Bacillus subtilis Vegetative Catalase Is an Extracellular Enzyme

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Strong catalase activity was secreted by *Bacillus subtilis* cells during stationary growth phase in rich medium but not in sporulation-inducing medium. N-terminal sequencing indicated that the secreted activity was due to the vegetative catalase KatA, previously considered an endocellular enzyme. Extracellular catalase protected *B. subtilis* cells from oxidative assault.

The molecular response to oxidative stress has been extensively studied as it occurs in both gram-negative (6, 17) and gram-positive (18) bacteria. Exposure of aerobically grown *Escherichia coli* cells to hydrogen peroxide results in the coordinate expression of nine genes that compose the *oxyR* global regulon (17). A member of this regulon is *katG*, the structural gene for hydrogen peroxidase I, an H_2O_2 -inducible catalase. The *katE* gene, coding for the second catalase, HPII, is expressed when cells enter stationary phase and is under the transcriptional control of a specific sigma factor (σ^s) of RNA polymerase (12). For *Bacillus subtilis* only a single catalase is found in cell extracts during vegetative growth (KatA). Expression of its structural gene, *katA*, is H_2O_2 and growth phase inducible (18). A second catalase is found only after the onset of sporulation (13).

Catalases and peroxidases in a number of microorganisms, including *Haemophilus influenzae* (1, 2), *Mycobacterium tuberculosis* (10), and *Streptomyces viridosporus* (16), have been studied. These enzymes play important roles in several microbial processes also independent of oxidative stress. Microbial peroxidases are involved in the oxidation of a large number of aromatic compounds, including recalcitrant substances (14). Catalases of pathogenic bacteria, like *Listeria monocytogenes*, *H. influenzae*, and *Helicobacter pylori*, are considered potential virulence factors which may counteract the oxygen-dependent defense mechanisms of infected phagocytes (1, 8, 15). It is thought that during food fermentation catalases from lactobacilli decrease hydrogen peroxide accumulation, which would severely affect the sensory quality of products (11). Catalases from *Aspergillus niger* are used to eliminate from milk hydrogen peroxide either endogenously produced or added in the process of cold pasteurization.

We have found a vegetative catalase activity secreted by *B. subtilis* cells during the stationary growth phase. We report here that this activity is due to KatA and that its secretion is essential to ensure complete protection of *B. subtilis* cells against oxidative stress. The finding that KatA is a secreted enzyme will facilitate its purification and the study of its mechanism of action, therefore enhancing interest in *B. subtilis* catalase.

KatA, the vegetative catalase of *B. subtilis*, is secreted during the stationary growth phase. Filter-sterilized cell-free super-

natants of the food-isolated strain *B. subtilis* GN99 were found to contain a high level of catalase activity. Table 1 shows that a basal amount of activity, secreted during the exponential growth phase, was strongly enhanced during the stationary phase in rich (TY) but not in sporulation-inducing (DS) media (7). The secreted enzyme represented 56% of the total catalase produced by strain GN99 (data not shown).

A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the various cell-free culture supernatants, eightfold concentrated by Centricon centrifugation (molecular weight cutoff, 3,000; Amicon), showed that during the late stationary growth phase several polypeptides were secreted in large amounts (Fig. 1). A good correspondence between the apparent molecular mass of the *B. subtilis* vegetative catalase, KatA (59.5 kDa [9]), and that of the most abundant protein secreted by GN99 (about 60 kDa) (Fig. 1) was observed. To determine whether that 60-kDa protein was KatA, after gel electrophoresis proteins were electrotransferred onto a ProBlott membrane and the protein band corresponding to 60 kDa was sliced from the blot and directly sequenced by using an automated Applied Biosystems 477A pulse-liquid phase sequencer equipped on-line with a model 120A phenylthiohydantoin amino acid analyzer. The NH_2 -terminal sequence, 21 residues long, was clearly determined and found to be identical to the deduced amino acid sequence of KatA (4). It is interesting that the *katA* gene does not code for a typical leader peptide, in most cases required for protein secretion. Thus, KatA secretion seems to be independent of the well-characterized Sec system.

Induction of KatA secretion by H_2O_2 . To test whether the secretion of KatA was H_2O_2 inducible, as previously reported for the expression of its structural gene (3, 5), exponentially

TABLE 1. Catalase activity secreted by strain GN99 in TY or DS medium at various stages of growth

Medium	Catalase sp act ^a at the following growth phase ^b :			
	Exponential	Early stationary	Mid-stationary	Late stationary
TY	257.1	267.0	608.8	1,108.5
DS	201.6	226.1	306.8	341.8

^a Catalase specific activity was determined as the decrease in the amount of H_2O_2 (in millimoles) per min per ml per U of optical density at 590 nm of the cell culture as previously described by Knauf et al. (11).

^b Exponential phase corresponded to an optical density of 0.7 at 590 nm; early stationary phase corresponded to the onset of stationary phase; and mid- and late stationary phase corresponded to 24 and 48 h of growth, respectively.

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FIG. 1. Proteins secreted by strain GN99 at various stages of growth. Lanes: 1, mid-exponential growth phase; 2, onset of stationary phase; 3, 20-h culture; 4, 40-h culture. The proteins were fractionated by SDS-12.5% PAGE and stained with Coomassie brilliant blue.

growing cells of strain GN99 were treated with sublethal concentrations of H_2O_2 (5). Samples were taken at 15, 30, and 45 min after H_2O_2 addition, and the cell-free supernatants were concentrated eightfold as previously described and analyzed by SDS-PAGE. Figure 2 shows that the secretion of a band of the size of KatA was strongly induced in strain GN99. The secretion of several other bands appeared to be induced by the H_2O_2 treatment. It is unlikely that the appearance of these proteins was a consequence of cell lysis, since the intensity of

FIG. 2. H_2O_2 -induced secretion of proteins from strain GN99. Samples were taken at the beginning of induction and 15, 30, and 45 min later (lanes 1, 2, 3, and 4, respectively). The proteins were fractionated by SDS-12.5% PAGE and stained with Coomassie brilliant blue.

FIG. 3. Effect of secreted proteins on cellular protection from treatment with various H_2O_2 concentrations. Percent survival was measured after H_2O_2 treatments in induced (closed symbols) and noninduced (open symbols) culture broth.

only a few discrete bands was increased. A possible explanation could be that these proteins represent part of the cellular response to the oxidative stress caused by the H_2O_2 treatment.

Identical results were obtained with the laboratory collection *B. subtilis* strain PY17, a derivative of strain 168 (19), thus eliminating the possibility that KatA secretion was a peculiarity of our food-isolated strain (data not shown).

KatA secretion is essential for resistance to oxidative stress.

To test whether catalase secretion has a physiological role in the resistance of the growing cells to oxidative stress, we investigated the ability of *B. subtilis* cells to survive a treatment with hydrogen peroxide. Strain GN99 was induced by exposure of exponentially growing cells to the presence of a sublethal concentration of H_2O_2 (50 μ M). Cells were centrifuged and resuspended in filter-sterilized broth from (i) their own culture and (ii) a parallel noninduced culture. Cells were then incubated for 15 min at 30°C with various concentrations of H_2O_2 , diluted, and plated on TY agar plates. Figure 3 shows that survival of GN99 cells was clearly reduced in the noninduced broth. Similar results were obtained with strain PY17 (data not shown).

This result rules out the possibility that the presence of catalase activity in the medium is due to trivial membrane leakage and suggests that (i) secretion of the enzyme is essential to prevent H_2O_2 from damaging the cells and (ii) cytoplasmic catalase activity alone does not provide protection from the oxidative assault. This could be explained if KatA activity found inside the cells, probably due to enzyme molecules not yet secreted, is not sufficient to protect cellular components or if oxidative damages occur very rapidly once H_2O_2 is within the cytoplasm.

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