrogen peroxide (*katA*) (13), have recently been identified in *B. subtilis*. A mutation in the *katA* gene was moved into strain YB886 by congression (8), yielding strain YB2001, and sensitivity to H_2O_2 was tested as described above for strain YB886. Without a functional inducible catalase, *B. subtilis* was significantly more sensitive to H_2O_2 (Fig. 1); thus, it appears as though the vegetative catalase gene $katA^+$ is necessary to provide resistance of growing *B. subtilis* to H_2O_2.

Repair-proficient *B. subtilis* YB886 grown in the presence of 50 μM H_2O_2 for two generations prior to a subsequent challenge with more substantial doses of peroxide and compared with naive cultures (Fig. 1) showed increased resistance to H_2O_2. Killing of the prechallenged cells decreased 2 to 3 orders of magnitude (at the higher doses) compared with

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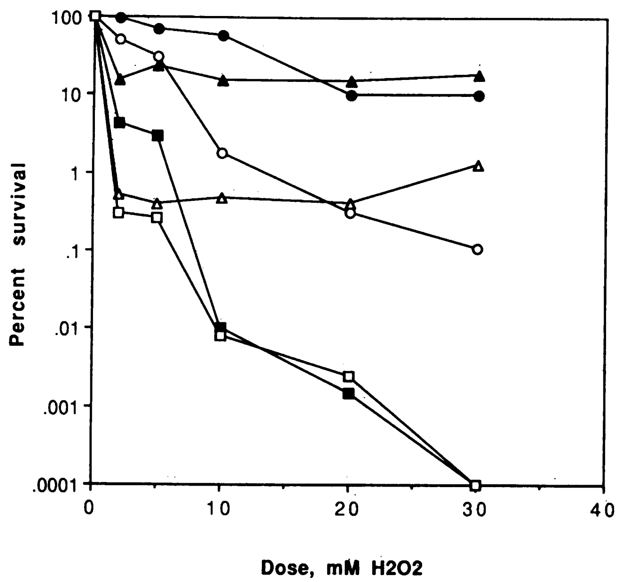

FIG. 1. Cell survival and reduced sensitivity. Strains were grown to mid-exponential phase (50 CFU/ml) in Penassay antibiotic medium no. 3 (Difco Laboratories) with aeration at 37°C before challenge for 15 min with various concentrations of hydrogen peroxide (Fisher Scientific Co.) (open symbols). For induction of the oxidative stress system (closed symbols), cultures were grown to the same phase, diluted 1:10, and exposed to 50 μM H_2O_2. Subsequent challenge with H_2O_2 occurred when the cultures reached 50 Klett units. Cell survival was determined by promptly diluting challenged cells into Spizizen salts (GIBCO Laboratories) and plating samples on tryptose blood agar base (Difco) immediately. The strains examined here are YB886 (○, ●), YB1015 (△, ▲), and YB2001 (□, ■).

untreated cells. Although the data shown here are from one representative experiment, cell killing at any one dose in an individual experiment deviated less than 1/2 order of magnitude from the average killing at that dose. In order to determine whether the protection induced by H_2O_2 in strain YB886 was RecE-dependent, strain YB1015 was similarly prechallenged with H_2O_2 for two generations. This pretreatment was sufficient to reduce cell killing by 2 to 3 orders of magnitude at higher concentrations of peroxide. Thus, the protective response induced by small doses of H_2O_2 is not dependent on a functional *recE*$^+$ gene product. Alternatively, the *katA* mutant strain YB2001 failed to show the response of reduced killing of pretreated cells shown in naive cultures (Fig. 1). Dependence on catalase for a protective response to H_2O_2 has been reported for *E. coli* (9, 10). The catalase-peroxidase enzyme HPI of *E. coli* is responsible for the protective response that is inducible by H_2O_2-ascorbate and is part of the *oxyR* regulon (7). A proposed mechanism for enhanced resistance during exposure to high concentrations of H_2O_2 is the protection of cellular transport components from H_2O_2 by catalase activity (4). The data presented in this paper demonstrate that *B. subtilis* has a similar system. Although a regulon like the OxyR system has not yet been discovered in *B. subtilis*, increased resistance to H_2O_2 and the correlation of this resistance with elevated catalase levels (13) indicate that a mechanism of activation or derepression of the catalase gene must exist. Since the *oxyR* gene product regulates genes identified in the heat shock response as well as resistance to oxidants, recent data suggest that the *katA*$^+$ gene of *B. subtilis* may be under the control of multiple stress regulons (2).

Induction of SOB characteristics. *B. subtilis* cells treated with H_2O_2 showed elevated levels of the RecE protein within 30 min after exposure (data not shown). The product of the *recE*$^+$ gene is a protein necessary for SOB induction, postreplication repair, and homologous recombination (17). RecE levels obtained after H_2O_2 treatment were comparable to those observed when cells were treated with other DNA-damaging agents that are known to induce the SOB response (14). Since induction of the *recE*$^+$ gene in the presence of DNA damage (17) or during competence (14) is correlated with the activation of the SOB response, the ability of H_2O_2 to induce the SOB system was tested by assaying for the production of β-galactosidase from transcriptional fusions at three DNA-damage-inducible (*din*) loci (6). Essentially, pro-

TABLE 1. SOB induction profile for hydrogen peroxide

Strain	Din induction[a] after treatment with:		Prophage induction[b] after treatment with:			Filamentation[c] after treatment with:	
	H_2O_2	MC[d]	NT[e]	H_2O_2	MC	H_2O_2	MC
YB886 DinA	+++	+++					
YB886 DinB	+++	+++					
YB886 DinC	+++	+++					
YB1015 DinA	—	—					
YB886 φ105			6.8×10^6	3×10^9	1.2×10^9		
YB1015 φ105			5×10^3	4×10^3	5.1×10^3		
YB886						+++	+++
YB1015						+++	+++

[a] Strains carrying Tn*917*::*lacZ* fusions to damage-inducible genes (*din*) were maintained on nutrient broth no. 2 (Oxoid Ltd.) with 1.5% purified Oxoid agar. Erythromycin and lincomycin (Sigma Chemical Co.) were added to maintain selection for the transposon Tn*917* in concentrations of 1 and 25 μg/ml, respectively. Cultures to be assayed were taken from overnight growth on Oxoid nutrient and in GM1 (0.5% glucose, 0.1% yeast extract, 0.05% casein hydrolysate, and Spizizen salts supplemented with tryptophan and methionine [50 μg/ml] [24]). Exponentially growing cells were induced with 3 mM H_2O_2 or 50 ng of mitomycin C per ml, and samples were assayed every 15 min for 2 h. β-Galactosidase levels were obtained as described previously (14). Basal levels were consistently less than 3 U per optical density of cells at 600 nm, and induction was as high as 40 U per optical denisty of cells at 600 nm.

[b] Values are in PFU per milliliter. Strains lysogenic for φ105 were grown to 35 Klett units in Oxoid nutrient broth no. 2 supplemented with 0.5% yeast extract and were induced with mitomycin C (0.5 μg/ml) or selected concentrations of hydrogen peroxide. After 20 min, cultures were diluted 1/100, incubated for 90 min, and centrifuged for 5 min (13,500 rpm [10,000 × *g*]), and supernatant titers were then dtermined (25). +++, Maximum level; —, no induction.

[c] Filamentation was determined by exposing mid-exponential-phase cells to 1 mM H_2O_2 or 50 ng of mitomycin C per ml and observing the cells 30 min later under a light-phase microscope (magnification, ×100).

[d] MC, Mitomycin C.

[e] NT, No treatment.

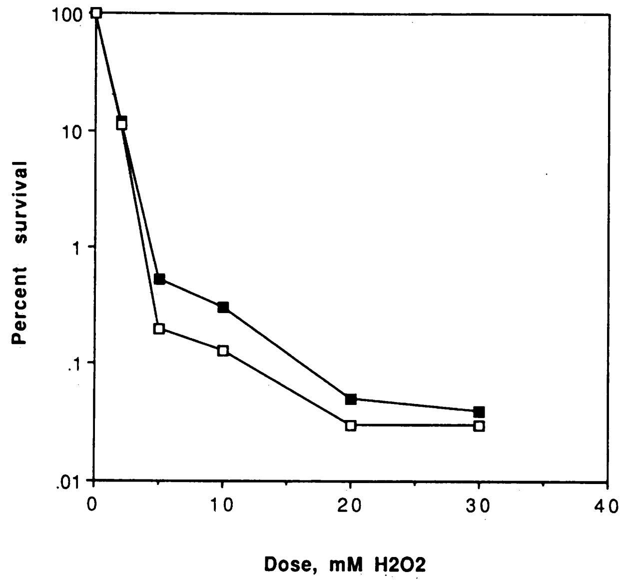

FIG. 2. Sensitivities of mitomycin C-treated cells to H_2O_2. Repair-proficient strain YB886 was grown exponentially for two generations in the presence of 50 ng of mitomycin C per ml prior to exposure to the indicated concentrations of H_2O_2. Survival of pretreated cells (■) is compared with that of naive cells (□).

moters of *dinA76*, *dinB7*, and *dinC17* were induced with hydrogen peroxide as well as with mitomycin C (Table 1). The results demonstrated that lack of a functional *recE*$^+$ gene renders all of the promoters noninducible by both H_2O_2 and mitomycin C (16). *din* gene induction was also examined in a dose-dependent fashion by exposing strain YB886, carrying an insertion at the *dinB17* locus, to various amounts of H_2O_2 (data not shown). In this case, β-galactosidase production was induced maximally from this fusion by 3 mM H_2O_2. These levels of enzyme activity were comparable to those induced by mitomycin C and were not increased by higher concentrations of H_2O_2. Another characteristic of the activation of the SOB response is the induction of resident prophage (15). *B. subtilis* prophage ϕ105 was easily induced by exposure of lysogenic strain YB886 to 1 mM H_2O_2 (Table 1). Again, the induction of the prophage did not occur in the absence of a functional *recE*$^+$ gene product (Table 1). However, H_2O_2 induced filamentation in both wild-type strain YB886 and *recE4* mutant strain YB1015 (Table 1). It has been demonstrated that filamentation is a *recE*$^+$-independent component of the SOB response (15). Taken collectively, these data demonstrate that hydrogen peroxide is an inducer of the SOB response. Since H_2O_2 is capable of inducing the SOB response, the possibility exists that H_2O_2 protection was merely the result of the induction of an unidentified repair mechanism that is *recE*$^+$ independent. However, when cells were treated with mitomycin C to fully induce the SOB system, no corresponding induction of oxidative stress resistance was observed (Fig. 2). There was a small increase in resistance; however, this is probably due to filamentation, in which killing kinetics are obscured by failure to septate.

The results clearly demonstrate that the inducible oxidative stress resistance of *B. subtilis* is independent of a functional *recE*$^+$ gene product. However, this system does require an inducible *katA*$^+$ gene. The regulatory components involved in the induction of *katA* and other oxidative stress resistance genes remain to be defined.

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