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The Global Transcriptional Response of *Bacillus subtilis* to Peroxide Stress Is Coordinated by Three Transcription Factors

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Bacillus subtilis **exhibits a complex adaptive response to low levels of peroxides. We used global transcriptional profiling to monitor the magnitude and kinetics of changes in the mRNA population after exposure to either hydrogen peroxide (H_2O_2) or *tert*-butyl peroxide (t-buOOH). The peroxide stimulons could be largely accounted for by three regulons controlled by the PerR, σ^B, and OhrR transcription factors. Three members of the PerR regulon (*katA*, *mrgA*, and *zosA*) were strongly induced by H_2O_2 and weakly induced by t-buOOH. The remaining members of the PerR regulon were only modestly up-regulated by peroxide treatment. Overall, the magnitude of peroxide induction of PerR regulon genes corresponded well with the extent of derepression in a *perR* mutant strain. The σ^B regulon was activated by 58 μM H_2O_2 but not by 8 μM H_2O_2 and was strongly activated by either t-buOOH or, in a control experiment, *tert*-butyl alcohol. Apart from the σ^B regulon there was a single gene, *ohrA*, that was strongly and rapidly induced by t-buOOH exposure. This gene, controlled by the peroxide-sensing repressor OhrR, was not induced by any of the other conditions tested.**

Treatment of bacteria with low levels of oxidants typically results in a classic adaptive response: the treated cells have a greatly enhanced ability to survive subsequent challenge with an otherwise lethal dose of the same oxidant or a related oxidant. This adaptive response is coordinated by the action of transcription factors that sense oxidative stress and regulate the expression of appropriate defensive and repair functions (34).

In *Escherichia coli* resistance to oxidative stress is largely coordinated by two systems. OxyR activates genes in response to either peroxide stress or changes in the thiol-disulfide status, while the SoxRS system controls genes in response to reactive free radicals, such as superoxide anion and nitric oxide (29, 35). Proteins induced by oxidative stress were initially catalogued by using one- and two-dimensional polyacrylamide gel electrophoresis (PAGE) (12, 15, 26). More recently, the OxyR and SoxRS regulons have been defined by using computational approaches to identify regulator binding sites together with transcriptional profiling (28, 39, 40).

Bacillus subtilis also displays an adaptive response to low levels of oxidants. Enzymatic assays have revealed that the major catalase present in growing cells (KatA) is strongly induced by treatment with peroxides (3, 25). With one-dimensional sodium dodecyl sulfate-PAGE several other proteins were also found to be strongly induced by 50 μM H_2O_2 (13). The most prominent members of the peroxide stimulon include the two subunits of alkyl hydroperoxide reductase (AhpC and AhpF), KatA, and the DNA-binding protein MrgA (9, 20). Similar conclusions were reached in studies of the protein profile of cells treated with H_2O_2, as visualized by two-dimensional PAGE (2).

Insight into the mechanisms controlling the inducible peroxide stress response in *B. subtilis* originally emerged from analysis of *mrgA*, which encodes a member of the Dps family of protective DNA-binding proteins (7, 9). Expression of *mrgA* is metalloregulated: repression is elicited by growth with either supplemental manganese or iron (8). This repression is mediated by PerR, a member of the ferric uptake repressor (Fur) family of metal-dependent DNA-binding proteins (5). The PerR regulon is now known to include *mrgA*, *katA*, *ahpCF*, a heme biosynthesis operon (*hemAXCDBL*), a zinc uptake system (*zosA*), *fur*, and *perR* itself (23). Studies with *lacZ* reporter fusions have demonstrated that expression of these genes is repressed by manganese and that some, but not all, of the genes are also repressed by iron (18). Some of these genes (*mrgA*, *katA*, and *zosA*) are strongly induced by H_2O_2, while others (*hemA* operon, *ahpCF*, and *perR*) show less induction. The *fur* gene is not induced by H_2O_2; thus, not all members of the PerR regulon are members of the peroxide stimulon (18).

To gain a global perspective on the transcriptional responses to peroxide stress, we monitored gene expression in stressed cells using DNA microarrays. Since many stress responses involve transient changes in gene expression, we isolated RNA at several times following imposition of stress and compared the resulting transcriptional profiles with the profiles of unstressed control cells. In addition, in this paper we describe a direct comparison between wild-type and *perR* mutant cells. A comparison of the responses to H_2O_2, *tert*-butyl peroxide (t-buOOH), and *tert*-butyl alcohol (t-buOH) and previously reported studies of the heat shock stimulon (22) revealed three distinct classes of induced genes: the PerR, σ^B, and OhrR regulons.

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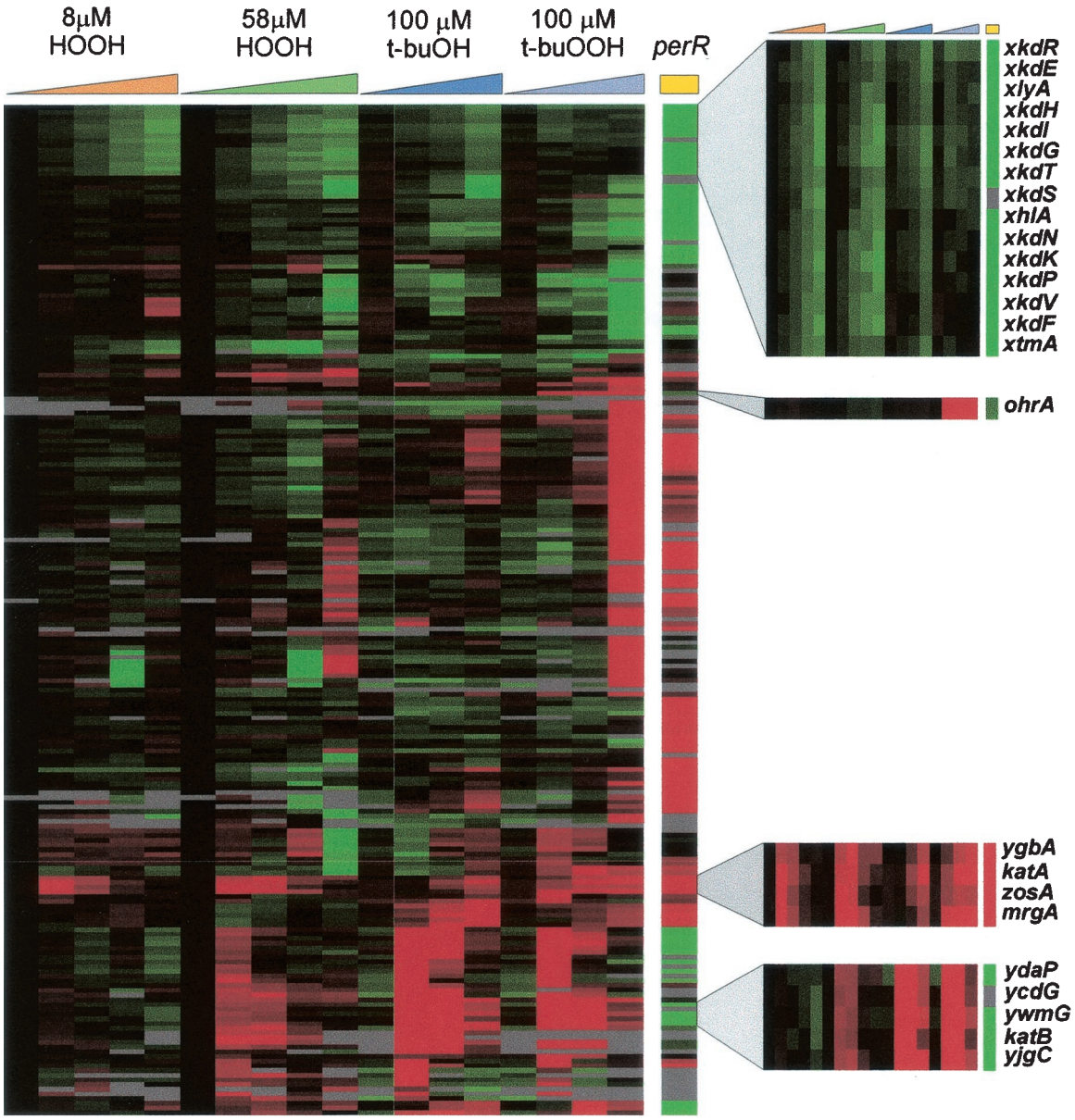

FIG. 1. Hierarchical cluster analysis (Eisen plot) of selected genes affected by peroxide stress. The hybridization ratios are displayed colorimetrically; genes induced by the experimental treatment are indicated by shades of red, and genes with reduced expression are indicated by shades of green. Genes were selected by being significantly altered in expression under at least one experimental condition (at least threefold induction or repression and SAM positive; the 40-min data were excluded in this analysis), and the data were filtered to remove genes that did not produce a measurable induction value (above the background value) in at least 10 of the 19 columns. Four clusters of genes discussed in the text are highlighted with zoom boxes; from top to bottom these are the *xkd* cluster of PBSX genes, the single gene strongly and selectively induced by t-buOOH stress (*ohrA*), the PerR regulon cluster (including *ygbA*) (see text), and a subset of the genes of the σ^B general stress regulon.

MATERIALS AND METHODS

Strains and growth conditions. *B. subtilis* CU1065 (W168 *attSP*β *trpC2*) was inoculated into 25 ml of prewarmed (37°C) Bacto Mueller-Hinton broth (MHB) (Difco, Detroit, Mich.) from a fresh (overnight) Luria-Bertani agar plate. The flask was shaken at 250 rpm on a rotary platform at 37°C until the optical density at 600 nm (OD_{600}) was 0.5 to 1.0. This seeder flask was used to inoculate a second flask of prewarmed MHB (50 ml in a 500-ml flask) to an OD_{600} of 0.2. This flask was grown until the OD_{600} was 1.0, at which time aliquots were removed and used as the time-zero samples. For the H_2O_2 stress experiments, 100 μl of appropriately diluted H_2O_2 was added immediately following removal of a time-zero aliquot to give a concentration of either 8 or 58 μM. Water (100 μl) was added to a mock treatment control flask. For the organic peroxide stress experiments, t-buOOH or t-buOH was added to a concentration of 100 μM (total amount added, 100 μl). Each flask was incubated on the shaker, and samples used for RNA isolation were removed at the times indicated below. The initial sampling of a culture was performed rapidly so that cells were frozen in liquid nitrogen within 40 s of removal from the culture.

Growth of the *perR* mutant strain. Cultures were inoculated as described above by using aliquots from an MHB plate containing either CU1065 or an isogenic *perR* strain (5) that had been grown overnight. The *perR* inoculum contained only small colonies, which was indicative of a lack of pseudorevertants (6). Each flask was shaken at 250 rpm on a rotary platform at 37°C. Samples used for RNA isolation were collected at an OD_{600} of 1.0. Prior observation had shown that the cultures exhibited exponential growth at this cell density (data not

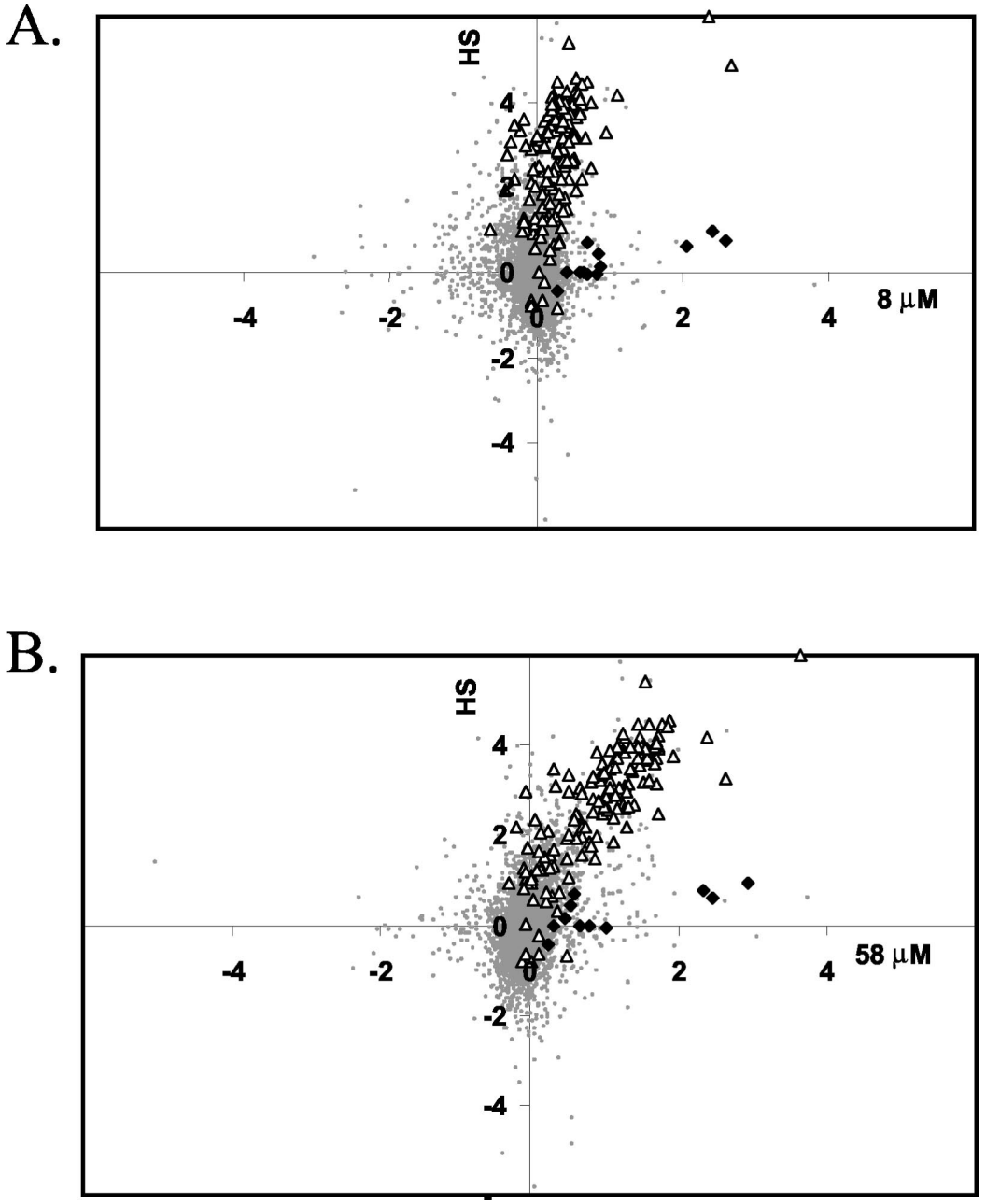

FIG. 2. Graphical comparison of the H_2O_2 and heat shock stimulons. The $\log_2$ of the induction ratio at 3 min is compared for a 48°C heat shock (HS) (*y* axis) and exposure to either 8 μM H_2O_2 (A) or 58 μM H_2O_2 (B) (*x* axis). Members of the PerR (solid diamonds) and σ^B (open triangles) regulons are highlighted and map, in general, to the upper right quadrant (corresponding to genes that have induction ratios of >1 for both heat shock and H_2O_2 treatment). All other gene signals are represented by small gray circles. Note that this and other two-dimensional graphical displays were generated without filtration of the data to remove low-quality and nonreproducible signals, so some of the background signals (small gray circles) that appear to represent highly regulated genes are not significant.

shown). CU1065 reached an OD_{600} of 1.0 in ~3 h, while a similar inoculum of the isogenic *perR* strain took ~7.5 h to reach a similar cell density. After the samples used for RNA isolation were removed, the cultures were diluted and plated to obtain single colonies on MHB agar. Following overnight growth the *perR* strain plate was examined for large pseudorevertant colonies, and individual colonies were tested for catalase activity to confirm that the *perR* strain culture contained undetectable levels of catalase-negative pseudorevertants (6). RNA was isolated as described above.

Microarray analysis, cDNA labeling, slide hybridization, data collection, and normalization. The procedures used for microarray analysis, cDNA labeling, slide hybridization, data collection, and normalization have been described previously (22). Time-zero cDNA samples were labeled with Cy3, and samples collected at subsequent times were labeled with Cy5. Slides were scanned and analyzed with a confocal laser scanner and a software package (Axon GenePix 4000A and GenePix Pro 3.0; Axon Instruments, Inc., Foster City, Calif.). Normalization was computed by using the algorithm incorporated into the Stanford Microarray Database (33), and all data are archived in this database. In general, fold induction was determined by comparison of the peroxide-treated samples and the time-zero control samples. However, where indicated below, the experimental data were corrected for time-dependent changes in the samples by using data for the parallel untreated samples. For example, the log-transformed gene induction ratios after mock treatment were subtracted from the log-transformed

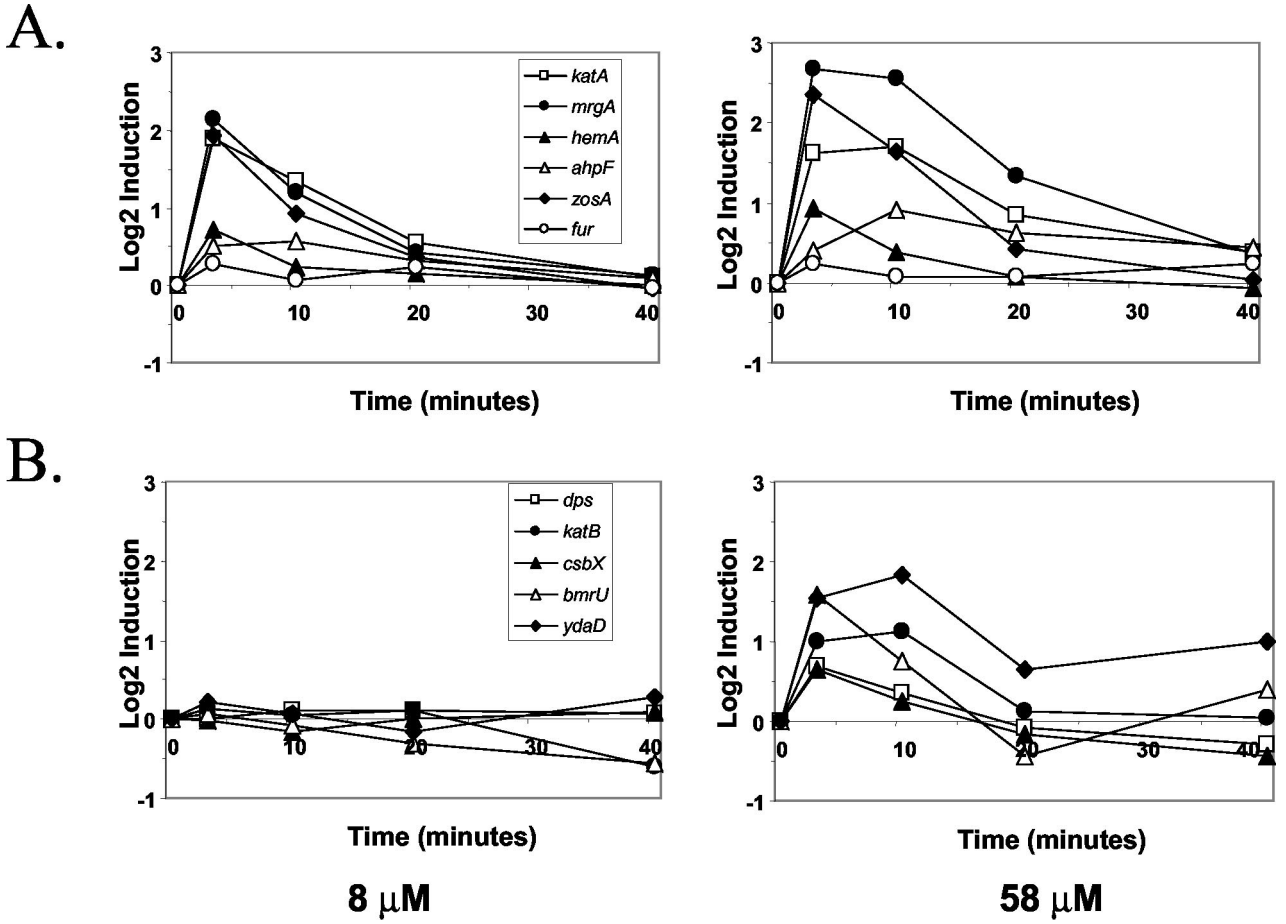

FIG. 3. Comparison of the time courses of expression for selected members of the PerR (A) and σ^B (B) regulons. The $\log_2$ fold induction (relative to the untreated control culture at the same time) is plotted for cells exposed to either a low (8 μM) or an intermediate (58 μM) level of H_2O_2.

induction ratios after H_2O_2 treatment. To identify and display groups of coregulated genes for this set of treatment regimens, we used hierarchical clustering as previously described (14). For the 8 and 58 μM H_2O_2 regimens, genes were considered to be differentially regulated if after 3, 10, or 20 min (i) the absolute value of the mean of the replicate $\log_2$ treatment ratios minus the mean of the $\log_2$ mock treatment ratios exceeded 1.585 (corresponding to a threefold difference) and (ii) significance analysis of microarrays (SAM) of all available treatment replicates versus mock replicates in a two-class, unpaired data design indicated statistical significance at the threefold level when the delta parameter was adjusted to give a median number of false significants typically less than 5% of the total number of selected genes but allowing for at least one false significant in a set regardless of its size (36). A similar analysis was carried out for the t-buOOH–versus–t-buOH experiments, with t-buOH being considered the mock treatment in the two-class design. In the *perR* mutant studies, reciprocal cDNA labeling was conducted with either CU1065 Cy3 versus *perR* Cy5 or CU1065 Cy5 versus *perR* Cy3. A total of six slides (three for each labeling regimen) were analyzed. In this case, two-class, unpaired data SAM was performed by using normalized intensity data rather than ratio data, and the delta parameter was adjusted to give a median number of false significants < 2. Supplemental tables and complete data sets are available at http://www.micro.cornell.edu/faculty.JHelmann.html.

RESULTS

Overview of experimental design. We monitored the global transcriptional profile of *B. subtilis* under peroxide stress conditions using DNA microarray-based measurements. To monitor changes in gene expression over time, we compared the mRNA profiles obtained 3, 10, 20, and 40 min after stress was imposed with the profile of a time-zero (prestress) control sample. Changes in gene expression were monitored for three different peroxide stress conditions: low (8 μM) and intermediate (58 μM) levels of H_2O_2 and 100 μM t-buOOH. Note that 58 μM H_2O_2 is designated an intermediate level of stress to distinguish our results from those of experiments performed by other groups with much higher levels of H_2O_2 (39). As controls we also monitored gene expression in parallel cultures treated with the corresponding alcohols (H_2O and t-buOH). In addition, we compared the transcriptional profile of a strain with a disruption in *perR* with the profile of the corresponding wild-type control. The majority of comparisons were performed on at least three independent slides, and while the SAM was based on all available replicates, the geometric means of the experimental ratios were, for reasons of convenience, used to generate the figures.

H_2O_2 stress stimulon. To define the major groups of genes most affected by peroxide stress, we performed a cluster analysis using the software developed by Eisen et al. (14). For this analysis we clustered the data by using data sets obtained for induction by H_2O_2 at concentrations of 8 and 58 μM (correct-

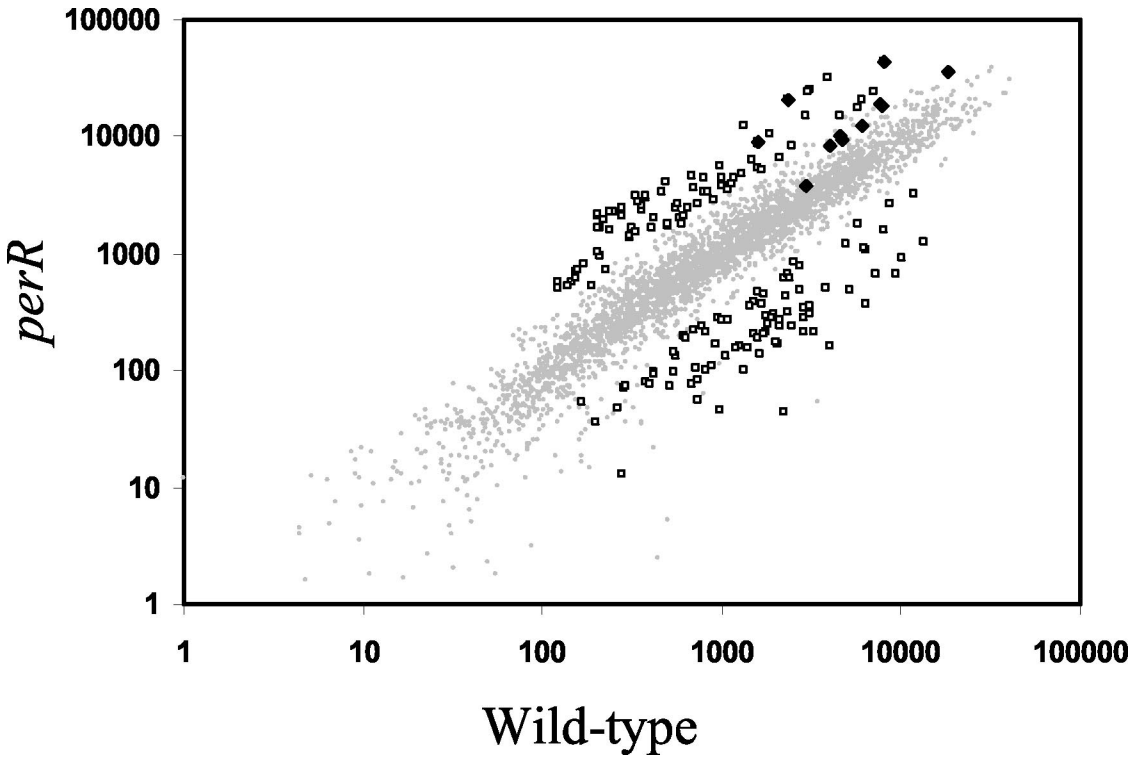

FIG. 4. Gene expression in the *perR* mutant compared to gene expression in the isogenic wild-type strain. Relative hybridization intensities (mean normalized hybridization quanta) are plotted for genes as measured for the wild-type (*x* axis) and *perR* mutant (*y* axis) strains. The genes that differ significantly (based on a threefold cutoff and SAM [see Materials and Methods]) in the two strains are indicated by open squares. The members of the PerR regulon are indicated by solid diamonds. Of these, only *katA*, *mrgA*, and *zosA* are derepressed more than threefold. Table 1 provides a summary of the genes that are significantly up-regulated in the *perR* mutant.

ed for changes in gene expression that occurred as a function of time in the parallel untreated cultures), by t-buOOH, and by t-buOH and for the changes noted in the *perR* mutant compared with data for the wild type. In addition, we included the previously described results obtained for the heat shock stimulon (22) to aid in clustering, although these data are not incorporated in Fig. 1.

The cluster analysis clearly grouped a subset of the PerR regulon genes as the genes that were strongly induced by both low and intermediate levels of H_2O_2 and were derepressed in the *perR* mutant (Fig. 1). Only four genes (*ygbA*, *katA*, *zosA*, and *mrgA*) were strongly and rapidly up-regulated by the low level of H_2O_2 (Fig. 1, third zoom box). Three of these genes are known members of the PerR regulon, whereas peroxide induction of *ygbA* has not been reported previously. The *ygbA* gene (also called *ssuA*) is the second gene in an operon involved in sulfonate transport that is transcribed divergently from the strongly induced *katA* gene (37). Since other genes in this operon are not peroxide inducible, the apparent induction noted here could have been an artifact (e.g., there could have been cross-hybridization or contamination of the *ygbA* PCR product with longer products extending into *katA*). A second, much larger cluster of genes was induced by the intermediate level of H_2O_2 but not by the low level of H_2O_2 and was further distinguished by strong and rapid induction in response to t-buOOH and t-buOH. This cluster corresponds to the large σ^B general stress response regulon (21, 27, 30, 31), and only a subset (*ydaP* though *yjgC*) is shown here (Fig. 1, fourth zoom box). Among the genes most dramatically down-regulated following imposition of peroxide stress are the genes comprising the PBSX phage-like element (*xkdR* through *xtmA*) (Fig. 1, first zoom box). Expression of these genes is also greatly reduced in a *perR* mutant (see below).

As an alternative method to visualize clusters of coregulated genes, we used a graphical approach to compare changes in gene expression under two different conditions. Since the general stress response is also strongly induced by heat shock, as we documented previously (22), we plotted the $\log_2$ values of the ratios of induction by H_2O_2 (relative to the untreated control at the same time) versus the $\log_2$ values of the ratios of induction by heat stress. Since the kinetic analyses revealed that both responses were strongly and selectively induced at the earliest time point (3 min), we focused on these data for comparison. The resulting graphs revealed two clusters of genes induced by both treatments (Fig. 2, upper right quadrants). One set of genes was strongly induced by either 8 or 58 μM H_2O_2 but only weakly induced by heat stress. These genes include *mrgA*, *katA*, and *zosA* and define a subset of the PerR regulon (23). A second large set of genes, representing the bulk of the heat shock stimulon, was induced by 58 μM H_2O_2 (Fig. 2B) but was only weakly affected by 8 μM H_2O_2 (Fig. 2A). This group includes many known and putative members of the large general stress regulon controlled by σ^B (21, 30).

Analysis of the kinetics of the transcriptional responses to

TABLE 1. Genes derepressed in the *perR* mutant strain

Gene(s)	Foldinduction[a]	Regulator(s)
comER	9.90	ComK
yybF	9.81	
mrgA	9.64	PerR
ywfM	8.94	
comEA	8.27	ComK
comG(*ABCDEFG*)	7.93 ± 1.74	ComK
melA	7.31	
cwlJ	7.64	σ^E
gbsAB	7.62 ± .52	Osmotic stress
msmRE amyDC	7.44 ± 1.67	
dppABCDE	7.04 ± .81	CodY
zosA	5.97	PerR
appDF	5.53 ± .34	Hpr
katA	5.34	PerR
ilvBC leuBC	5.01 ± .71	
ureABC	4.96 ± 1.4	CodY, GlnR, TnrA
yckDE	4.96 ± .10	
yybI	4.92	
appBC	4.89 ± .90	
yxbC	4.60	
yxbBA	4.50 ± .10	
yufN	4.44	
yvaWY	4.36 ± .20	
yhdG	4.27	
sipT	4.25	
ygbA	4.18	
ppsABCDE	3.93 ± .52	
yuiA	3.90	
ykvKLM	3.83 ± .60	
yheK	3.76	
yisS	3.67	
ykfBCD	3.67 ± .34	
ywcE	3.50	
yoeB	3.29	
yhdC	3.27	
bioAFDB	3.26 ± .11	
rapH	3.21	
yybG	3.14	
opuBB	3.14	Osmotic stress
ytpQ	3.10	
gltB	3.07	
ywfH	3.07	
yqzE	2.88	

[a] Induction values for genes in the same operon were used to determine the mean and standard deviation for the operon. All individual gene values are averages of induction ratios computed from six separate slides (see Materials and Methods).

H_2O_2 revealed that both the PerR and σ^B components of the H_2O_2 stimulon were induced transiently (Fig. 3). Most σ^B-dependent general stress genes were maximally induced at 3 min following exposure to 58 μM H_2O_2, and the mRNA levels declined by 10 min following exposure. This was consistent with the transient induction of the σ^B regulon in response to heat shock and likely reflected the rapid induction of negative regulatory factors, including RsbX (38). The PerR-controlled stress response was also transient, with the maximal mRNA levels occurring at either 3 or 10 min following exposure.

PerR regulon. The role of PerR in the regulation of catalase (*katA*), *mrgA*, *ahpCF*, *zosA*, and the heme biosynthesis operon has been reported previously (4, 9, 19). To identify additional members of the PerR regulon, we compared the transcriptional profiles of *perR* mutant and wild-type cells grown to the late logarithmic phase (OD_{600}, ~1.0) (Fig. 4). Our slides contained DNA probes for 90% (3,703 of ~4,100) of *B. subtilis* genes (22), and hybridization signals significantly above the background level were detected for 60 to 70% of these genes. In the comparisons of the *perR* mutant and the wild type, we calculated expression levels for ~2,400 genes, and 8% of these levels were significantly altered in the mutant strain (Fig. 4). Expression of ~75 genes (including known PerR regulon members *katA*, *mrgA*, and *zosA*) was significantly elevated in the *perR* mutant (Table 1), while expression of another ~120 genes was decreased.

To identify additional members of the PerR regulon, we compared the effects of 58 μM H_2O_2 and the *perR* mutation in a two-dimensional plot (Fig. 5). Known members of the PerR regulon were induced by H_2O_2 and derepressed by the *perR* mutation (Fig. 5, upper right quadrant). Remarkably, there was an excellent correlation between induction by 58 μM H_2O_2 (at 3 min) and the extent of derepression in the *perR* mutant (correlation coefficient [R^2], 0.9). This suggests that PerR repression was completely relieved under these conditions, and the difference in the magnitude of induction may have resulted primarily from differences in the extent to which PerR affected gene expression. Several other genes (including *ygbA*, *yerL*, *opuBC*, and *ureB*) also mapped to this region of the graph, but these genes were not associated with Per boxes and the effects may have been indirect. Note that the majority of the genes up-regulated in the *perR* mutant were not peroxide inducible (Fig. 5). Furthermore, the expression of many genes that were induced by 58 μM H_2O_2 (including many members of the σ^B regulon, as noted above) was decreased in the *perR* mutant, and these genes clustered in the lower right quadrant in Fig. 5.

Many of the observed changes in the *perR* mutant are likely to be indirect effects (Table 1). For example, the *perR* mutant may be stressed by the production of high levels of catalase, which could deplete heme pools or affect global iron homeostasis. In addition, the lower growth rate of the mutant affects the expression of many metabolic genes. Many of the 75 genes up-regulated in the *perR* mutant, including genes associated with competence, nutrient uptake, and the CodY regulon, are associated with the transition phase. The *perR* mutant strain also had significantly decreased expression of many genes, including the σ^B regulon genes (Fig. 5), several genes of the Fur regulon (*ykuNO*, *yxeB*, *ywbL*, and *ywbN*) (1), and the PBSX defective prophage (24). While the origins of the latter effect are unclear, PBSX is known to be induced by 100 μM H_2O_2 (12). Our results suggest that PerR or a PerR-regulated gene may act as a regulator of gene expression for PBSX.

t-buOOH stimulon. We next investigated the transcriptional response elicited by exposure of cells to t-buOOH. Within 3 min of exposure to t-buOOH, more than 100 genes were induced at least threefold compared to the expression in the time-zero sample. At subsequent times many more changes in gene expression became apparent (Fig. 1), but these appeared to be secondary effects and were not analyzed in detail. As a control, we also determined the time course of transcriptional changes elicited by exposure to the corresponding alcohol, t-buOH. A two-dimensional comparison of the responses revealed a remarkable pattern: the vast majority of the genes induced by t-buOOH were induced equally by t-buOH (Fig. 6). Not surprisingly, most of these genes belong to the σ^B regulon, which is strongly induced by ethanol (31).

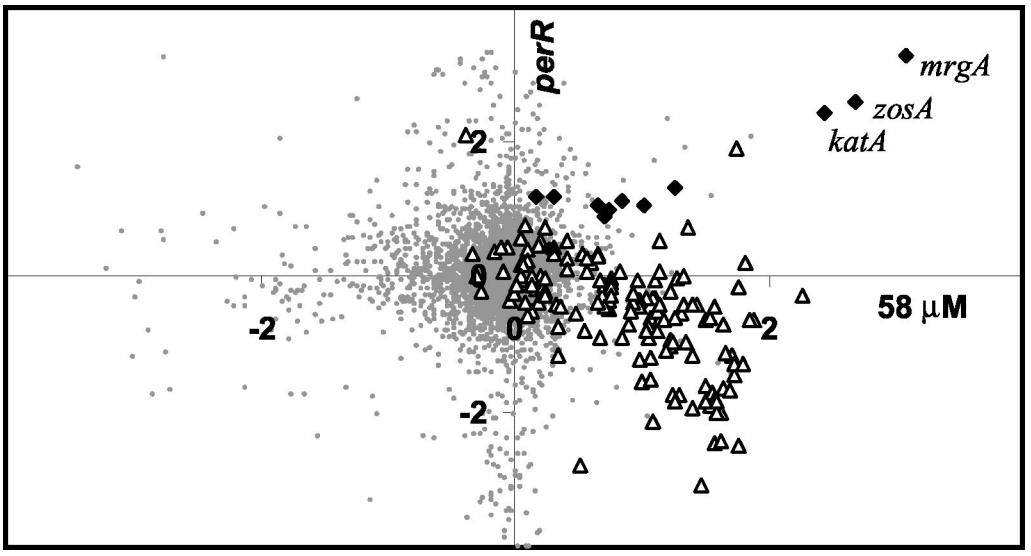

FIG. 5. Graphical comparison of the H_2O_2 stimulon and the effects of the *perR* mutation. The $\log_2$ of the induction ratio at 3 min after exposure to 58 μM H_2O_2 (*x* axis) is compared with the $\log_2$ of the ratio of induction in the *perR* mutant to induction in the wild type (*y* axis). Members of the PerR and σ^B regulons are indicated by solid diamonds and open triangles, respectively. The three most strongly regulated members of the PerR regulon are labeled. Note that PerR-regulated genes are in the upper right quadrant (these genes derepressed in the *perR* mutant and peroxide inducible in the wild type).

There are also several genes that were induced by t-buOOH but not by t-buOH, and these genes are therefore candidates for an organic peroxide stimulon. Most notably, the *ohrA* gene was strongly and specifically induced by t-buOOH (Fig. 1, second zoom box, and Fig. 6). Expression of *ohrA* is regulated by the peroxide-sensing transcription factor, OhrR (16, 17). Other genes that were selectively induced by t-buOOH (although no gene was induced as dramatically as *ohrA*) include the PerR regulon member *mrgA*, the thioredoxin B gene (*trxB*), and at least two genes of the ArsR-regulated *ars* operon (32).

Three peroxide-induced regulons. Overall, our analyses led us to conclude that the peroxide stimulon has three major components: the PerR, σ^B, and OhrR regulons. These three regulons can be clearly visualized by comparing the transcrip-

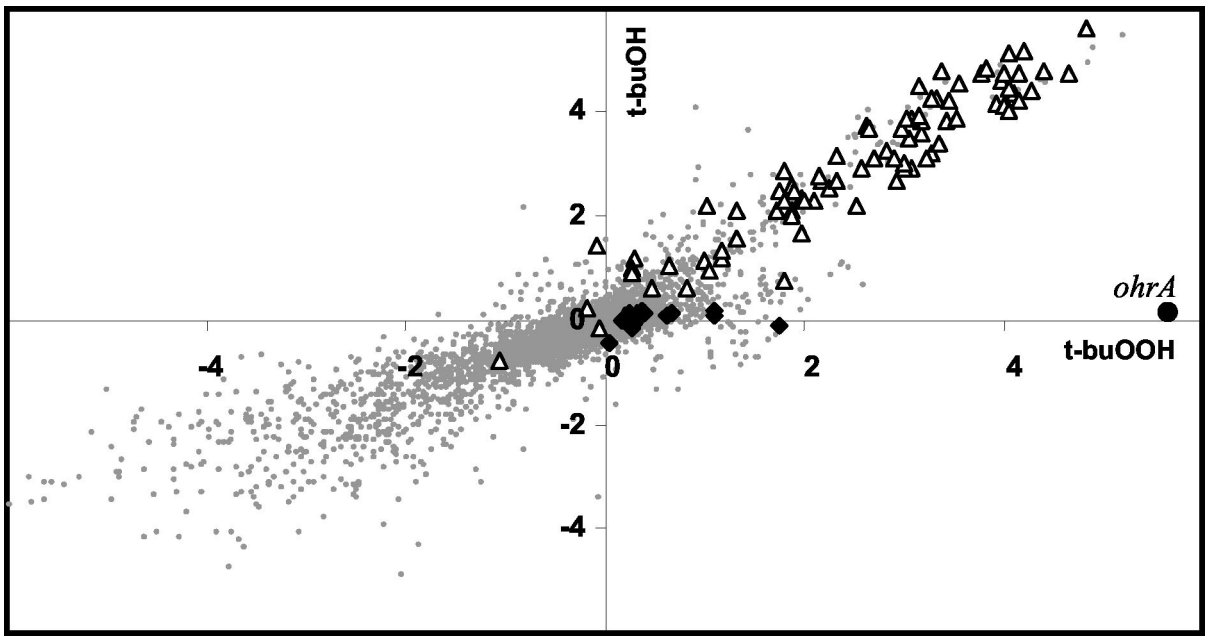

FIG. 6. Graphical comparison of the t-buOOH and t-buOH stimulons. The $\log_2$ of the induction ratio at 3 min after exposure to 100 μM t-buOOH (*x* axis) is compared with the $\log_2$ of the induction ratio after exposure to 100 μM t-buOH (*y* axis). Members of the PerR and σ^B regulons are indicated by solid diamonds and open triangles, respectively. The *ohrA* gene (indicated by a solid circle) is strongly and specifically induced by t-buOOH. Note that the σ^B regulon defines a line with a slope near 1, which is indicative of equivalent induction by t-buOOH and t-buOH.

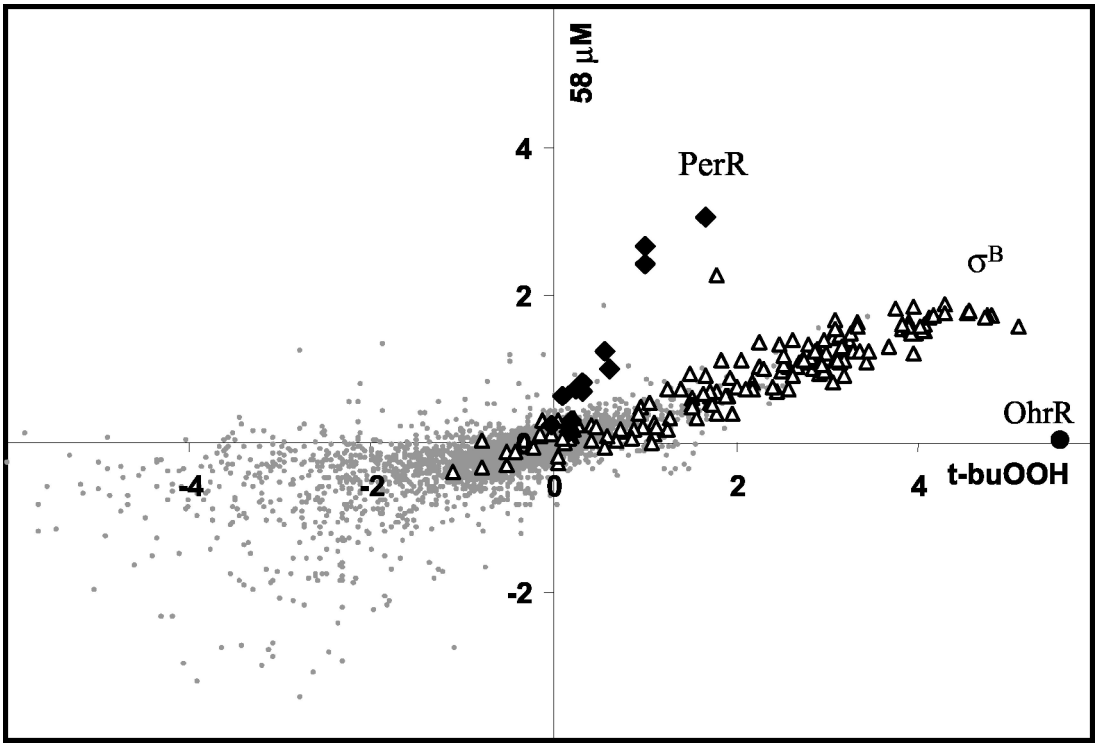

FIG. 7. Graphical comparison of the t-buOOH and H_2O_2 stimulons. The $\log_2$ of the induction ratio at 3 min after exposure to 100 μM t-buOOH (*x* axis) is compared with the $\log_2$ of the induction ratio after exposure to 58 μM H_2O_2 (*y* axis). Genes of the PerR and σ^B regulons are indicated by solid diamonds and open triangles, respectively, and *ohrA* is indicated by a solid circle. The regulators responsible for controlling the clusters of genes (PerR, σ^B, and OhrR) are indicated.

tional responses (after 3 min) to 58 μM H_2O_2 with the responses elicited by 100 μM t-buOOH (Fig. 7). Three distinct sets of genes clearly emerged, as follows: the OhrR-regulated gene, *ohrA*, was strongly induced by t-buOOH but not by H_2O_2; the σ^B regulon was induced by t-buOOH more than it was induced by H_2O_2; and the PerR-regulated genes were induced by H_2O_2 more than they were induced by t-buOOH. We summarized the selectivity of these transcriptional responses by comparing the efficacies of various inducers for representative members of each regulon (Fig. 8).

DISCUSSION

Here we provide a global overview of the major transcriptional changes elicited by various peroxidative stress conditions. Our findings indicate that PerR is the major regulator of genes induced by low levels of H_2O_2, while OhrR regulates the single gene that is most rapidly and selectively induced by the model organic peroxide t-buOOH. However, the largest single group of genes induced by these treatments is the σ^B-dependent general stress regulon (30). Indeed, knowledge about the general stress response regulon, as deduced both from previous studies of the heat shock stimulon (22) and from extensive characterization of the σ^B regulon (21, 30), provided important background for our analysis.

Our work also illustrates the value of monitoring the kinetics of global changes in gene expression following application of a stress. Many of the most dramatic transcriptional effects were maximal within 3 min of application of the stress conditions, and there was an often rapid return to lower expression levels within 10 to 20 min. The transient nature of the σ^B-dependent general stress response is well known, but transient induction of the PerR and OhrR regulons has not been well documented previously. Thus, studies based on a single time point may miss major parts of the transcriptional response.

Our results emphasize the importance of control experiments for interpreting transcriptional profiles. We found, for example, that the vast majority of the transcriptional changes noted 40 min after H_2O_2 treatment were also observed in mock (H_2O)-treated samples and were due to a growth phase transition rather than any effect of the peroxide treatment. Similarly, the comparison of the t-buOOH and t-buOH stimulons (Fig. 6) highlighted the need to use appropriate controls when the effects of particular classes of compounds are investigated. Indeed, we cannot exclude the possibility that t-buOOH is an inducer of the σ^B regulon because it is reduced in vivo to t-buOH. However, the rapid kinetics of these transcriptional responses make it seem unlikely that reduction to the alcohol is necessary for induction.

While transcriptional profiling is an extremely powerful tool for monitoring changes in gene expression after a change in culture conditions, the comparison of mutant and wild-type strains presented additional challenges. In this case, the *perR* mutant and wild-type strains differed significantly in growth rate and physiological state. Despite these differences, the *perR* mutant clearly revealed derepression of known PerR-repressed target genes. Remarkably, the extent of derepression

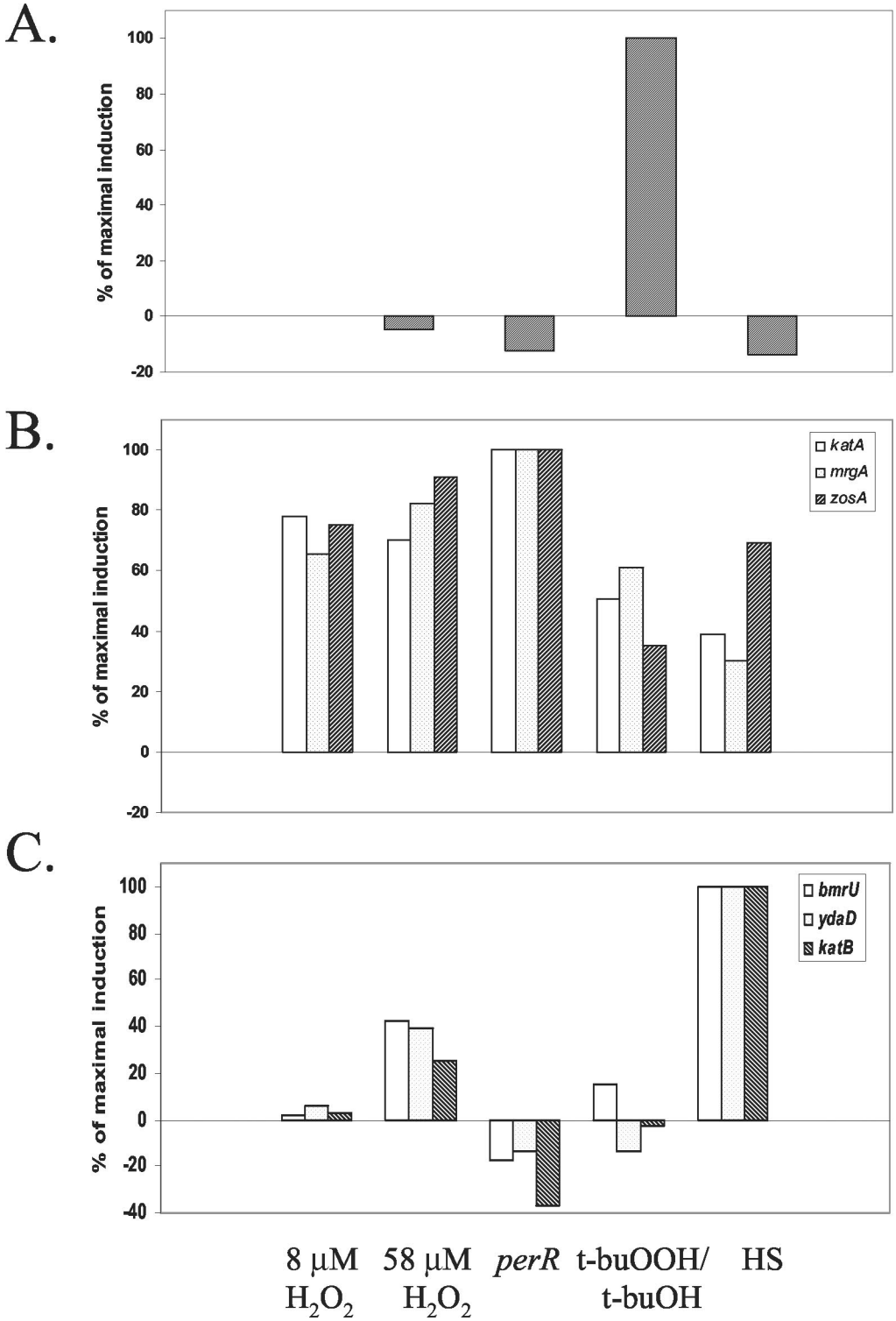

FIG. 8. Selectivity of induction of different genes by various stress conditions. The extent of induction (percentage of the maximal value) is compared for *ohrA* (A), three members of the PerR regulon (*katA*, *mrgA*, and *zosA*) (B), and three members of the σ^B regulon (*bmrU*, *ydaD*, and *katB*) (C). The conditions tested included 8 and 58 μM H_2O_2, the *perR* mutant strain, t-buOOH (induction by t-buOH was first subtracted), and heat shock (HS).

in the samples corresponded very well with the fold induction 3 min after treatment with H_2O_2 (Fig. 5). This correlation suggests that the magnitude of peroxide induction of different genes reflects the extent to which PerR represses gene expression. As noted elsewhere, the *fur* gene is unusual among PerR-repressed genes in that it is not peroxide inducible under these or any other conditions tested (18). While not apparent from the transcriptional profiling data, PerR does mediate an approximately fourfold repression of *fur* in response to Mn(II) (18).

It is instructive to compare the *B. subtilis* and *E. coli* peroxide stress responses. In *E. coli*, transcriptional profiling was performed with cells treated with much higher levels of H_2O_2 (1 mM), in part because lower levels led to only transient changes in gene expression (39). Under these conditions 140 genes were induced approximately fourfold in the wild-type strain, and an even greater number of genes were induced in an *oxyR* mutant strain. The OxyR regulon includes genes with protective and detoxification functions, such as *katG* (hydroperoxidase I), *ahpCF* (alkyl hydroperoxide reductase), and *dps* (DNA-binding protein). In addition, OxyR activates transcription of genes that maintain intracellular thiols in their reduced states, including *gorA* (gluthathione reductase), *grxA* (glutaredoxin), and *trxA* (thioredoxin 2). In aerobically growing *E. coli* the concentration of intracellular H_2O_2 is maintained at levels near 20 nM by the potent peroxidase activity of Ahp (10, 11). Catalase peroxidase appears to be most important in detoxifying higher levels of peroxides and has the added advantage of being active even in energy-depleted cells that may lack sufficient reducing capacity to maintain optimal Ahp activity. Ahp itself was originally characterized as the major resistance factor protecting cells against alkyl peroxides. However, Ahp is also active with H_2O_2, and this may be the physiologically relevant substrate (10).

The PerR regulon differs from the regulon controlled by OxyR in several respects. PerR does not appear to control functions that might be involved in maintaining intracellular thiols in a reduced state. Although *B. subtilis* encodes several predicted peroxiredoxins, including a thiol-dependent peroxidase (*tpx*) and the product of *ygaF* (the gene immediately upstream of *perR*), only the *ahpCF* genes are members of the peroxide stimulon characterized in this study. In future work, it will be important to define the roles of catalase, Ahp, peroxiredoxins, and the organic hydroperoxide resistance proteins in *B. subtilis*. While all of these enzymes may act to detoxify reactive oxygen species in the cell, genetic studies indicate that they are not redundant.

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