NOTES

Analysis of the Dual Regulatory Mechanisms Controlling Expression of the Vegetative Catalase Gene of *Bacillus subtilis*

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The expression of a vegetative catalase gene, *katA* (formerly the *kat-19* gene), is necessary to protect *Bacillus subtilis* from H_2O_2 , presumably by removing the oxidant from the environment. Genetic analysis of *katA* revealed that this gene is under two distinct forms of regulation, temporal and H_2O_2 inducible. The results reported here demonstrate that (i) the H_2O_2 -inducible regulation of *katA* gene is not a component of the SOS regulon, (ii) the regulatory genes *spo0A* and *abrB* are involved in the temporal regulation but not the H_2O_2 -specific induction of *katA* gene expression, and (iii) transcription initiation for the *katA* gene occurs at the same site under both forms of regulation.

Molecular responses, such as those induced as a result of oxidative stress, have been studied predominantly in gram-negative organisms (26). These studies have identified inducible stress regulons. For instance, induced expression of the *oxyR* regulon by H_2O_2 results in increased resistance of enteric organisms to H_2O_2 compared with noninduced cells (7, 8). Similarly, the constitutive overexpression of the genes in this regulon results in enhanced resistance to H_2O_2 (7). OxyR has

been shown to be a positive regulator that binds to specific regions of the promoters of at least two genes in this regulon, *aphC* and *katG* (24). One of these genes, *katG*, encodes a catalase (24).

Further capability to protect *Escherichia coli* from H_2O_2 is found in the expression of another catalase gene, *katE* (13). Production of this catalase is regulated by a sigma factor (σ^S ; the product of the *katF* gene [16, 20, 23]) and is characterized

FIG. 1. Induced protection in mutant *B. subtilis* strains. Exponentially growing cultures (35 Klett units; Klett-Summerson colorimeter, no. 66 filter; 66, 1 Klett unit $\approx 10^6$ CFU/ml) were divided, either pretreated with 50 μM H_2O_2 or incubated in PAB (antibiotic medium #3, Difco) prior to challenge with higher concentrations of H_2O_2 as described previously (1), and plated on tryptose blood agar base agar. Strains: YB886, induced (■) and noninduced (□); YB1072, induced (●) and noninduced (○); YB2003, induced (▲) and noninduced (△). The data presented are representative of experiments performed at least three times for each strain. While the actual numbers can vary as much as 10%, the survival trends, i.e., the relative resistance to H_2O_2 , of the strains remain constant.

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TABLE 1. Bacterial strains used

Strain ^a	Relevant genotype ^b or phenotype	Reference
YB886	<i>metB5 trpC2 xin-1 amyE rpoB</i> , repair-proficient, SPβ ⁻	28
YB2003	<i>katA::lacZ</i> (Tn917)/YB886	2
YB1072	<i>spo0AΔ677</i> /YB886	29
YB2007	<i>recA260 katA::lacZ</i> (Tn917)/YB886	This study
YB2008	<i>spo0AΔ677 katA::lacZ</i> (Tn917)/YB886	This study
YB2009	<i>spo0AΔ677 abrB::Tn917 katA::lacZ</i> (Tn917)/YB886	This study

^a All strains were maintained as previously described (1). The procedures for DNA isolation and transformation have also been described (4).

^b Mutations of interest were placed into YB886 via DNA-mediated transformation. DNA carrying the *abrB* and *spo0A* mutations were initially obtained from J. A. Hoch. The *spo0A* mutant strains YB2008 and YB2009 were maintained on tryptone blood agar base medium containing 1% dry milk in order to detect the presence or absence of protease production as previously described (21).

by increased expression as cultures enter stationary phase or if the bacteria are grown in media lacking glucose (13, 15). Thus, the genes coding for these two catalases, *katG* and *katE*, are independently regulated.

The response of the gram-positive bacterium *Bacillus subtilis* to H₂O₂ during exponential growth appears to be similar to the response just described for *E. coli* (1, 9). For instance, upon exposure to H₂O₂ *B. subtilis* induces DNA damage-responsive genes and resident prophage (SOS regulon) as well as *katA* (formerly referred to as *kat-19*), encoding catalase-1 (2, 14). Additionally, exponentially growing *B. subtilis* cells demonstrate enhanced protection from killing by H₂O₂ when pretreated with sublethal concentrations of this oxidizing agent (Fig. 1) (1, 9). However, as the bacteria enter stationary-phase growth, the basal level of resistance to H₂O₂ increases significantly (9). Coincidentally, the expression of the vegetative catalase of *B. subtilis* (catalase-1) also increases as cells enter stationary-phase growth (14). On the other hand, in contrast to catalase expression in *E. coli* (in which there is an H₂O₂-inducible enzyme and a growth stage-regulated enzyme), *B. subtilis* has only one vegetative catalase (14). Furthermore, the production of this enzyme appears to be under both H₂O₂-inducible and growth stage regulation (14).

Resistance of *B. subtilis* to H₂O₂ is a dynamic characteristic of this organism. While cells are able to respond to the levels of oxidative stress in the environment, this aerobic organism also appears to regulate the components needed for resistance to H₂O₂ in coordination with the growth cycle (9). As cultures of *B. subtilis* proceed from exponential growth to stationary phase, resistance to oxidative stress increases. This resistance is maximized by the formation of the mature spore. The molecular mechanisms that regulate the resistance of *B. subtilis* peroxide have not been characterized. However, Hartford and Dowds (11) have isolated an H₂O₂-resistant mutant of *B. subtilis* that overproduces a catalase. The characterization of this gene and its product should provide important information as to the nature of the oxidative stress system(s) of this bacterium. Similarly, in this study we used a Tn917::lacZ transcriptional fusion to the *B. subtilis* *katA* promoter (2) to aid in the elucidation of the molecular mechanisms associated with resistance to H₂O₂. The strain carrying this transposon fusion (YB2003) has increased sensitivity to H₂O₂, is deficient in the induced protective response (Fig. 1), and fails to evolve O₂ when exposed to 8 M H₂O₂ (unpublished observation) and thus it apparently is deficient in catalase activity.

Regulation of the H₂O₂-specific induction of *katA::lacZ*

FIG. 2. Induction of the *katA::lacZ* transcription fusion. (A) Strain YB2003 was grown in GM1 medium to a turbidity of 35 Klett units, treated with 50 μ M H₂O₂ or 500 ng of mitomycin (mitC) per ml, or left untreated. The β -galactosidase activities for aliquots taken 15, 30, and 45 min posttreatment were then quantitated as described previously (17). (B) H₂O₂ induction of transcription from the *katA* promoter was observed for induced (I) and noninduced (NI) strains YB2007, YB2008, and YB2009. The data are presented as β -galactosidase activity (Miller units) of the induced cells divided by the activity of the noninduced or naive cells. The data presented are representative of experiments performed three times for each strain. The relative increase in β -galactosidase activity remained constant (with a variation of $\leq 10\%$).

fusions. The strains used are described in Table 1. Exponentially growing cells of strain YB2003 carrying the *katA::lacZ* fusion exposed to a sublethal concentration of H₂O₂ (50 μ M) expressed as much as sevenfold more β -galactosidase than did uninduced cells (Fig. 2a). Thus, this catalase gene is induced following exposure to oxidative reagents such as H₂O₂. However, in addition to the oxidative stress system, H₂O₂ also induces the SOS regulon of *B. subtilis* (2, 27).

The SOS regulon consists of a set of DNA damage-inducible (*din*) genes regulated by the RecA protein (27). To determine whether induction of the *katA* promoter was independent of the SOS system and thus part of the oxidative stress regulon,

FIG. 3. Temporal expression of the *katA::lacZ* transcriptional fusion. The amount of β -galactosidase activity was determined from aliquots of *B. subtilis* strains carrying the *katA::lacZ* fusion collected in early exponential phase (at 35 Klett units [KU] and 30 min later) as well as during stationary phase (90 and 120 min after 35 KU was obtained). The 0-min time point represented the sample taken at 35 KU (under the growth conditions used, the doubling time was ≈ 34 min and exponential growth ceased at ≈ 110 KU). Thus, the β -galactosidase expression in each strain was determined at the same point in the growth cycle. Strains shown are YB2003 (■), YB2008 (●), and YB2009 (▲). The data presented are representative of experiments performed at least three times for each strain.

the *recA260* mutation was introduced into YB2003 (5), yielding strain YB2007. Following the exposure of strain YB2007 to 50 μ M H_2O_2 , there was a 2.5-fold induction of the amount of β -galactosidase produced (Fig. 2B). The reduced level of transcription induction in strain YB2007 is most probably attributable to the extreme sensitivity of strains carrying a *recA* mutation to H_2O_2 . Furthermore, mitomycin C (a potent inducer of all known SOS phenomena) failed to induce β -galactosidase production in strain YB2003 (*katA::lacZ* *recA*⁺; Fig. 2A). Finally, the Cheo box, a regulatory sequence essential for the induction of SOS-regulated genes (4, 18), was not found in the sequence upstream of the *katA* gene (unpublished observation). These results support the conclusion that the H_2O_2 induction of *katA* expression is independent of the SOS regulon and that the peroxide-induced expression from the *katA* promoter is a component of the *B. subtilis* oxidative stress regulon.

Developmental regulation of the *katA* gene. Coordinate regulation of the oxidative stress system and the developmental cycle of *B. subtilis* has been demonstrated by the coincident increase in H_2O_2 resistance with entry into stationary phase (9). Appropriately, as the bacteria enter stationary phase, there is an increase in the amount of transcription that occurs from the *katA* promoter (Fig. 3). During exponential growth, as much as 17% of the oxygen used for metabolism can be in the form of oxygen radicals (3). At the end of the exponential phase of growth, these oxidants may accumulate to toxic levels. Thus, the increased resistance to H_2O_2 that appears when *B. subtilis* enters stationary phase could be the result of the induction of the oxidative stress response.

The regulation of many genes expressed during the transition from exponential growth to stationary phase depends on the multifunctional product of the *spo0A* gene (22). Mutations in the *spo0A* gene are known to produce pleiotropic effects as *B. subtilis* enters the stationary phase of growth (12). To examine the control of the oxidative stress response with respect to bacterial development, the role of *spo0A* gene in

resistance to peroxide was examined. Accordingly, the resistance of strain YB1072 (*spo0A*) to H_2O_2 was determined (Fig. 1). The results demonstrated that exponentially growing cells of strain YB1072 were more sensitive to H_2O_2 than were wild-type cells. Interestingly, pretreatment of strain YB1072 with 50 μ M H_2O_2 resulted in increased resistance of these cells to higher concentrations of this oxidant than that demonstrated by nonpretreated cells (Fig. 1). Similarly, the presence of the *spo0A* mutation in a strain carrying the *katA::lacZ* fusion (YB2008) did not abolish the H_2O_2 -induced expression from this promoter (Fig. 2). Thus, a functional *spo0A* gene is not required for the H_2O_2 -inducible stress response system. Previously, Dowds and Hoch (10) had shown that a *spo0A* mutation increased the resistance of *B. subtilis* to H_2O_2 . However, these authors noted variations in their results that they attributed to growth stage differences and to different alleles of the *spo0A* gene. While the data related to the peroxide sensitivity of strains carrying *spo0A* mutations might be confusing, clearly our results demonstrate that the H_2O_2 -induced expression from the *katA* promoter is independent of a functional *spo0A* gene product. On the other hand, the results demonstrated that the absence of a functional *spo0A* gene does eliminate the growth stage-induced expression of the *katA* gene (Fig. 3).

A mutation (*abrB*) that suppresses some of the effects of mutant *spo0A* alleles has been identified (25). The presence of the *abrB* allele in strains carrying *spo0A* mutations restores the ability of the bacteria to produce secondary metabolites. Accordingly, the ability of strain YB2009 (*spo0A* *abrB*) to induce expression from the *katA* promoter during growth stage transition was examined. The results (Fig. 3) clearly showed that the *abrB* mutation suppresses the loss of growth stage regulation of the *katA* gene associated with mutations within the *spo0A* gene.

Promoter/operator structure of the *katA* gene. To dissect the mechanism of regulation of *katA* gene expression, the promoter region of this gene was determined. Using the sequence

FIG. 4. Analysis of *katA* mRNA from strain YB886. RNA was obtained as previously described (6) and quantitated spectrophotometrically (19). The primer extension assays were executed by using a modification of the procedure described previously (6). One hundred nanograms of oligonucleotide 5'-TTATCTCCAACCGGAGCG-3' was end labeled by incubation with 200 μ Ci of [32 P]dATP and 5 μ g of T4 polynucleotide kinase in T4 PNK buffer (Promega, Chicago, Ill.) for 1 h at 37°C. The labeled oligonucleotide was precipitated by adding sodium acetate (final concentration, 0.3 M) and then ethanol (3 volumes). (A) Primer extension experiments were performed on 20 μ g of mRNA from exponentially growing cells exposed to 50 μ M H_2O_2 for 20 min (lane 1) and from uninduced cells that were entering the stationary phase of growth (lane 2). The reverse transcripts were run on a 6% polyacrylamide-8 M urea sequencing gel with a sequencing reaction as a size standard (19). nt, nucleotides. (B) Slot blot analysis of 5 μ g of mRNA from exponentially growing cells induced with 50 μ M H_2O_2 (slot 1), exponentially growing uninduced cells (slot 2), and stationary-phase cells (slot 3). The probe used was an internal fragment of the *katA* open reading frame (1) which was labeled with 32 P by the random primer method as described previously (19).

of *katA* (2), we synthesized an oligonucleotide (5'-ATCTCC AACCGGAGCG-3') that would anneal to the transcript of this gene, 29 nucleotides downstream from what was the suspected site of translation initiation. Total RNA was isolated from H_2O_2 -induced and noninduced exponentially growing cultures of YB886 as well as from uninduced cells in early stationary phase. Primer extension assays were performed on transcripts from cells under these three conditions (Fig. 4a). The presence of the same single band generated by primer extension on transcripts isolated from H_2O_2 -induced and from the stationary-phase cells makes it likely that both of these mechanisms lead to transcription initiating from the same start site.

In conclusion, although the oxidative stress response regulation and the transition stage regulation of the *katA* gene are controlled by two independent mechanisms, the transcription of this gene seems to depend on the same promoter. The results presented also indicate that the developmental regulation of the *katA* gene is similar to those previously described for the production of secondary metabolites (12, 22), i.e., dependent on the presence of functional *spo0A* and *abrB* genes. Finally, the oxidative stress response of *B. subtilis* is separate from the SOS regulon, and the induced expression from the *katA* promoter is part of the oxidative stress response.

We thank David Cheo and Ken Bayles for their help. J. A. Hoch generously provided strain JH575.

This research was supported by grants from the National Science Foundation (MCB-9219436) and the NIH (DE10547). Financial support was also provided by the Maryland Agricultural Experiment Station.

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