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Differential Gene Expression in Response to Hydrogen Peroxide and the Putative PerR Regulon of *Synechocystis* sp. Strain PCC 6803‡

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We utilized a full genome cDNA microarray to identify the genes that comprise the peroxide stimulon in the cyanobacterium *Synechocystis* sp. strain PCC 6803. We determined that a gene (slr1738) encoding a protein similar to PerR in *Bacillus subtilis* was induced by peroxide. We constructed a PerR knockout strain and used it to help identify components of the PerR regulon, and we found that the regulatory properties were consistent with the hypothesis that PerR functions as a repressor. This effort was guided by finding putative PerR boxes in positions upstream of specific genes and by careful statistical analysis. PerR and sll1621 (*ahpC*), which codes for a peroxiredoxin, share a divergent promoter that is regulated by PerR. We found that *isiA*, encoding a Chl protein that is induced under low-iron conditions, was strongly induced by a short-term peroxide stress. Other genes that were strongly induced by peroxide included *sigD*, *sigB*, and genes encoding peroxiredoxins and Dsb-like proteins that have not been studied yet in this strain. A gene (slr1894) that encoded a protein similar to MrgA in *B. subtilis* was upregulated by peroxide, and a strain containing an *mrgA* knockout mutation was highly sensitive to peroxide. A number of genes were downregulated, including key genes in the chlorophyll biosynthesis pathway and numerous regulatory genes, including those encoding histidine kinases. We used PerR mutants and a thioredoxin mutant (TrxA1) to study differential expression in response to peroxide and determined that neither PerR nor TrxA1 is essential for the peroxide protective response.

The appearance of oxygen on earth from oxygenic photosynthesis resulted in the production of potentially deleterious reactive oxygen species, such as superoxide and peroxide (19). Superoxide is not extremely reactive by itself, but the hydroxyl radicals formed can damage many biological macromolecules. All organisms have developed ways of protecting themselves against reactive oxygen species, including specific defenses and global responses that enable cells to survive oxidative stress. These defenses include superoxide dismutases, which convert superoxides to hydrogen peroxide, and the related catalases and peroxides, which degrade simple and organic peroxides. Bacteria display a classic adaptive response after treatment with low levels of oxidants, such as hydrogen peroxide; the treated cells have an enhanced ability to withstand subsequent treatment with a dose that normally would be lethal to the cells. This type of adaptive response is coordinated by transcriptional factors that sense the oxidative stress stimuli and control subsequent gene expression (60, 61). *Escherichia coli* and *Bacillus subtilis* are the microorganisms in which oxidative stress defense regulation has been studied most. In *E. coli*, significant progress has been made in elucidating the mechanisms by which the activities of the SoxR and OxyR transcription factors respond to $O_2^{\cdot -}$ and H_2O_2 stress, respectively. OxyR reacts with H_2O_2 to form an intramolecular disulfide bond, and the resulting conformational change activates the protein (8, 49, 61, 75). Similarly, in *Streptomyces coelicolor* oxidation of the RsrS anti-sigma factor by H_2O_2 or diamide leads to disulfide bond formation and results in release of σ^R and activation of target genes (28). However, not all bacteria have an OxyR homologue. Recently, a Fur-like peroxide-sensing repressor, PerR, was found in some organisms that lack OxyR. The PerR regulon in *B. subtilis* includes *katA*, *ahpCF*, *mrgA*, *hemAXCDBL*, *fur*, and *perR* itself. Moreover, PerR is a metalloprotein, and its regulation involves both oxidation and metal ions (17, 20, 21, 40).

PerR functions as the central regulator of the inducible peroxide stress response in certain gram-positive and gram-negative bacteria (17, 21, 40). PerR regulation was first described in *B. subtilis*, in which it regulates the peroxide stress response in regard to iron, manganese, and peroxide stress conditions (4). *B. subtilis* contains three Fur homologues that coordinate gene expression in response to iron (Fur), zinc (Zur), or H_2O_2 (PerR). All three proteins are dimeric, DNA-binding repressors that contain a single Zn(II) atom per monomer and a second regulatory metal ion, either Mn(II) or Fe(II), that acts as a cofactor that is necessary for binding to the target operator sites (17). When PerR binds to Mn(II), it is not readily dissociated from DNA by peroxide, whereas when PerR binds to iron, it can be readily dissociated from DNA by low levels of H_2O_2 (21).

The adaptive response to peroxide stress in *B. subtilis* is coordinated by at least two other transcription factors, σ^B and OhrR (20). The general stress response, coordinated by the σ^B protein, includes paralogs of many of the stress proteins, including two additional catalases (KatB and KatX). OhrR is

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TABLE 1. Strains, plasmids, and primers used in this work

Strain, plasmid, or primer	Relevant genotype, description, or sequence	Source or reference
Synechocystis strains		
PCC 6803 (wild type)		Laboratory collection
Δ*perR* mutant	PCC 6803 slr1738::Kan^r	This study
Δ*trxA1* mutant	PCC 6803 slr0623::Sp^r	This study
Plasmids		
P1738	*perR* of *Synechocystis* sp. strain PCC 6803 in pGEM-T vector	This study
P0623	*trxA1* of *Synechocystis* sp. strain PCC 6803 in pGEM-T vector	This study
P1738- Kan^r	Kanamycin gene cloned in P1738 plasmid	This study
P0623- Sp^r	Spectinomycin gene cloned in P0623 plasmid	This study
Primers		
slr1738-forward	5′-CCATTCTGAGGGAATAACACTGCC-3′	
slr1738-reverse	5′-ACAGGCAACAGGGATAATCAAGGC-3′	
slr0623-forward	5′-CAGATCGGTCAACTGGGCGTTTGG-3′	
slr0623-reverse	5′-CGCTATGGTCTTACCGACAAAGACC-3′	

representative of a recently identified class of peroxide sensors and acts as a repressor of the OhrA organic peroxide resistance gene (16, 62). Given the wide range of oxidative damage to the cell, it is conceivable that DNA repair, protein degradation, metabolic energy generation, cell division, and other cellular activities are all coordinately regulated. Together, these activities form an oxidative stress response network or stimulon.

Because cyanobacteria are the oldest known organisms that perform oxygenic photosynthesis, their responses to oxidative stresses have been studied extensively. However, until recently, even the identities of the protective enzymes were poorly characterized. Advances in this area were greatly aided by the complete genomic sequences of strains such as *Synechocystis* sp. strain PCC 6803 and *Anabaena* sp. strain PCC 7120, and the occurrence and biochemistry of cyanobacterial hydroperoxidases have been reviewed recently (31, 43, 48, 53). These studies have led to a better understanding of the role of catalase peroxidases (63), peroxiredoxins, glutathione peroxidases (53), and thioredoxin peroxidase (71) in these cyanobacteria. It is now recognized that there is a thioredoxin superfamily (proteins that have a thioredoxin-like fold and that interact with either thiol- or disulfide-containing substrates) that includes six subclasses: glutathione peroxidase, glutathione *S*-transferase, thioredoxin, glutaredoxin, Dsb, and peroxiredoxin (54, 55). In addition, insightful studies of the inhibition by oxidative stress of the repair of photodamage to photosystem II (PSII) have led to the conclusion that peroxide affects the translational machinery and not PSII directly (43).

To identify the genes that are involved with or regulate the anti-oxidative stress and repair system, we performed DNA microarray experiments with the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (50). In this paper, we describe some of the key genes that are differently regulated by hydrogen peroxide. We immediately identified a gene encoding a putative PerR homologue in *Synechocystis* sp. strain PCC 6803 that responded strongly to peroxide stress, and below we outline our current view of the PerR regulon. We also constructed a *perR* knockout mutant and used microarray and physiological analyses to determine how this mutant responded to peroxide stress. We are also interested in redox regulation, and we performed a related analysis with a *Synechocystis* sp. strain PCC 6803 mutant that lacked one of the thioredoxins. We show here that some of the iron-stress-related genes were highly expressed under oxidative stress conditions, indicating that there is some coupling between the oxidative stress response and regulation of iron metabolism. Based on these experiments, we developed a general outline of the peroxide stimulon and the PerR regulon in this photosynthetic phototroph.

MATERIALS AND METHODS

Strains, growth conditions, and stress induction. The bacterial strains and plasmids used in this work are listed in Table 1. *Synechocystis* sp. wild-type strain PCC 6803 and mutants were cultured at 30°C with 40 μmol of photons m^{-2} s^{-1} in a modified BG-11 medium as the basal medium. These growth conditions are referred to below as the control. The cell density of a culture was determined by absorption at 730 nm as previously described (10, 39).

We used microtiter plates in order to analyze the impact of oxidative stress conditions on growth. Cultures of wild-type and mutant strains were grown to the exponential phase (~7×10^7 cells ml^{-1}) or the stationary phase (~2.0×10^8 cells ml^{-1}) and diluted with fresh BG-11 medium to obtain 4×10^7 cells ml^{-1}. Aliquots (0.5 ml) were placed in a 48-well microtiter plate. Different concentrations of H_2O_2 (0, 0.6, 0.9, 1.2, 1.5, and 1.8 mM) or methyl viologen (0, 0.5, 1, 2, 3, and 4 μM; Sigma, St. Louis, Mo.) were added to the wells. The microtiter plates were incubated at 30°C with 40 μmol of photons m^{-2} s^{-1} and shaking for approximately 2 to 3 days. Cell growth was measured in 96-well UV-transparent microplates (BD Biosciences, San Jose, Calif.) by using a $Spectra_{max}$ $Plus^{384}$ (Molecular Devices Corporation, Sunnyvale, Calif.) with 100 μl of cells in each well. The absorbance at 730 nm provided a way to monitor cell density and allowed us to calculate an approximate concentration required for 50% inhibition of the control for each oxidative stress treatment. The final results were recorded by digital photography. Growth experiments were repeated at least three times for each of the parameters measured.

To determine the effect of oxidative conditions on gene expression, cells were grown in BG-11 medium at 30°C until the late logarithmic phase (~1.0×10^8 cells ml^{-1}), 1.5 mM H_2O_2 was added to one-half of the culture, and the cells were harvested after 30 min.

Mutant construction. PCR products that contained *perR* and *trxA1* (Table 1) were cloned into the pGEM-T vector (Promega, Madison, Wis.). Δ*trxA1* was constructed by replacing a 252-bp fragment with the 2.0-kb spectinomycin resistance cassette (from plasmid pRL453), and *trxM1* was inactivated by replacing a 590-bp fragment with the 1.1-kb kanamycin resistance cassette. *Synechocystis* sp. strain PCC 6803 was transformed with these plasmid constructs, and transformants were selected on plates containing antibiotics (40 μg ml^{-1}). Segregation of the mutants was performed under low light (10 μmol of photons m^{-2} s^{-1}) in the presence of 5 mM glucose. Colonies were streaked five times prior to analysis, and complete segregation was confirmed by Southern blot, PCR, and Western blot analyses (data not shown). It was also important to ensure that each result-

ing segregant was based on a single mutation in the gene of interest and that no second-site mutations were present. Therefore, complementation of the single and double mutants was performed, and the complemented strains responded like the wild type (data not shown). Overall, the results demonstrated that there were fully segregated knockout mutations in each of the genes. Although we obtained mutants with knockout mutations of *trxA1* and *trxM1*, we were unable to obtain fully segregated *trxM2* (sll1057), *trxA2* (slr1139), and *trxA3* (sll1980) mutants.

RNA isolation and Northern analysis. Total RNA was extracted and purified by using phenol-chloroform extraction and $CsCl_2$ gradient purification as previously described (52, 59). Five micrograms of total RNA was fractionated by electrophoresis on a 1.0% agarose gel with 0.6 M formaldehyde. RNA was transferred to a nylon membrane and fixed by baking the membrane at 80°C for 2 h in a vacuum oven. The blots were hybridized with α-^{32}P-labeled DNA probes prepared by random primer labeling by using a Ready-To-Go kit (Pharmacia Biotech, Piscataway, N.J.). Hybridization was performed at 42°C with 50% formamide. Staining of the rRNA with ethidium bromide was used to standardize the loading of total RNA.

Microarrays and differential expression experiments. A complete description of array construction has been provided previously by Postier et al. (50). PCR products corresponding to the 3,165 genes identified in the *Synechocystis* genome on the Kazusa website (prior to May 2002) were amplified by using a two-stage process. The experimental loop design is shown in Fig. 1A. The cDNA labeling, glass treatment, prehybridization, and hybridization protocols have been described in detail previously by Singh et al. (58). Biological variation was sampled by extracting RNA from three separate experiments and pooling the RNA prior to hybridization. This strategy has been used in many microarray experiments (1, 47) and simplifies the final analysis.

Data acquisition and statistical analysis. Spot intensities of the images were quantified by using Quantarray 3.0 (Packard BioChip Technologies, Boston, Mass.). Data for the six slides in the Δ*perR* experiment and the eight slides in the Δ*trxA1* experiment were then collated into two data sets (one for each experiment) by using SAS (version 8.02; SAS Institute, Cary, N.C.). Previous testing has demonstrated that the results obtained with Quantarray are reliable and similar to the results obtained with Imagene 6.0 (41). The local background was subtracted from each spot.

For each replicate block on a slide, there were empty spots (422 or 326 spots depending on the specific slide batch), and there were three replicates per slide. We examined the distribution of spot intensities for these empty spots and declared data from a nonempty spot to be detected if the background-corrected intensity of the spot was greater than that for 95% of the empty spots. We examined the data for the Δ*perR* and Δ*trxA1* experiments separately. If all the spots for a given gene were not detected on all the slides in an experiment, then the gene was considered to be off and was not analyzed further (797 genes in Δ*perR* experiments and 864 genes in Δ*trxA1* experiments). We then calculated the log of the background-corrected signals that were normalized to the slide median (the median for all noncontrol spots detected).

Each experiment contained two genotypes (mutant and wild-type controls) and two stimuli (mutant and wild-type peroxide) for a total of four treatment combinations. The effects of the mutant and the peroxide stimulus were examined in an analysis of variance (ANOVA) modeling framework (9, 12, 29, 30, 44, 58, 68, 70). The model $Y_{ijklm} = \mu + t_i + d_j + \omega_k + \omega_k(\rho_l) + \varepsilon_{ijklm}$ was fit, where Y is the intensity of the spot after correction for the local background signal, normalization, and log transformation, μ is the overall mean of the normalized values for the gene, and ω_k and $\omega_k(\rho_l)$ are the random effects of slide and of replicates within a slide, respectively. The fixed effects of treatment (t_i), where $i = (1,...4)$ for each of the four combinations of genotype and peroxide, and dye (d_j), where $j =$ (red, green), were also included in the model. To test the null hypothesis that a gene's expression level was not different across treatments, an F test of the effect of treatment for each gene was conducted, and a P value was calculated. We examined the model for conformation to the assumption of normality of the residuals by testing the null hypothesis that the residuals for each gene were normally distributed by using the Shapiro-Wilkes test. Additional tests comparing the effects of the genotype (mutant versus wild type) and peroxide stimulus (peroxide versus control) and the interaction between the genotype and peroxide stimulus were performed. All analyses were performed with SAS (SAS Institute).

We used a Bonferroni significance level as an initial criterion for rejecting the null hypothesis of a significant treatment effect ($0.05/2{,}368 = 2.11 \times 10^{-5}$ for Δ*perR* and $0.05/2{,}301 = 2.17 \times 10^{-5}$ for Δ*trxA1*). As type I and type II errors are inversely related, with decreases in false positives (type I) associated with increases in false negatives (type II), and as the Bonferroni correction is overly conservative as tests are correlated (11, 38, 69), we used a second nominal threshold of 0.001 to balance type I and type II results, and we bounded our considerations with an overly liberal threshold (0.05). In addition, we considered the test for dye effects and normality of the residuals. If the test of the null hypothesis of difference across times was rejected at the Bonferroni level and we had no evidence for dye effects or departure from normality of the residuals, we declared the gene differentially expressed across treatments and examined the contrasts. If the P value for the test of differences over time was less than or equal to 0.05 but larger than the Bonferroni level and we had no evidence for dye effects or departure from normality of the residuals, we considered the gene interesting. When dye effects were present or residuals showed evidence of departure from normality, we used caution in interpreting the results. Once the analysis was completed, we focused our attention on statistically significant and interesting genes ($P < 0.001$) that exhibited a change of at least 1.4-fold (68). In some cases, we included genes with a P value of <0.05 because they augmented or completed a functional category. Our objective was to identify genes that exhibited differential expression for further experimentation. Thus, we bracketed our interpretation of the results with a conservative (Bonferroni) threshold and a liberal 0.001 criterion, and we used a fold change filter to focus our efforts. The raw P values are shown in Table S1 in the supplemental material.

RESULTS

Array data and statistical analysis. The effects of H_2O_2 on gene expression in the wild-type strain and two mutants of *Synechocystis* sp. strain PCC 6803 were monitored by using full genome microarrays printed in triplicate on each slide. Microarray experiments were performed as previously described (58) by using the loop design, as shown in Fig. 1A. For each RNA sample, either eight slides (Δ*trxA* experiment) or six slides (Δ*perR* experiment) were used for hybridization; since each slide contained three replicates, 12 and 9 measurements per gene, respectively, could be used for the statistical analysis. The loop design allowed comparison of all conditions by using the ANOVA model (9, 58, 72). We could analyze the effect of peroxide on the wild type, the effect of peroxide on the mutant, the effect of peroxide on the specific genotype used in the loop, and the relationship between the wild type and each mutant. To obtain the results presented below, we used 1.5 mM H_2O_2 for 30 min to elicit stress, although we investigated the effects of peroxide concentrations ranging from 75 μM to 3 mM and times as short as 15 min. We found that 75 μM H_2O_2 for 15 min was sufficient to result in changes in expression of some of the genes but that other genes exhibited a temporal response to the stress and that it took some time for the amounts to increase or decrease (Li, Singh, and Sherman, data not shown).

The scatter plot in Fig. 1B shows the relationship between the average hybridization intensities of the wild-type control cells and cells that were treated with peroxide. This simple procedure provided an overview of the data and indicated that most of the spots fell along the diagonal and were equally labeled. The spots that were not on the diagonal were candidates for genes with expression changes, and lines indicating twofold and threefold changes are shown in Fig. 1B. In some of the previous microarray analyses the workers used an arbitrary cutoff of a twofold change to identify differentially expressed genes. However, it has been shown that changes in gene expression less than a twofold change can be reliably identified (1, 26, 35, 44, 72, 74), and this aspect has been discussed in detail previously (58). We selected genes for additional consideration using a significance level of 0.05 from the full ANOVA model (see Table S1 in the supplemental material) and at least a 1.4-fold change in transcript level intensity.

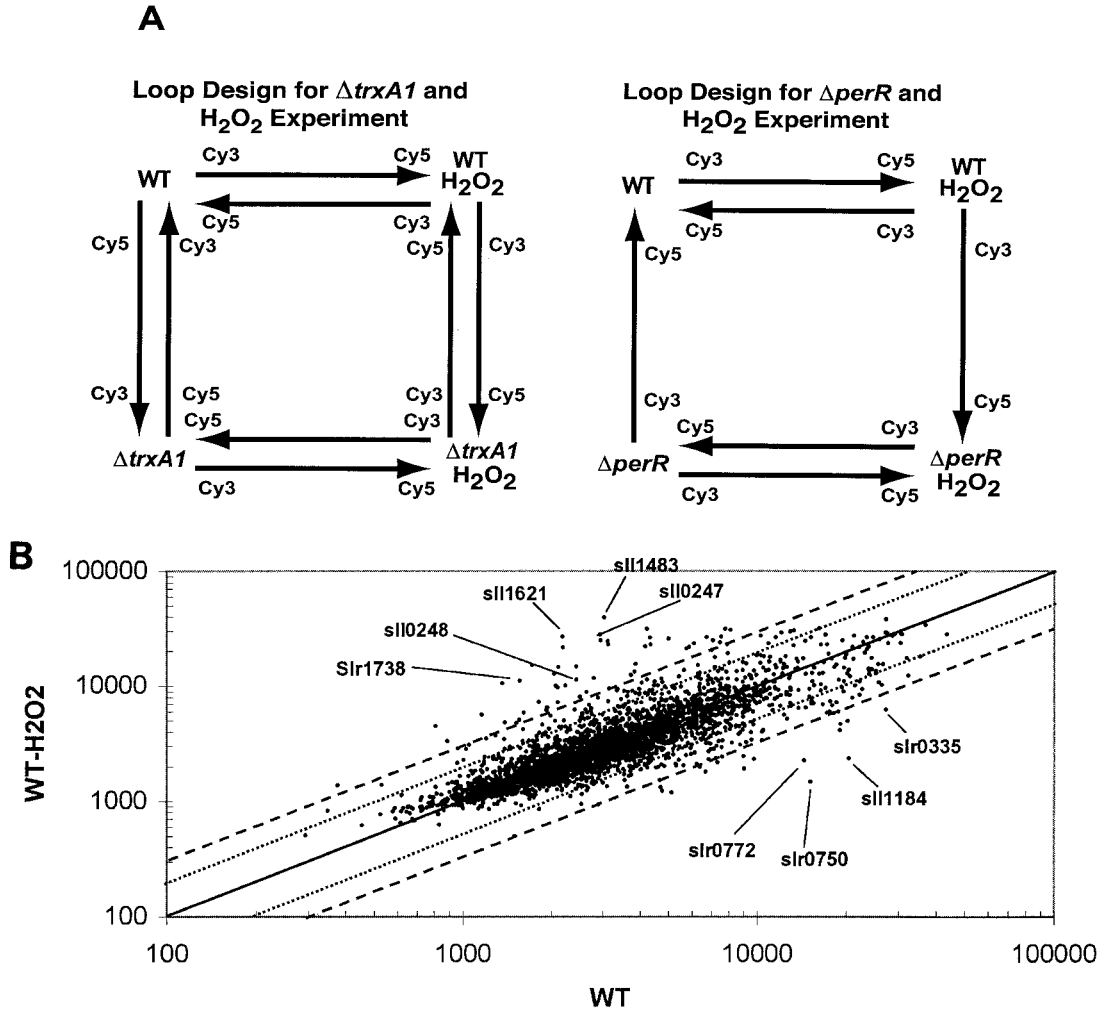

FIG. 1. (A) Diagrammatic representation of the loop designs utilized for identification of differentially expressed genes in response to peroxide stress. A total of eight slides were used with dye swaps for the experiment with and without peroxide with the wild type and the Δ*trxA1* mutant (left side), and six slides were used for the related experiment with the Δ*perR* mutant and the wild type (right side). (B) Scatter plot comparing the mean spot intensities of wild-type *Synechocystis* sp. strain PCC 6803 in the absence (*x* axis) and in the presence (*y* axis) of 1.5 mM H_2O_2. Data were normalized, and the mean signal intensities were plotted. The solid line indicates equal labeling for the two samples, whereas the dotted line (twofold) and the dashed line (threefold) identify genes that exhibited large labeling differences during the hybridization experiment. WT, wild type.

Genes differentially regulated by peroxide stress: the peroxide stimulon. The effect of peroxide on gene expression based on the functional categories defined in Cyanobase is shown in Table 2. Table 2 also shows the differences in expression among the wild type and the Δ*trxA1* and Δ*perR* mutants. In all three experiments, approximately 18 to 20% of all genes showed some evidence of changes in levels of transcript accumulation that were at least 1.4-fold ($P < 0.05$). The use of a liberal filter, along with the requirement for similar significance in both experiments, provided a comprehensive overview of the impact of peroxide on gene expression for the different functional categories. We analyzed the statistically significant genes ($P < 0.001$) in detail, focusing particularly on the genes whose statistical significance was below the Bonferonni threshold. There were 158 and 198 genes in the Δ*perR* and Δ*trxA1* experiments, respectively, whose *P* values were under the Bonferonni threshold; for 103 of the 158 genes in the PerR experiment the difference also met the most stringent criterion in the TrxA experiment, and most of the other 55 genes had a *P* value of <0.001 (Table 3).

When the effect of peroxide were examined, in some categories, such as central intermediary metabolism and DNA replication and related processes, few genes were affected, whereas more than one-third of the genes encoding photosynthesis and respiration proteins and cellular processes were affected. In the case of categories such as photosynthesis or biosynthesis of cofactors, most of the differentially expressed genes were in specific functional categories involved in pigment-protein complexes (Table 3). In most cases, there were few differences between the levels in the mutants and the levels in the wild type. However, there were a few significant exceptions. Many fewer genes involved in energy metabolism were affected in the Δ*trxA1* mutant than in the wild type and the Δ*perR* mutant. Conversely, the number of regulatory genes

TABLE 2. Functional categories of peroxide-responsive genes in *Synechocystis* sp. strain PCC 6803 and two mutants[a]

General pathway	No. of genes	No. of differentially regulated genes in:		
		Wild type	Δ*perR* mutant	Δ*trxA1* mutant
Amino acid biosynthesis	97	21	20	15
Biosynthesis of cofactors, prosthetic groups, and carriers	124	32	34	24
Cell envelope	67	9	14	12
Cellular processes	76	28	26	26
Central intermediary metabolism	31	7	8	6
DNA replication, restriction, recombination, and repair	60	8	7	10
Energy metabolism	132	29	31	15
Hypothetical	1,076	189	148	160
Other categories	306	74	46	85
Photosynthesis and respiration	141	63	50	47
Purines, pyrimidines, nucleosides, and nucleotides	41	3	5	7
Regulatory functions	146	28	24	39
Transcription	30	8	8	10
Translation	168	37	41	32
Transport and binding proteins	196	39	37	28
Unknown	474	79	75	67
Total	3,165[b]	654	574	583

[a] Genes were considered differentially regulated when the *P* value was <0.05 and the change was >1.4-fold.
[b] Total number of genes based on Kazusa annotation prior to May 2002.

affected by peroxide was greater in the Δ*trxA1* mutant than in the other two strains. Finally, many fewer genes categorized as "other" showed differential expression in the Δ*perR* mutant compared to the expression in the wild type and the Δ*trxA1* mutant. It should be noted that the total numbers of differentially expressed genes in these experiments were quite similar; these findings indicate the reproducibility and correspondence of the data.

One objective of this study was to determine the genes that were most responsive to peroxide stress in our wild-type strain, and we identified some of these key genes. A gene with one of the greatest increases in transcript level in every experiment was *isiA*, which encoded a Chl protein that was also highly induced by low-iron conditions (58). In the wild type, this gene was induced 11.3-fold ($P = 1 \times 10^{-12}$). The adjacent genes *isiB* (4.7-fold; $P = 4 \times 10^{-8}$) and sll0249 (2.2-fold; $P = 2 \times 10^{-5}$), were also upregulated (Table 3), which was also observed under low-iron conditions (58). For convenience, we placed all three of these genes in the PSI group in Table 3, although the functions of all three genes remain to be clarified. When cells were treated with 75 μM H_2O_2, the *isiAB* operon was still the most peroxide-responsive operon, although the transcript levels did not increase as much when cells were stressed with a lower peroxide concentration (data not shown). Another highly responsive gene, *sigD* (sll2012), encoding a group 2 sigma factor, was induced eightfold ($P = 9 \times 10^{-11}$). In addition, the slr1738 gene was upregulated sevenfold ($P = 2 \times 10^{-8}$). Analysis of the protein sequence encoded by this gene indicated that the protein is very closely related to the PerR protein that was first identified in *B. subtilis* as a repressor inactivated by peroxide (reference 20 and references therein). Therefore, we designated this gene *perR* in *Synechocystis* sp. strain PCC 6803. Some of the other highly upregulated genes are shown in Table 3. The effects of peroxide stress on Δ*perR* and Δ*trxA1* are also shown in Table 3. Most of the key genes were induced to different degrees in the absence of TrxA1, and the differences are discussed below. In addition, several gene clusters in which all genes were either upregulated or downregulated in the wild type were identified from the microarray results, as described by Singh et al. (57).

A selected subset of the genes that were either up- or downregulated by peroxide are listed by functional category in Table 3. The entire data set is shown in Table S1 in the supplemental material, which includes the fold changes and the *P* values from the ANOVA analysis. Some of the more important categories are discussed below.

Pigment and photosynthesis genes. Peroxide stress led to large changes in the transcript levels of genes encoding components of the photosynthetic apparatus. This was especially noticeable for genes involved in pigment biosynthesis and for genes associated with phycobilisome synthesis and assembly; the transcript levels of these genes were decreased substantially by peroxide stress. The largest changes were in the genes associated with the conversion of divinyl protochlorophyllide *a* to monovinyl protochlorophyllide *a*, and the genes encoding protochlorophyllide reductase (slr0749, slr0750, and slr0772) were downregulated approximately four- to ninefold (see Table S2 in the supplemental material for a comparison of changes due to growth under low-iron conditions with changes due to peroxide stress in the heme and pigment biosynthesis pathways). The transcript levels of the sll1214 and sll1874 genes, both of which exhibit strong homology to *Arabidopsis* CHL27, also decreased approximately two- to threefold (see Table S2 in the supplemental material). CHL27 is required for synthesis of protochlorophyllide and is a prime candidate for the cyclase in chlorophyll biosynthesis (64). The transcript levels of the genes encoding phycobilisome components decreased approximately two- to threefold. The one gene in this category whose transcript level increased was the gene encoding the phycobilisome degradation protein, NblA (ssl0453); in this case the transcript level increased approximately fourfold. The transcript levels of the structural genes of the photosynthetic and respiratory apparatus did not change greatly during the short peroxide stress. The obvious exception was *isiA*,

TABLE 3. Selected differentially regulated genes of the peroxide stimulon in *Synechocystis* sp. strain PCC 6803 and Δ*perR* and Δ*trxA1* mutants[a]

Gene	Function	*P*(treatment)	Fold change[b]		
			Wild type	Δ*perR* mutant	Δ*trxA1* mutant
Biosynthesis of cofactors, prosthetic groups, and carriers					
Carotenoid					
slr1254	Phytoene dehydrogenase (phytoene desaturase)	2.0E-05	2.2	2.5	2.4
Cobalamin, heme, phycobilin, and porphyrin					
sll1184	Heme oxygenase (*ho1*)	1.0E-10	−7.7	−5.4	−5.0
slr0772	Light-independent protochlorophyllide reductase subunit (*chlB*)	3.0E-10	−6.3	−6.5	−4.0
slr0750	Light-independent protochlorophyllide reductase subunit (*chlN*)	9.0E-10	−9.2	−6.8	−3.8
slr0749	Light-independent protochlorophyllide reductase subunit (*chlL*)	1.0E-09	−4.5	−3.8	−1.8
sll1091	Geranylgeranyl hydrogenase (*chlP*)	6.0E-07	−3.2	−2.2	−1.6
sll1185	Coproporphyrinogen III oxidase, aerobic (*hemF*)	3.0E-05	−2.2	−1.7	−2.2
sll1876	Coproporphyrinogen III oxidase, anaerobic (*hemN*)	1.0E-04	−2.7	−2.6	−3.5
slr0839	Ferrochelatase (*hemH*, *scpA*)	4.0E-04	1.3	1.6	1.9
sll1685	Light-induced Na^+-dependent proton extrusion (*pxcA*, *cotA*)	1.0E-03	−1.4	−1.4	−1.3
slr1055	Mg protoporphyrin IX chelatase subunit H (*chlH*)	3.0E-03	−1.5	−1.8	−2.1
slr0905	Mg-protoporphyrin IX ester oxidative cyclase (*bchE*)	9.0E-03	−1.8	−1.8	−2.7
Thioredoxin, glutaredoxin, and glutathione					
slr0623	Thioredoxin (*trxA1*)	2.0E-06	−2.1	−2.4	−1.5
ssr0330	Ferredoxin-thioredoxin reductase, variable chain (*ftrV*)	8.0E-06	−3.1	−2.1	−2.0
slr1846	Hypothetical protein (*ycf64*)	5.0E-05	1.6	1.6	1.4
sll1057	Thioredoxin M (*trxM2*)	7.0E-04	−1.8	−1.8	−2.9
Cellular processes					
Cell division					
slr0228	Cell division protein FtsH (*ftsH*)	1.0E-08	4.5	4.6	2.4
slr1604	Cell division protein FtsH (*ftsH*)	8.0E-06	2.8	2.3	2.8
sll1633	Cell division protein FtsZ (*ftsZ*)	1.0E-03	−2.0	−1.7	−2.0
slr1267	Cell division protein FtsW (*ftsW*)	5.0E-03	−1.3	−1.6	−2.0
Cell killing					
slr1747	Cell death suppressor protein Lls1 homolog	1.0E-06	4.1	3.2	4.0
Chaperones and chemotaxis[c]					
sll1533	Twitching mobility protein (*pilT2*)	4.0E-08	−2.5	−3.9	−3.5
sll0041	Phytochrome-like photoreceptor (*pixJ1*, *pisJ1, taxD1*)	2.0E-05	2.7	2.0	2.1
slr1929	Type 4 pilin-like protein (*pilA6*)	6.0E-05	−2.0	−2.1	−2.5
slr1456	*gspG* or *pilA4*	3.0E-06	2.0	2.0	3.1
slr2015	Type 4 pilin-like protein, essential for motility (*pilA9*)	5.0E-03	1.6	1.1	1.5
slr1930	Type 4 pilin-like protein (*pilA7*)	1.7E-02	−1.7	−1.3	−2.0
slr1928	Type 4 pilin-like protein (*pilA5*)	3.4E-02	−1.4	−1.2	−2.6
Detoxification					
slr1516	Superoxide dismutase (*sodB*)	3.0E-06	1.9	1.9	2.3
sll1615	Thiophen and furan oxidation protein	7.0E-03	1.8	1.6	1.8
Energy metabolism: fatty acid, phospholipid, and sterol metabolism					
sll0330	Sepiapterine reductase	6.0E-12	8.4	4.0	4.5
sll1655	Similar to biotin (acetyl coenzyme A carboxylase) ligase	6.0E-11	−1.6	−2.4	−1.4
slr1020	Sulfolipid biosynthesis protein SqdB (*sqdB*)	3.0E-05	−2.1	−2.3	−2.0
slr1672	Glycerol kinase (*glpK*)	5.0E-05	−2.5	−2.4	−2.2
Hypothetical					
slr1687	Hypothetical protein	9.0E-12	11.0	4.4	2.7
sll1483	Periplasmic protein	2.0E-11	15.3	14.4	6.5
sll2013	Hypothetical protein	4.0E-11	6.4	5.7	4.1

Continued on following page

TABLE 3—*Continued*

Gene	Function	*P*(treatment)	Fold change[b]		
			Wild type	Δ*perR* mutant	Δ*trxA1* mutant
slr0270	Hypothetical protein	3.0E-10	8.6	5.1	4.2
sll1620	Hypothetical protein	4.0E-10	5.8	−1.3	4.7
slr1215	Hypothetical protein	7.0E-10	4.4	4.5	2.5
sll0185	Hypothetical protein	1.0E-09	4.9	4.4	2.4
slr0967	Hypothetical protein	2.0E-09	5.4	5.6	2.1
slr1235	Hypothetical protein	3.0E-09	2.6	2.5	1.9
slr0888	Hypothetical protein	4.0E-09	−2.6	−5.5	−2.4
sll0939	Hypothetical protein	5.0E-09	6.4	4.8	3.6
Other categories					
Adaptations and atypical conditions					
sll0947	Light-repressed protein A homolog (*lrtA*)	6.0E-06	−2.6	−3.6	−1.6
ssl2542	HliA, CAB/ELIP/HLIP superfamily (*hliA*, *scpC*)	2.0E-05	2.8	1.6	2.0
ssr1789	CAB/ELIP/HLIP-related protein HliD (*hliD*, *scpE*)	1.3E-02	1.6	1.4	2.0
Drug and analog sensitivity					
sll0086	Putative arsenical pump-driving ATPase	3.0E-08	2.4	2.2	2.0
slr0946	Arsenate reductase (*arsC*)	7.0E-08	3.5	3.2	2.7
sll1159	Probable bacterioferritin comigratory protein	1.0E-07	2.8	5.2	5.9
slr1198	Antioxidant protein	9.0E-05	−2.0	−2.0	−1.6
sll1154	Putative antibiotic efflux protein	6.0E-04	2.1	1.5	1.4
Other					
sll1621	AhpC-peroxiredoxin	5.0E-14	13.1	−1.0	2.3
slr0381	Lactoylglutathione lyase	1.0E-10	8.3	4.5	2.7
slr0298	FraH protein homolog	7.0E-08	9.4	5.1	3.1
sll1534	Probable glycosyltransferase	3.0E-07	−3.6	−4.6	−2.7
sll1245	Cytochrome *cM* (*cytM*)	2.0E-06	−2.5	−2.0	−1.6
slr2094	Fructose-1,6-sedoheptulose-1,7-bisphosphatase (*fbpl*)	4.0E-06	−1.9	−2.5	1.1
slr1942	Circadian clock protein KaiC homolog (*kaiC3*)	7.0E-05	−2.4	−2.2	−2.1
Photosynthesis and respiration					
NADH dehydrogenase					
slr1291	NADH dehydrogenase subunit 4 (*ndhD2*)	3.0E-08	4.2	2.9	6.4
slr0331	NADH dehydrogenase subunit 4 (*ndhD1*)	7.0E-05	−1.7	−2.2	−1.6
PSI					
sll0247	Iron stress chlorophyll-binding protein (*isiA*)	1.0E-12	11.3	9.9	5.8
sll0248	Flavodoxin (*isiB*)	4.0E-08	4.7	3.8	4.9
sll0249	Hypothetical	2.0E-05	2.2	3.9	3.3
ssr2831	PSI subunit IV (*psaE*)	1.0E-05	−1.9	−1.8	−1.3
ssl0563	PSI subunit VII (*psaC*)	7.0E-05	−1.9	−2.5	−2.3
PSII					
slr1181	PSII D1 protein (*psbA1*)	1.0E-05	1.6	1.6	1.2
ssl2598	PSII PsbH protein (*psbH*)	7.0E-05	−1.7	−1.7	1.1
sml0003	PSII reaction center M protein (*psbM*)	1.0E-04	2.2	1.6	2.1
slr1739	PSII 13-kDa protein homolog (*psb28-2*)	2.0E-02	1.7	1.1	1.7
sml0005	PSII PsbK protein (*psbK*)	2.1E-02	−1.6	−1.5	−1.9
Phycobilisome					
slr0335	Phycobilisome core membrane linker protein (*apcE*)	4.0E-10	−4.5	−4.6	−2.1
slr2067	Allophycocyanin alpha subunit (*apcA*)	1.0E-09	−4.4	−3.1	−2.0
sll0928	Allophycocyanin B (*apcD*)	2.0E-07	−1.8	−1.6	−1.7
slr1986	Allophycocyanin beta subunit (*apcB*)	3.0E-07	−2.9	−3.0	−1.5
ssl0453	Phycobilisome degradation protein NblA (*nblA2*)	4.0E-07	4.3	3.3	4.0
sll1579	Phycobilisome rod linker polypeptide (*cpcC2*)	1.0E-05	−2.0	−2.0	−2.5
sll1577	Phycocyanin beta subunit (*cpcB*)	2.0E-05	−3.0	−2.3	−1.4
sll1471	Phycobilisome rod-core linker polypeptide (*cpcG2*)	5.0E-05	−2.3	−1.4	−2.0
sll1580	Phycobilisome rod linker polypeptide (*cpcC1*)	9.0E-05	−2.0	−2.3	−1.8
slr1459	Phycobilisome core component (*apcF*)	2.0E-04	−1.3	−2.0	−2.7

Continued on following page

TABLE 3—*Continued*

Gene	Function	*P*(treatment)	Fold change[b]		
			Wild type	Δ*perR* mutant	Δ*trxA1* mutant
ssr3383	Phycobilisome small core linker polypeptide (*apcC*)	3.0E-04	−1.5	−1.6	−1.2
ssl0452	Phycobilisome degradation protein NblA (*nblA1*)	8.0E-03	1.4	1.3	−1.7
slr1878	Phycocyanin alpha-subunit phycocyanobilin lyase	2.8E-02	−1.5	−1.3	−1.9
Soluble electron carriers					
ssl3044	Probable ferredoxin	1.0E-04	2.7	1.5	4.4
slr1828	Ferredoxin, PetF-like protein (*petF*, *fdx*)	2.0E-04	−1.5	−1.6	−2.0
slr1643	Ferredoxin-NADP oxidoreductase (*petH*)	3.0E-04	−1.9	−2.2	−1.8
ssr3184	4Fe-4S-type iron-sulfur protein	5.0E-04	−1.6	−1.5	−1.6
slr0150	Ferredoxin, PetF-like protein (*petF*, *fdx*)	8.0E-04	−1.5	−1.7	−2.3
ssl0020	Ferredoxin I, essential for growth (*petF*)	3.0E-03	−1.9	−1.7	−1.5
sll0199	Plastocyanin (*petE*)	5.0E-03	−1.6	−1.4	−1.5
sll1382	Ferredoxin, PetF-like protein (*petF*, *fdx*)	2.0E-02	−1.4	−1.6	−2.0
Regulatory functions					
slr1738	Transcription regulator, Fur family	2.0E-08	7.0	1.3	4.0
sll1161	Probable adenylate cyclase	2.0E-06	3.4	3.7	2.9
sll1742	Transcription antitermination protein (*nusG*)	3.0E-06	1.3	1.9	2.0
slr1214	Two-component response regulator, PatA subfamily	1.0E-05	2.2	2.3	1.5
sll1626	LexA repressor	2.0E-05	−2.3	−2.3	−1.5
slr0474	Regulator for phytochrome 1 (Cph1) (*rcp1*)	2.0E-05	−1.8	−2.8	−1.9
sll1005	MazG protein homolog	7.0E-05	−1.9	−1.8	−1.8
slr0152	Serine/threonine protein kinase	9.0E-05	−2.0	−2.0	−1.3
sll0998	LysR family transcriptional regulator (*ycf30*)	9.0E-05	−1.3	−2.2	−2.0
sll0789	Two-component response regulator, OmpR subfamily	1.0E-04	1.8	1.8	−1.1
sll1672	Two-component hybrid sensor and regulator (*hik12*)	1.0E-04	−1.7	−1.6	−1.6
sll2014	Sugar fermentation stimulation protein	2.0E-04	1.8	2.5	3.0
sll0790	Two-component sensor histidine kinase (*hik31*)	6.0E-04	−2.1	1.2	−1.6
slr1760	Two-component response regulator	1.0E-03	−1.3	−1.3	−3.5
sll1544	Two-component response regulator, NarL subfamily	2.0E-03	2.0	1.8	3.2
sll1329	Inositol monophosphate family protein	4.0E-03	1.3	1.3	2.2
sll1353	Two-component sensor histidine kinase (*hik15*)	5.0E-03	−1.4	−1.5	−2.6
slr0081	Two-component response regulator, OmpR subfamily	5.0E-03	−1.3	−1.4	−1.4
slr1584	Two-component transcription regulator, OmpR subfamily	7.0E-03	−1.6	−1.4	−1.8
sll0750	KaiC-interacting protein (*hik8*, *sasA*)	1.1E-02	1.8	1.7	−2.1
sll1871	Two-component system sensory histidine kinase (*hik6*)	1.3E-02	−1.7	−1.2	−2.1
slr1414	Two-component sensor histidine kinase (*hik11*)	1.7E-02	1.6	1.4	2.2
slr2099	Two-component hybrid sensor and regulator (*hik40*)	2.1E-02	−1.5	−1.2	−1.5
sll1292	Two-component response regulator, CheY subfamily	2.8E-02	−1.3	−1.4	−2.0
Transcription: RNA synthesis, modification, and DNA transcription					
sll2012	Group2 RNA polymerase sigma factor (*sigD*)	9.0E-11	7.9	5.5	5.7
sll0306	RNA polymerase group 2 sigma factor (*sigB*)	3.0E-08	2.7	3.0	1.7
sll1818	RNA polymerase alpha subunit (*rpoA*)	6.0E-06	−1.7	1.8	1.4
sll1689	Group2 RNA polymerase sigma factor (*sigE*)	2.0E-04	1.2	−1.1	1.6

Continued on following page

TABLE 3—*Continued*

Gene	Function	*P*(treatment)	Fold change[b]		
			Wild type	Δ*perR* mutant	Δ*trxA1* mutant
sll1787	RNA polymerase beta subunit (*rpoB*)	3.0E-03	−1.6	−1.3	1.3
sll0184	Group2 RNA polymerase sigma factor (*sigC*)	4.0E-03	1.7	1.7	2.4
Translation: degradation of proteins, peptides, and glycopeptides					
slr0008	Carboxyl-terminal processing protease (*ctpA*)	5.0E-06	2.0	2.3	2.1
slr1204	Protease	1.0E-04	2.6	1.2	3.0
slr0156	ClpB protein (*clpB2*)	2.0E-03	1.5	1.7	1.5
slr1641	ClpB protein (*clpB1*)	4.4E-02	1.4	1.5	4.8
Transport and binding proteins					
slr0513	Iron transport system substrate-binding protein	6.0E-10	2.4	5.7	4.2
slr0447	ABC-type urea transport system (*urtA*)	2.0E-09	−2.6	−2.2	−1.9
sll0771	Glucose transport protein (*glcP,gtr*)	1.0E-07	−3.8	−3.7	−1.4
slr1740	Oligopeptide binding protein of ABC transporter	4.0E-07	3.2	2.6	3.9
Unknown					
slr1544	Unknown protein	1.0E-09	9.1	6.9	6.1
sll1135	Unknown protein	7.0E-09	7.7	3.3	5.6
sll1830	Unknown protein	2.0E-08	−3.6	−4.9	−2.5
slr0108	Unknown protein	3.0E-08	3.7	4.2	2.8
slr1484	Unknown protein	6.0E-08	3.3	4.9	4.7
slr1855	Unknown protein	9.0E-08	−1.9	−2.6	−1.6
sll1515	Glutamine synthetase-inactivating factor IF17 (*gifB*)	3.0E-07	4.5	2.6	3.1
sll1009	Unknown protein	6.0E-07	−2.5	−2.7	−1.8
slr1854	Unknown protein	2.0E-06	−2.4	−1.9	−2.7
slr0572	Unknown protein	3.0E-06	−3.0	−2.5	−2.2
sll1851	Unknown protein	7.0E-06	3.0	1.4	1.3
sll0923	Unknown protein	1.0E-05	3.9	2.7	6.6

[a] Genes were considered differentially regulated when the *P* value was <0.05 and the change was >1.4-fold. The genes are listed in descending order of *P*(treatment) within each category.

[b] The changes for the wild type and the Δ*perR* and Δ*trxA1* mutants were calculated by dividing the normalized mean intensities of peroxide-treated strains by the normalized mean intensities of nontreated strains.

[c] See Table 5.

which was the most peroxide sensitive of all of the genes. The only other gene in this category whose transcript level increased significantly was slr1291 (*ndhD2*), which encodes a component of the NADH dehydrogenase complex; the transcript level of this gene increased approximately fivefold. The other major changes in transcription involved genes encoding the soluble electron transfer components, such as ferredoxins and plastocyanin, all of whose transcript levels decreased approximately twofold.

Detoxification genes. Peroxide stress induced expression of a number of genes thought to be involved in detoxification. The main gene in this category was the peroxiredoxin gene, *aphC* (sll1621; ~13-fold induction; $P = 5 \times 10^{-14}$). Expression of another putative peroxiredoxin gene, sll1159, was also induced (approximately threefold; $P = 1 \times 10^{-7}$). This was part of a four-gene cluster whose members were all strongly expressed in the presence of peroxide and that included a gene encoding a putative adenyl cyclase (sll1161) (57). Two other detoxification genes were upregulated in both experiments; expression of both slr1516 (*sodB*) and sll1615 was induced approximately twofold. Other genes that may belong in this category that were upregulated by peroxide included *arsC* (slr0946; arsenate reductase), whose expression was induced approximately threefold, and a series of genes encoding flavoproteins and putative oxidoreductases (Table 3). Interestingly, the two genes normally associated with peroxide scavenging, *katG* (sll1987) and *tpx* (sll0755), were not significantly upregulated under these oxidative stress conditions (63).

Thioredoxin and redox regulation genes. One of the objectives of our experiments was to map the functions of the different thioredoxins that have an impact on oxidative stress. Thioredoxins are small disulfide-containing redox proteins that serve as general protein disulfide oxidoreductases (54). We hypothesized that defects in a thioredoxin and the resulting inability to form disulfide bonds could have a negative impact on the cellular response to oxidative stress. The thioredoxin gene superfamily in *Synechocystis* sp. strain PCC 6803 is comprised of at least six genes, and attempts to construct mutants with mutations in some of these genes have been unsuccessful (42). The effects of the oxidizing agents H_2O_2 and methyl viologen on Δ*trxA1*, Δ*trxM1*, and other mutants used in this study are shown in Table 4. The Δ*trxA1* mutant was sensitive to both peroxide and methyl viologen, whereas the Δ*trxM1* mutant was sensitive only to methyl viologen. The response of the Δ*perR* mutant was similar to that of the wild type, whereas the Δ*mrgA* (slr1894) mutant was highly sensitive to peroxide but not to methyl viologen.

Figure 1A shows the experimental loop design utilized to

TABLE 4. Effects of peroxide and methyl viologen on the exponential-phase growth of *Synechocystis* sp. wild-type strain PCC 6803 and mutants

Strain	Concn of[a]:	
	H_2O_2 (mM)	Methyl viologen (μM)
Wild type	1.5 ± 0.6	2.0 ± 0.5
Δ*trxA1* mutant	1.0 ± 0.5	1.0 ± 0.25
Δ*trxM1* mutant	1.5 ± 0.6	1.0 ± 0.25
Δ*perR* mutant	1.5 ± 0.6	2.0 ± 0.5
Δ*mrgA* mutant	0.075 ± 0.025	2.0 ± 0.5

[a] The values are the concentrations that permitted cellular growth that was ~50% of the growth of the untreated controls. The values are means ± standard deviations ($n \geq 6$).

study the effect of peroxide stress in the Δ*trxA1* and Δ*perR* mutants. In general, the individual genes responded to peroxide qualitatively in the same way in the Δ*trxA1* mutant as in the wild type, but the changes in the Δ*trxA1* strain were generally smaller quantitatively. This was particularly true of genes encoding energy metabolism proteins, as shown in Table 3. In addition, the transcript levels of some other thioredoxin genes were decreased upon peroxide stress; the transcript levels of *trxA1* (slr0623) and *trxM2* (sll1057) both decreased approximately twofold. At the same time, three genes that have not been studied much but that appear to encode thioredoxin-like proteins were induced; these genes were slr1846 (ycf64), ssl2667 (*cnfU*), and sll0621 (*dsbD*-like). More importantly, the latter two genes appeared to be under control of PerR (see below).

Cellular processes, including chaperones: defining the heat shock regulon. The chaperones represented one of the most prominent of the categories with many induced genes. We also analyzed the effect of heat shock on gene expression in *Synechocystis* sp. strain PCC 6803, and the responses of the chaperone genes to heat shock are shown in Table 5. The results of the heat shock experiment, in which cells were shifted from 35 to 45°C for 15 min, were analyzed on microarrays by using a separate loop. The experiment was performed with a mutant with a mutation in *sigB*, which may encode the heat shock sigma factor in *Synechocystis* sp. strain PCC 6803 (data not shown). From the results, it is evident that many stress-related genes were induced upon peroxide stress and that these genes in the peroxide stimulon were indirectly regulated by the specific stress.

In addition to the chaperones, a number of genes coding for cellular processes were up- or downregulated by peroxide. Especially prominent were two of the *ftsH* genes (slr0228 and slr1604), both of which were upregulated approximately two- to fourfold. Conversely, the *ftsZ* (sll1633) and *ftsW* (slr1267) genes were downregulated approximately twofold. In addition, genes involved with pilins or motility were strongly downregulated (sll1533 and slr1928 to slr1931), as were many genes involved with the synthesis of surface polysaccharides (see Table S1 in the supplemental material). Also, transcription of the *secDF* genes and the leader peptidase gene increased after peroxide stress (see Table S1 in the supplemental material).

Regulatory genes. The regulatory genes provided the richest panorama of changes in transcript levels of all the functional categories. The transcript levels of approximately 40 genes involved in regulatory and transcriptional activities were changed in the Δ*trxA1* mutant experiments, and the transcript levels of 24 of these genes were changed in both experiments (Table 3). The transcript levels of most of the regulatory genes decreased, whereas the transcript levels of one-half or more of the RNA synthesis genes increased (especially in the Δ*trxA1* mutant). Ten of the regulatory genes that displayed differential expression were genes encoding histidine kinases, and the transcript levels of all but one of these genes decreased; the transcript level of hik11 (slr1414) increased 1.4- to 2.2-fold in the two experiments. Marin et al. (37) demonstrated that Hik41 (sll1229) was involved in the response of *Synechocystis* sp. strain PCC 6803 to salt stress. The Hik41gene was upregulated (~2.5-fold) in the Δ*perR* mutant experiment, as were slr0967 (~5.5-fold), sll0939 (~5.5-fold), and sll0938 (~1.7-fold), the three genes shown to be under the control of Hik41 in response to salt stress. The genes encoding two group 2 sigma factors were strongly upregulated by peroxide in both experiments; these genes were *sigD* (sll2012; 8-fold upregulation; $P = 9 \times 10^{-11}$) and *sigB* (sll0306; ~2.7-fold upregulation; $P = 3 \times 10^{-8}$).

Putative PerR regulon in *Synechocystis* sp. strain PCC 6803. We used three criteria to tentatively determine the PerR regulon: the presence of a potential PerR binding motif, the effect

TABLE 5. Chaperone genes in *Synechocystis* sp. wild-type strain PCC 6803 and Δ*perR* and Δ*trxA1* mutants that are differentially regulated by peroxide stress and high temperature

Gene	Function	*P*(treatment)[a]	Fold change			
			Wild type	Δ*perR* mutant	Δ*trxA1* mutant	Heat shock[b]
sll1514	16.6-kDa small Hsp	8.0E-12	5.9	5.0	2.9	5.1
sll1666	DnaJ-like protein	5.0E-10	5.1	3.8	3.5	
slr2075	10-kDa chaperonin (*groES*)	6.0E-10	3.6	3.5	3.8	8.7
sll0416	60-kDa chaperonin 2 (*groEL2*)	2.0E-08	4.0	2.5	6.1	11.6
sll0430	HtpG, Hsp90	4.0E-08	2.7	2.9	3.5	9.9
slr2076	60-kDa chaperonin (*groEL1*)	5.0E-06	3.2	2.4	2.1	8.2
sll0897	DnaJ protein, Hsp40	3.0E-04	1.8	1.8	2.9	2.2
sll0170	DnaK protein 2, Hsp70	7.0E-04	1.5	2.3	3.3	5.6
slr0093	DnaJ protein, Hsp40	5.0E-03	2.5	1.5	2.5	4.0
sll0058	DnaK protein 1, Hsp70	1.3E-02	1.4	1.2	1.4	

[a] *P*(treatment) is the loop design for the Δ*perR* experiment.
[b] In the heat shock experiment, the change after a shift from 35 to 45°C for 15 min was examined. All *P* values for this experiment were <0.05.

TABLE 6. Putative genes that may be under PerR control

Cyano designation	Gene name	Putative *perR* box	Position (bp)[a]	Function	Fold change[b]			*P*(genoperoxide)
					Wild type	Δ*perR* mutant	Δ*trxA1* mutant	
slr1738	*perR*	ATAATTATTCTATCTAATA	−186	Peroxide regulon repressor	7.0	1.3	4.0	4.0E-07
sll1621	*ahpC*	ATAATTATTCTATCTAATA	−150	Alkyl hydroperoxide reductase	13.1	1.0	2.3	5.0E-13
sll1620				Unknown	5.8	1.0	4.7	7.0E-07
slr1739	*psbW*-like			Unknown	1.7	1.0	1.7	6.0E-02
ssl2667	*cnfU*			NifU-like C terminal	5.0	1.0	1.4	2.0E-07
sll0621	*dsb*-like			*c*-type cytochrome biogenesis	3.1	1.0	1.2	5.0E-06
slr0513	*idiA*	AATAATTTATGGATTATT	−202	IdiA homologue	2.4	5.7	4.2	2.0E-05
sll0247	*isiA*	ATAAATTCTCATTTAT	−246	Inducible Chl-binding protein	11.3	10.0	5.8	1.6E-01
sll1135		TTAATT TTAATT	−28	Unknown	7.7	3.3	5.6	2.0E-02
sll1483		ATAATTTGTCATAATA	−50	Fasciclin-like domain	15.3	14.4	6.5	4.3E-01
slr1894	*mrgA*	ATATTATTTCTATCTAATAT	−188	Metal-regulated gene	2.2	2.0	2.3	9.7E-01
slr1204	*htrA*			HtrA protease	2.6	1.2	3.0	1.6E-03

[a] The position of the putative *perR* box is defined as the distance between the ATG and the first nucleotide of the putative box.
[b] The changes for the wild type and the Δ*perR* and Δ*trxA1* mutants were calculated as described in Table 2 by dividing the normalized mean intensities of peroxide-treated strains by the normalized mean intensities of nontreated strains.

of deleting PerR on gene transcription in the presence of peroxide, and an interaction term from the statistical analysis. The availability of the genome sequence of *Synechocystis* sp. strain PCC 6803 allowed preliminary prediction of the PerR regulon of this organism. We identified putative PerR binding sites in *Synechocystis* sp. strain PCC 6803 based on the 8-bp inverted repeat sequence ATAATTAT. A preliminary search revealed 66 sites that contained the 8-bp consensus sequence. Some of these candidate sites were found upstream of the coding sequences of genes whose protein products were likely to have a role in oxidative stress or metal ion storage, including genes that were strongly upregulated by peroxide stress (Table 6). The *perR* (slr1738) and *ahpC* (sll1621) genes share a divergent promoter with an excellent putative PerR box, and both of these genes are upregulated by peroxide. In addition, *ahpC*, which codes for a peroxiredoxin-like protein, is even more strongly induced by methyl viologen (a generator of singlet oxygen) than *perR* is (data not shown).

We utilized Northern blots to verify the microarray results and to determine transcriptional regulation of certain genes in the absence of PerR (Fig. 2). Strong induction by peroxide in the wild type was evident for *isiA*, *perR*, and *ahpC*, as was the approximately twofold increase in the level of slr0513. As reported previously, there was excellent quantitative and qualitative correspondence between the microarray and Northern blot data (57, 58). Differences in the induction pattern were evident between the Δ*perR* strain and the wild type. Transcription of *isiA* was similar in the wild type and the Δ*perR* mutant, and transcription of slr0513 was actually reduced in the Δ*perR* mutant in the absence of peroxide. This led to a greater increase compared to the wild type and was similar to the 2.4- and 5.7-fold changes revealed by the microarray results (Table 6). Transcription of *perR* and *ahpC* exhibited a different pattern. The results were consistent with the hypothesis that PerR is a transcription factor (a repressor) that regulates these two genes and that slr1738 and sll1621 are fully transcribed when PerR is not present. Analysis by using both microarrays and Northern blots in the Δ*perR* mutant experiment indicated that two genes that were contiguous to *perR*/*ahpC* (sll1620 and slr1739) also acted as if they were under PerR control, although neither had a PerR box motif in the promoter. Thus, this regulation might reflect a structural interaction involving PerR binding to the *perR* promoter on this neighboring set of genes.

We then determined the set of genes for which there were large changes in the wild-type strain when there was peroxide stress but only approximately 1.0-fold changes in the Δ*perR* mutant. This was consistent with the action of a repressor; when PerR was absent, gene expression would be expected to be as high in the control as it is in the presence of peroxide (Table 6). This set of genes was then analyzed statistically for *P*(genoperoxide). This was the probability that there was an interaction between the stress and the mutant strain (i.e., that there was a significant change in the response of a particular gene to the stress in the mutant compared to the response in the wild type). Six genes fit these criteria and had *P*(genoperoxide) values of $<2 \times 10^{-5}$ (Table 6). These genes included slr1738 (*perR*) and sll1621 (*ahpC*), genes which have a good putative PerR box in a divergent promoter region. The neigh-

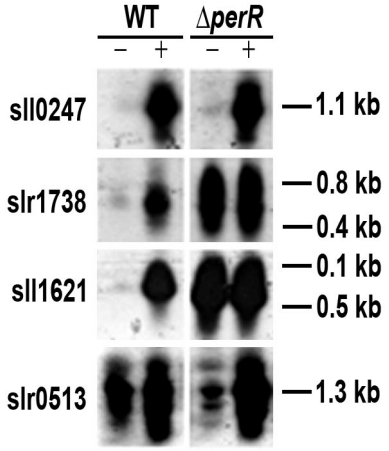

FIG. 2. Northern blots showing the effect of peroxide on gene transcription in *Synechocystis* sp. wild-type strain PCC 6803 and the Δ*perR* mutant. RNA was isolated from cells that were treated with 1.5 mM peroxide for 30 min (+) or from untreated controls (−).

boring genes sll1620 and slr1739 were also in this set of genes, although slr1739 did not have a good *P*(genoperoxide) value.

Based on these criteria, our current understanding of the putative PerR regulon is shown in Table 6. We tentatively propose that *perR*, *ahpC*, sll1620, slr1739, *cnfU* (ssl2667), sll0621, and sll1135 comprise the PerR regulon. In addition, there are four other genes that may be under PerR control. Of the genes in Table 5, *perR*, *ahpC*, *mrgA*, and the putative thioredoxin reductase gene *ahp* (sll1135) represent genes that have previously been identified as components of oxidative stress regulons in *B. subtilis* (19). The *cnfU* and *dsbD*-like (sll0621) genes code for proteins that may be involved in protein assembly and/or disulfide redox control. The CnfU protein has a thioredoxin motif (C-X-X-C), and sll0621 encodes a protein with four membrane-spanning segments that closely resembles DsbD from *E. coli* (54). However, *isiA*, sll1483, and slr0513 are genes that seem to be more specific to cyanobacteria. *isiA* and slr0513 were both first identified as inducible genes under iron-deficient conditions in a number of cyanobacteria and have some involvement in photosynthesis (58, 73). The sll1483 gene has not been studied previously, and it appears to have repeats of a fascicilin domain and may encode a protein involved with the cell envelope. Since induction of slr0513 actually increased in the Δ*perR* mutant and the transcript levels of *isiA* and sll1483 changed very little in the Δ*perR* mutant, we tentatively describe these genes as part of the regulon based on the presence of a PerR box and their strong induction by peroxide stress. The transcript levels of *mrgA* did not change much in either the Δ*perR* or Δ*trxA1* mutant, but there are additional reasons for placing this gene in the PerR regulon, as discussed below.

DISCUSSION

Reactive oxygen species, such as hydrogen peroxide, superoxide, and hydroxyl radicals, are inevitably produced during growth of organisms that carry out aerobic respiration and/or oxygenic photosynthesis. Survival of these organisms depends on the efficient scavenging of these oxygen species. In the present work, we utilized a full genome cDNA microarray to identify the genes that are regulated in response to hydrogen peroxide stress in the cyanobacterium *Synechocystis* sp. strain PCC 6803, which can carry out photosynthesis and respiration simultaneously. The effect of H_2O_2 on gene expression in the wild type was compared with the effect in two strains that lacked either a thioredoxin (TrxA1) or a transcriptional regulator (PerR). Whereas thioredoxin was chosen for its role in maintaining the cellular redox status of cells that might interfere with responses to H_2O_2, studies on PerR were based on our microarray results. The majority of genes regulated by peroxide stress in the three strains were similar, which strongly suggests that gene regulation occurred in response to peroxide stress rather than due to growth phase transitions.

The pathway leading to induction of the peroxide stimulon in cyanobacteria is not well understood. Our data demonstrated that the majority of genes that responded to oxidative stress are PerR and TrxA1 independent or are indirectly regulated by these two proteins. Interestingly, many genes that responded to peroxide stress also responded to high light (22), redox changes (23), high salinity (27), and high osmolality (27). For example, some of the pigment genes were downregulated under all of these stress conditions. The sll0306 (*sigB*), sll2012 (*sigD*), sll0790 (*hik31*), sll1621 (*ahpC*), slr1544 (unknown gene), heat shock, and chaperone genes are all induced under these conditions. The similar responses of these genes were expected, because these stresses often disrupt the cellular homeostasis of cells, leading to enhanced production of reactive oxygen species. It is also possible that the need to coordinate gene expression effectively in changing environmental conditions has led to the evolution of a set of signal transduction pathways which sense and respond to the various stress conditions. In this regard, it is possible that reactive oxygen species, such as hydrogen peroxide, can act as signaling molecules to mediate responses to these stress conditions. We do not yet know if H_2O_2 acts as a separate signal or as part of a systemic response in cyanobacteria. It is likely that the redox poise of cells may control cellular events through interactions with H_2O_2 and redox-sensing molecules, such as glutathione, thioredoxins, and peroxiredoxins. Thus, the largest single group of genes regulated by peroxide stress likely is part of a general stress regulon.

The heme and Chl biosynthesis genes represented a key difference between the oxidative stress responses in *Synechocystis* sp. strain PCC 6803 and *B. subtilis*. In *Synechocystis* sp. strain PCC 6803, genes in the first half of the heme pathway were transcribed similarly in the presence and absence of peroxide. However, many of the genes in the second half of the pathway, leading to Chl and bilin production, were significantly downregulated, as they were under low-iron growth conditions (Table 3; see Table S2 in the supplemental material). From the results in Table 3, it does not appear that PerR is directly involved in the regulation of these genes.

One objective of this study was to determine the importance of thioredoxins in oxidative stress. We found that the transcript levels of the genes encoding two putative thioredoxins, TrxA1 and TrxM2, decreased upon peroxide stress and that the Δ*trxA1* mutant was somewhat more sensitive to peroxide than the wild type. In fact, the increases in the transcript levels of many genes encoding proteins involved in the response to peroxide were smaller in the Δ*trxA1* mutant than in the wild type, and the genes acted as if TrxA1 provided a more oxidizing environment when it was present. This could be accomplished by other thioredoxin genes that play more active roles in the absence of TrxA1 and may explain the smaller increases in the transcript levels. For example, the *E. coli* Trx2 protein is required for the viability of cells that lack *trxA* and *gshA* (51). At the same time, two genes encoding proteins with thioredoxin or Dsb-like properties (ssl2667 and sll0621) were induced by peroxide and may be under PerR control (Table 6). We also pursued the possibility that the Δ*trxA1* mutant had higher transcript levels of many genes even prior to administration of the peroxide stress. However, a careful analysis of the intensity values for the genes listed in Table S1 in the supplemental material indicated that this was not generally true. The apparent lack of a difference in the responses to H_2O_2 in the wild type and the Δ*trxA1* mutant suggest that *trxA1* might be functionally redundant. One objective for future studies is to determine the response of *Synechocystis* sp. strain PCC 6803 to disulfide stress and the relationship to peroxide stress, as was accomplished recently for *B. subtilis* (34).

To date, PerR binding sites have been identified in only *B. subtilis* (21), and the studies made good use of the PerR mutants. These binding studies confirmed the role of the proposed *B. subtilis* PerR binding site, 5′-TTATAATNATTATAA. A problem in predicting Fur and PerR boxes lies in the extreme similarity of the consensus sequences. Experimental evidence of Fur- or PerR-mediated regulation is necessary, although the analysis is complicated due to overlaps between the Fur and PerR regulons (24, 65). Our initial studies have shown that purified PerR binds to the promoters that carry a putative PerR binding site (data not shown). We utilized three criteria to identify the PerR regulon: induction in response to H_2O_2 treatment in the wild type and either the presence of a putative PerR binding motif or loss of inducibility in the PerR mutant. However, it is important to recognize that not all components of the PerR regulon in *B. subtilis* can be induced by peroxide and that the metalloregulation of different PerR regulon genes is distinct; some components can be repressed by either Mn or Fe, whereas others are Mn specific. In *Staphylococcus aureus*, PerR is selective for Mn(II) (25), whereas the *Campylobacter jejuni* orthologue responds to Fe (65). Thus, complete determination of the PerR regulon in *Synechocystis* sp. strain PCC 6803 will require additional experimentation.

One of the striking findings was that transcription of the operon that encoded the CP43-like iron stress-induced protein (IsiA) and a flavodoxin (IsiB) was highly induced by peroxide. Although these proteins were discovered almost two decades ago (5, 32, 33, 45), their precise functions are not completely understood. Recently, two groups of workers have shown that 18 copies of the IsiA protein form a ring around the PSI reaction center in *Synechocystis* sp. strain PCC 6803 (2, 3). These workers suggested that the additional chlorophylls in this antenna ring of a PSI supercomplex increased the light-harvesting capacity of the PSI reaction centers. This can be an important factor under long-term iron stress conditions (3 to 4 days of growth) when the numbers of PSI centers and phycobilisomes have been reduced. In addition to its response to iron limitation, IsiA of *Synechocystis* sp. strain PCC 6803 responded to salt stress (2, 3, 66, 67), and we show here that *isiA* and *isiB* are highly induced by short-term oxidative stress, even in the presence of low peroxide concentrations (data not shown). This implies that induction of *isiA* is initiated long before IsiA is used as an antenna for a smaller number of PSI units. We could not detect significant increases in the level of the IsiA protein under our oxidative stress conditions (data not shown), nor did we see the absorption changes that are typical of IsiA (5). Unexpectedly, Δ*isiA* or Δ*isiB* mutants were generally no more sensitive to peroxide than the wild type under normal growth conditions (50 μmol of photons m^{-2} s^{-1}), although this was not the case at higher light intensities (100 μmol of photons m^{-2} s^{-1}). These results suggested that IsiA has multiple functions; it may not only have a role as a structural protein but may also be involved in signaling.

Studies with the cyanobacterium *Synechococcus elongatus* strain PCC 7942 have important ramifications for understanding the cyanobacterial response to peroxide and the functions of proteins like IsiA. Yousef et al. (73) and Lundrigan et al. (36) presented evidence which suggested that there is a relationship between iron homeostasis and peroxide stress in this strain. In particular, they showed that many genes known to be iron inducible, including *isiA* and *idiA*, were also inducible by short-term peroxide or methyl viologen stress. They proposed that IdiA was involved in the protection of PSII and that an enhanced cyclic electron transport activity around PSI was part of the cyanobacterial adaptive response to oxidative stress. Interestingly, there are two potential IdiA orthologues in *Synechocystis* sp. strain PCC 6803, slr1295 and slr0513. Although slr1295 has the greatest similarity to the IdiA gene, this gene shows little responsiveness to changes in the iron concentration (58) or to peroxide (this study). On the other hand, slr0513 was strongly induced under both conditions, and thus the gene product might have the functional properties of IdiA. However, the differences indicate that caution must be used before such models are extrapolated to all cyanobacteria.

We constructed mutants with mutations in a number of the genes that were induced by peroxide and studied their sensitivity to this stress. Interestingly, many of these mutants showed little change in peroxide sensitivity, and some of the mutants were actually more resistant to peroxide; this was most noticeable for the Δ*isiA* mutant. However, the Δ*mrgA* mutant was hypersensitive to peroxide (Table 4) (~20-fold more sensitive), and MrgA currently represents the most critical protein studied to date that is needed for peroxide protection. In *B. subtilis*, MrgA forms highly stable, multimeric protein-DNA complexes that accumulate in stationary-phase cells and protect against oxidative killing (15, 18). A Per box was identified in the promoter region, suggesting that *mrgA* is a member of the *B. subtilis* peroxide regulon (6, 7). In *Synechococcus* sp. strain PCC 7942, *dpsA* (the *mrgA* homologue) transcripts accumulated in the stationary phase and under most nutrient stress conditions (13, 46, 56), and a strain lacking *dpsA* was more sensitive to peroxide (14). The *mrgA* gene in *Synechocystis* sp. strain PCC 6803 has a good putative PerR box (Table 6), although the transcript levels were about the same after peroxide treatment in the wild type and the Δ*perR* mutant. Thus, although we cannot definitively place *mrgA* in the PerR regulon, the sensitivity of the Δ*mrgA* mutant to peroxide and its relationship to orthologous genes in other bacteria emphasize its importance to the response of *Synechocystis* sp. strain PCC 6803 to peroxide stress.

These microarray experiments provided a vast amount of new data on the response of *Synechocystis* sp. strain PCC 6803 to short-term, exogenous peroxide stress. A number of key regulatory genes were induced, and understanding the induction of these genes and identifying the genes that they control are important tasks. Toward this goal, we studied the effect of loss of PerR on the H_2O_2 response and showed that the PerR regulon controls only a small subset of genes. The strong induction of *sigD* by peroxide suggested that this gene could play an important role in peroxide protection, and evaluating the importance of SigD in the response to peroxide stress is one of our short-term goals. Since approximately 40% of the peroxide-regulated genes have unknown functions, this system should help us answer some of the most pertinent questions related to oxidative stress in cyanobacteria, including what regulatory networks control the production and scavenging of reactive oxygen species. For example, are there novel scavenging mechanisms that operate in cyanobacteria? It is interesting that Perelman et al. (48) identified a novel H_2O_2 detoxification activity in *Synecochoccus* sp. strain PCC 7942. Tichy and Ver-

maas (63) also showed that the absence of catalase led to a 30-fold decrease in H_2O_2 decomposition, although the mutant strain had a normal phenotype and the same resistance to H_2O_2 and methyl viologen as the wild type. Identification of such novel detoxification pathways and additional regulatory networks and characterization of the redox-sensing mechanisms are some of the important directions for future studies.

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