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Oxidative Stress and Growth Temperature in *Bacillus subtilis*

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Pretreatment of *Bacillus subtilis* with low concentrations of hydrogen peroxide protected the cells against the lethal effects of higher levels of oxidative stress. During the period of adaptation, eight proteins were induced, as detected by one-dimensional gel electrophoresis. Four of these proteins were the same size as four of the proteins induced by the temperature upshift. The range of proteins synthesized in response to an elevation in temperature depended both on the starting (lower) temperature and on the temperature to which the cells were shifted. Both catalase and superoxide dismutase were present at high levels in *B. subtilis*, but neither was induced by oxidative stress or temperature upshift. In fact, catalase activity was reduced after the temperature upshift.

Stress responses are characterized by the adaptation of cells to normally lethal levels of stress by their pretreatment with low, nonlethal levels of stress. Such an adaptive response can be induced on exposure to a diverse group of agents, including heat, ethanol, arsenite, oxidizing agents, anaerobiosis, agents which induce the SOS response, and amino acid analogs. A set of proteins, characteristic for each agent, is rapidly induced on pretreatment. There is, however, a degree of overlap in the proteins that are induced (8, 12, 17).

The most extensively studied procaryotic stress response is the response of *Escherichia coli* to heat shock. In this organism, induction of heat shock proteins occurs at the transcriptional level and is mediated by the *htpR* locus, which codes for a minor sigma factor (sigma^{32}) of RNA polymerase (12, 16). Several additional proteins are induced following a temperature upshift but are not regulated by *htpR* (15). It is not yet clear whether *Bacillus subtilis* has a similar heat shock response. *B. subtilis* contains a minor sigma factor, sigma^{28}, which shares an overlapping promoter specificity with sigma^{32} of *E. coli* (4). However, transcription in *E. coli* from cloned sigma^{28}-specific promoters of *B. subtilis* does not depend on heat shock.

The adaptation of *E. coli* (6) and *Salmonella typhimurium* (20) to lethal levels of H_2O_2 by pretreatment with lower levels has been reported. The resistance to H_2O_2 toxicity in *E. coli* occurs at least partially at the level of DNA repair (6), and that in *S. typhimurium* can be partially attributed to higher levels of catalase (20). Thirty proteins were induced on pretreatment of *S. typhimurium* with the protective level of H_2O_2 (5). A hydrogen peroxide-resistant *oxyR1* mutant constitutively expressed nine proteins that are normally induced by H_2O_2 pretreatment. This strain also had increased levels of catalase, superoxide dismutase (SOD), glutathione reductase, and NAD(P)H-dependent alkyl hydroperoxide reductase. Mutants with the *oxyR* locus deleted fail to induce the nine proteins that are overexpressed in *oxyR1*. Many of the proteins induced by H_2O_2 are also induced by a variety of other stress agents (14).

In this report we describe the response of *B. subtilis* to hydrogen peroxide and temperature shifts. We found that pretreatment with low levels of hydrogen peroxide enabled the cells to withstand normally lethal concentrations of this oxidizing agent. We identified eight proteins that were induced by low levels of hydrogen peroxide and found that some of them were the same size as those induced by the temperature upshift. Finally, we found that the profile of proteins synthesized after the temperature upshift depended on the particular pair of temperatures at which the cells were grown.

MATERIALS AND METHODS

Materials. Acrylamide ammonium persulfate, *N,N,N',N'*-tetramethylenediamine, and hydrogen peroxide (Analar) were obtained from BDH Chemicals Ltd. (Poole, England). L-[^{35}S]methionine (>800 Ci/mmol) and ^{14}C-methylated protein molecular weight markers (molecular weights, 14,300 to 200,000) were obtained from Amersham Corp. (Arlington Heights, Ill). En^{3}Hance autoradiography enhancer was obtained from New England Nuclear Corp. (Boston, Mass.), and *N,N'*-methylenebisacrylamide was purchased from Sigma Chemical Co. (St. Louis, Mo.). X-ray film (RX) was from Fuji, and X-ray developer (LX-24) and fixer (FX-40) were from Eastman Kodak Co. (Rochester, N.Y.).

Strains. *B. subtilis* JH642 (*pheA1 trpC2*) was obtained from the Bacillus Genetic Stock Center, Ohio State University (Columbus, Ohio).

Induction of protection against oxidative stress. *B. subtilis* was grown to the mid-log phase in Luria broth (LB). A total of 1 ml was diluted into 100 ml of LB or minimal medium (Spizizen salts [1] supplemented with glucose [0.5%], L-phenylalanine [0.035%], and L-tryptophan [0.025%]) and grown to an optical density at 550 nm of 0.15. Fractions of 1 ml were removed into four 10-ml culture tubes, two of which (samples 3 and 4) contained hydrogen peroxide to a final concentration of 50 μM. All four tubes were shaken at 220 rpm at 37°C for 1 h. Hydrogen peroxide was then added to samples 2 and 3 to a final concentration of 10 mM. The cells were again shaken at 200 rpm in a Gallenkamp orbital incubator at 37°C for 1 h. Appropriate dilutions were then plated onto LB plates, which were incubated at 37°C overnight. The doubling time for the cultures was 1 h in minimal medium and 0.5 h in LB.

Metabolic labeling of proteins after oxidative stress. *B. subtilis* was grown in minimal medium as described above. At an optical density at 550 nm of 0.35, nine 1-ml fractions were removed into culture tubes. Hydrogen peroxide was added to seven samples (samples 3 to 9) to a final concen-

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tration of 50 μM, while two samples (samples 1 and 2) were untreated. Two samples (samples 1 and 3) were labeled immediately, while the others were labeled at various times up to 1 h after oxidative stress. Samples 2 and 9 were labeled between 60 and 65 min. All labelings were done for 5 min with 30 μCi of L-[^{35}S]methionine. After 5 min of shaking at 37°C in the presence of label, cold L-methionine (0.33 ml of 50 mg/ml added to 1-ml cultures) was added. The cells were then immediately centrifuged for 1 min in an Eppendorf centrifuge at 4°C. The pellets were stored at −20°C before they were processed as described below.

Metabolic labeling of proteins at elevated temperatures. *B. subtilis* was grown in minimal medium at 30 or 37°C as described above. At an optical density at 550 nm of 0.35, 1-ml fractions were taken and shaken at 200 rpm at 30, 37, or 48°C to give the following temperature shifts: 30 to 30°C (control), 30 to 48°C, 37 to 37°C (control), and 37 to 48°C. Samples were labeled at 5 to 10 min and 20 to 25 min after the temperature shift, as described above.

Solubilization of labeled cells for one-dimensional gel electrophoresis. The labeled cell pellets were stored at −20°C for 1 h after centrifugation. The pellets from 1-ml cultures were suspended in 45 μl of 10 mM Tris (pH 8)–5 mM EDTA–500 μg of lysozyme per ml–100 μg of DNase I per ml. Then phenylmethylsulfonyl fluoride was added (1 μl of 10 mM), and the suspensions were incubated at 37°C for 8 min, which was found to be just sufficient to lyse the cells. An equal volume of 2× gel sample buffer described by Laemmli (11) was then added to the samples, after which they were boiled for 10 min. The samples were then spun in an Eppendorf microfuge for 1 min, and the supernatants were stored at −20°C before they were run on polyacrylamide gels. Levels of incorporation were estimated by precipitation of 1 μl of extract with trichloroacetic acid and counted in the ^{14}C channel of a scintillation counter TriCarb 3375; (Packard Instrument Co., Inc., Rockville, Md.). Typical incorporation levels were 2×10^5 to 5×10^5 cpm/μl of extract.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The labeled samples (10^6 cpm of each sample) were run on 8 and 12.5% sodium dodecyl sulfate-polyacrylamide gels by the method described by Laemmli (11). ^{14}C-methylated protein mixture (molecular weights, 14,300 to 200,000; Amersham) was coelectrophoresed as a molecular weight marker. After electrophoresis, the gels were soaked overnight in En^{3}Hance autoradiography enhancer, treated according to the instructions of the manufacturer, and dried on a gel dryer. The dried gels were autoradiographed for 10 and 18 h before they were developed.

Enzyme assays. Cells were grown to the mid-log phase in minimal medium and lysed by French pressing. SOD activity was localized in 8% polyacrylamide gels as described by Beauchamp and Fridovich (2). SOD liquid assays on cell extracts were performed as described by Marklund and Marklund (13). Liquid assays of catalase activity were carried out on cell extracts (3). Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

One unit of catalase decomposes 1 μmol of H_2O_2 per min per mg of protein at pH 7 and 20°C. One unit of SOD is equal to 50% inhibition of pyrogallol oxidation per mg of protein.

RESULTS

Survival of *B. subtilis* after treatment with hydrogen peroxide. *B. subtilis* JH642 was treated with various concentrations of hydrogen peroxide for 1 h, and appropriate dilutions of each sample were plated. The highest concentration of H_2O_2 which had negligible effects on the culture titer compared with the untreated control was found to be 50 μM. A concentration of 10 mM, however, caused 3 to 4 log units of killing.

TABLE 1. Percent survival of JH642 after H_2O_2 treatment

Expt	% Survival after the following H_2O_2 treatments[a]:		
	10 mM	50 μM, 10 mM	50 μM
1	0.01	20	100
2	0.04	5	107
3	0.004	0.3	29
4	0.02	0.4	20
5	0.01	0.3	54
6	0.01	2.5	55

[a] With no treatment there was 100% survival.

B. subtilis JH642 was pretreated with 50 μM H_2O_2 for 1 h, followed by 10 mM H_2O_2 for a second hour. The percent survival was determined (Table 1). It can be seen that this pretreatment reduced the degree of killing by the higher concentration of H_2O_2 by approximately 100-fold. Thus, 50 μM H_2O_2 is referred to as the protective concentration and 10 mM as the killing concentration of hydrogen peroxide. Some variation was observed between experiments, particularly in the degree of protection obtained by pretreatment with 50 μM H_2O_2. This variation can be partially explained by the dramatic changes in sensitivity observed during the growth cycle (7). While the cell density at the time of the 50 μM H_2O_2 addition was constant, slight daily differences in the growth rate meant that at the time of 10 mM H_2O_2 addition, the cells were at different phases of the growth cycle.

To minimize the amount of cell growth that occurred during the H_2O_2 treatment period, cells were assayed to determine the minimum time required for optimum protection and killing. Samples were treated with 10 mM H_2O_2 and diluted and plated for survival at different times between 10 min and 1 h after H_2O_2 addition. The percent survival of cells was the same at 10 min as at 1 h of treatment, indicating that a 10-min treatment time is adequate for maximal killing with 10 mM H_2O_2.

To determine the optimal protection time, replicate samples were treated with 50 μM H_2O_2 for various times between 5 min and 1 h. This was then followed by a 10-min treatment with 10 mM H_2O_2. The percent survival was the same for cells protected for 5 min as for cells protected for 1 h, indicating that protection is complete after 5 min of pretreatment.

Induction of proteins by the protective concentration of H_2O_2. *B. subtilis* JH642 was pulse-labeled at various times after treatment with 50 μM H_2O_2. Labeled proteins were separated by one-dimensional gel electrophoresis. The experiment was performed on four occasions. To obtain maximum resolution, each set of labeled samples was run on 8 and 12.5% gels, and two different exposures were taken of each autoradiogram. Approximately equal numbers of counts were loaded onto each lane. Band intensities were compared among lanes. In addition, inductions were checked by comparing the intensity of induced bands with that of noninduced bands within lanes. A representative autoradiogram of cells labeled for 5 min in the absence of oxidative stress and at 0 to 5 min after stress is shown in Fig. 1. Composite results are illustrated in Fig. 2. Levels of induction were judged visually. All inductions represented

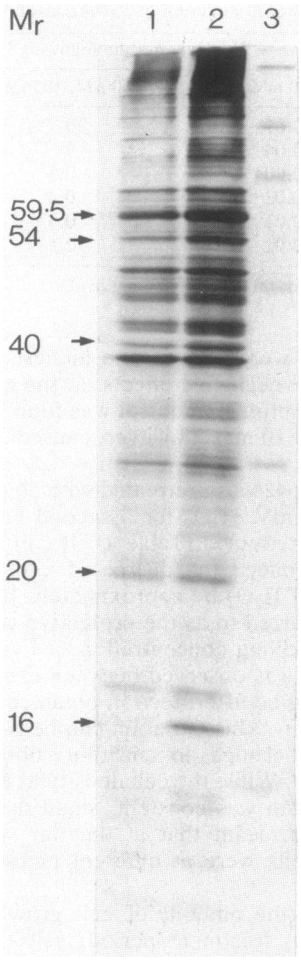

FIG. 1. One-dimensional polyacrylamide gel (12.5%) of [^{35}S]-methionine-labeled proteins from *B. subtilis* JH642. Lane 1, Proteins produced by an untreated 1-ml culture; lane 2, protein production 0 to 5 min after treatment with 50 μM H_2O_2; lane 3, molecular weight markers, from top to bottom, as follows: 200,000, 92,000, 69,000, 46,000, 30,000, 14,300. The inductions of five proteins in lane 2 are indicated by the arrows. Three further inductions are not visible on this gel, two of which are characterized by late induction.

here were observed in all sets of labeled samples, although the time of induction sometimes varied by 5 min. All inductions noted in Fig. 2 cannot be seen in the particular gel shown in Fig. 1, since many gels were required to maximally resolve all the proteins.

Eight proteins were induced by this treatment (Fig. 1 and 2). All of the proteins were maximally induced within 5 min following the initiation of stress, with the exception of the 49-kilodalton (kDa) protein, which was maximally induced between 15 and 20 min, and the 88-kDa protein, which was maximally induced between 20 and 35 min, following H_2O_2 addition. Of the eight proteins, the induction of the 16-kDa protein was strikingly stronger than that of the others (Fig. 1).

Proteins induced by temperature shifts. To study the relationship between cellular responses to different environmental stresses, we compared the proteins induced by temperature upshift to those induced by protective concentrations of H_2O_2. A temperature of 48°C was arbitrarily chosen as the upshift temperature. Since *B. subtilis* can grow at a wide range of temperatures, both 30 and 37°C were chosen as the preshift growth temperatures.

JH642 cells grown at 30 and 37°C were shifted to 48°C and were pulse-labeled with [^{35}S]methionine at 5 to 10 and 20 to 25 min after upshift. As controls, cells were transferred from 30 to 30°C and 37 to 37°C and similarly labeled. Results (Fig. 3) show that the protein synthetic profile of cells grown at 30°C is different from that of cells grown at 37°C (in Fig. 3, compare lane 1 with lane 6 for the 5- to 10-min labeling and lane 2 with lane 7 for the 20- to 25-min labeling). When cells were upshifted to 48°C, the protein synthetic profiles changed from that observed at the respective preshift temperatures (e.g., in the case of the 20- to 25-min postshift labeling; in Fig. 3 compare lane 2 [37°C] with lane 4 [48°C] and lane 7 [30°C] with lane 9 [48°C]). In addition, the protein synthetic profile observed at 48°C depended on whether the cells were grown at 30 or 37°C before upshift (in Fig. 3, compare lane 9 [30°C preshift] with lane 4 [37°C preshift]). We conclude that growth temperature has a profound effect on protein synthetic profiles in *B. subtilis*.

When the proteins induced by the shift from 37 to 48°C were compared with those induced by 50 μM H_2O_2 (at 37°C), proteins of 54, 40, 20, and 16 kDa were seen to have been induced by both stresses. The levels of induction observed on temperature upshift were less than those observed with H_2O_2 induction. In addition, the kinetics of induction of these proteins differed under the two stress conditions. However, these proteins were induced to a relatively minor extent compared with the dramatic increase in synthesis of proteins of 80, 74, 65, 41, and 25 kDa after 5 min at 48°C. The 65-kDa protein may be the same as the major heat shock protein reported by Streips and Polio (18). This protein may be the *B. subtilis* equivalent of hsp 70, the heat shock protein which has been conserved from bacteria to mammals (12).

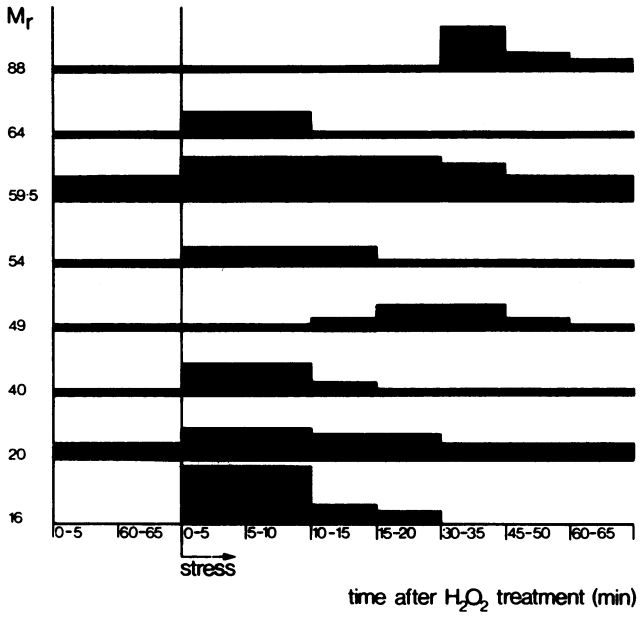

FIG. 2. Diagrammatic representation of the eight protein inductions observed following treatment of *B. subtilis* JH642 with 50 μM H_2O_2. The molecular weight of the proteins is given on the left. The level of expression, before and after stress, was judged by the intensity of bands on one-dimensional polyacrylamide gels, as shown in Fig. 1. The kinetics of induction was revealed by labeling at the times indicated during 1 h of growth following stress.

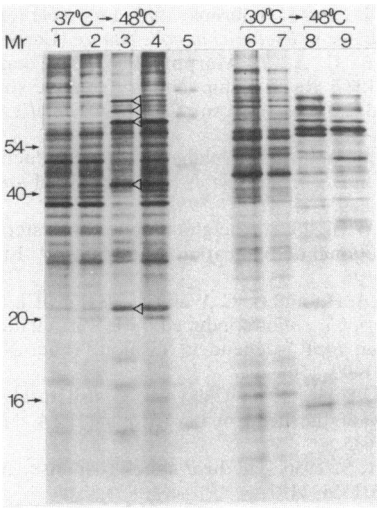

FIG. 3. One-dimensional polyacrylamide gel (12.5%) of ^{35}S-labeled proteins produced by *B. subtilis* JH642 following temperature shifts. The temperature shifts and the times of labeling after the shifts in each of the lanes are as follows: lane 1, 37 to 37°C, 5 to 10 min; lane 2, 37 to 37°C, 20 to 25 min; lane 3, 37 to 48°C, 5 to 10 min; lane 4, 37 to 48°C, 20 to 25 min; lane 5, molecular weight markers, as in lane 3 of Fig. 1; lane 6, 30 to 30°C, 5 to 10 min; lane 7, 30 to 30°C, 20 to 25 min; lane 8, 30 to 48°C, 5 to 10 min; lane 9, 30 to 48°C, 20 to 25 min. Proteins induced by both H_2O_2 treatment and heat treatment are indicated by arrows. Major proteins induced by heat alone are marked with open triangles.

Levels of catalase, SOD, and glutathione *S*-transferase in unstressed and stressed cells. Bacteria are protected from oxidative damage by several enzymes, including catalase and SOD. In addition, the enzyme glutathione *S*-transferase plays a role in inactivating free radicals in some organisms (9).

B. subtilis JH642 cells were separately treated with (i) 50 μM H_2O_2 for 10 and 30 min and (ii) a heat shock at 48°C (from a preshift temperature of 37°C) for 10 min. Cytosolic fractions were assayed for catalase, SOD, and glutathione *S*-transferase. Both catalase and SOD were present in *B. subtilis* (Table 2), whereas no glutathione *S*-transferase activity could be detected. The levels of catalase were extremely high; they were 1,000-fold higher than those found in *S. typhimurium*. Neither catalase nor SOD was induced by hydrogen peroxide or temperature upshift. On the contrary, levels of catalase activity were lower in cells upshifted to 48°C for 10 min compared with those in cells maintained at 37°C. This effect cannot be explained by temperature sensitivity of the enzyme, as incubation of the cell extract at 48°C for 10 min did not lead to a decrease in catalase activity. Two isozymes of SOD were detected in *S. typhimurium*, an iron-containing and a manganese-containing form (5). In contrast, *B. subtilis* was found to contain only one type of SOD, as observed on a nondenaturing gel (data not shown).

DISCUSSION

Characteristic features of the response of organisms to environmental stress are (i) adaptation of cells to normally lethal levels of stress by pretreatment with sublethal levels of stress and (ii) the rapid and generally transient synthesis of proteins on this pretreatment. The response of *B. subtilis* to oxidative stress caused by H_2O_2 observed in this study displays both features of the classic stress response. It was observed that pretreatment of log-phase cells with 50 μM H_2O_2 protected cells against death when treated with 10 mM H_2O_2. The pretreatment level of 50 μM H_2O_2 was the maximal level which conferred protection without causing significant cell death. This level is similar to the protective levels of H_2O_2 used in the case of *E. coli* (50 μM [6]) and *S. typhimurium* (60 μM [5]).

Synthesis of eight proteins was induced by the adaptive levels of 50 μM H_2O_2 in *B. subtilis*. Induction of six of these proteins was observed early, i.e., within 5 min of the application of stress, whereas synthesis of 49- and 88-kDa proteins was observed late, at 20 to 25 and 30 to 35 min poststress, respectively. In *S. typhimurium*, 30 proteins were induced by H_2O_2 stress. This difference in the number of proteins induced presumably reflects the greater resolution of two-dimensional gel analysis rather than a significant difference in the response of the organisms. The division of protein inductions into early and late groups in *B. subtilis* has also been observed in the response of *S. typhimurium* to H_2O_2 stress and has been observed for other stresses, including heat shock, in *E. coli* (5, 12).

Heat shock proteins are synthesized when cells are shifted to a higher temperature. Some of these proteins are induced by a wide variety of stresses and can be termed stress proteins. The protein synthetic profiles of *B. subtilis* cells exposed to a temperature upshift were compared with those of H_2O_2-stressed cells. It was apparent that four proteins with similar molecular weights were induced by oxidative stress and temperature upshift. The extent and kinetics of induction of these four proteins differed, however, in the two stress responses. Our results indicate the complex nature of the response of *B. subtilis* to a temperature upshift. Dramatically different protein synthetic profiles were obtained with cells shifted from 30 to 48°C compared with those of cells shifted from 37 to 48°C. The induction of heat shock proteins in *B. subtilis* by temperature shifts from 30 to 43°C (19) and 37 to 48°C (18) has been observed. However, an intermediate temperature which confers protection against thermal death at a higher temperature remains to be defined in *B. subtilis*.

Our results indicate that the protection of cells against killing by lethal levels of H_2O_2 is not mediated by induction of catalase or SOD. The same result is found for *E. coli*, unlike that for *S. typhimurium*, in which protection correlates with induction of these enzymes (5, 20). The level of SOD in *B. subtilis* is similar to that in aerobically grown *S. typhimurium*, but the level of catalase is 1,000-fold higher than that in either *E. coli* or *S. typhimurium*. In addition, our results show only one isozyme of SOD in *B. subtilis* compared with two in *S. typhimurium* (5). These differences may be due to the fact that *B. subtilis* is an obligate aerobe, whereas *E. coli* and *S. typhimurium* are facultative anaerobes.

It is not yet clear whether there is a close mechanistic

TABLE 2. Activities of SOD and catalase after H_2O_2 and heat treatment

Treatment	Activities (U) of:			
	SOD		Catalase (10^3)	
	Expt 1	Expt 2	Expt 1	Expt 2
None	6.76	7.11	1.03	1.30
H_2O_2 (10 min)	8.52	7.36	1.50	0.75
H_2O_2 (30 min)	8.54	6.46	0.54	1.20
37 to 48°C (10 min)	10.64	7.68	0.09	0.09

relationship between various stress responses or whether the same proteins can simply be induced by a number of different mediators. In the case of the SOS and heat shock responses in *E. coli*, the *groEL* and *dnaK* gene products are induced by both stresses. The induction of these proteins by UV light and nalidixic acid (inducers of the SOS response), however, is independent of *recA* and *lexA*, which control the induction of other proteins in response to these stimuli. Instead, induction of these genes is mediated by the *htpR* locus, which regulates induction of other heat shock genes (10). Similarly, overlapping sets of proteins are induced by oxidative stress, heat shock, and other stresses in *S. typhimurium*. Some of these are regulated by the *oxyR* gene, and others are not (14). These data suggest that any one environmental stress can switch on a number of different regulatory systems, each of which controls its own set of stress genes. It is not yet known whether any of these stress genes can respond to more than one of the regulatory loci. In the accompanying paper (7), we show that the induction of proteins by oxidative stress is controlled by at least two regulatory loci.

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