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Regulation of Hydrogen Peroxide-Dependent Gene Expression in *Rhodobacter sphaeroides*: Regulatory Functions of OxyR▽†

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Genome-wide transcriptome profiling was used to reveal hydrogen peroxide (H_2O_2)-dependent regulatory mechanisms in the facultatively photosynthetic bacterium *Rhodobacter sphaeroides*. In this study we focused on the role of the OxyR protein, a known regulator of the H_2O_2 response in bacteria. The transcriptome profiles of *R. sphaeroides* wild-type and *oxyR* mutant strains that were exposed to 1 mM H_2O_2 for 7 min or were not exposed to H_2O_2 were analyzed. Three classes of OxyR-dependent genes were identified based on their expression patterns in the wild type of *oxyR* mutant strains with differing predicted roles of oxidized and reduced OxyR as activators of transcription. DNA binding studies revealed that OxyR binds upstream of class I genes, which are induced by H_2O_2 and exhibit similar basal levels of expression in the wild-type and *oxyR* mutant strains. The effect of OxyR on class II genes, which are also induced by H_2O_2 but exhibit significantly lower basal levels of expression in the wild-type strain than in the mutant, is indirect. Interestingly, reduced OxyR also activates expression of few genes (class III). The role of reduced OxyR as an activator is shown for the first time. Our data reveal that the OxyR-mediated response is fast and transient. In addition, we found that additional regulatory pathways are involved in the H_2O_2 response.

Microorganisms are exposed to various external factors in their natural environments and show general and specific stress responses. Oxidative stress is caused by reactive oxygen species (ROS) that are generated endogenously by autooxidation of components of the respiratory chain (6, 8, 15), by other cellular processes (25), or by exposure to metals, redox-active chemicals, or radiation in the presence of oxygen. The purpose of the oxidative stress response is to prevent, counteract, or repair damage caused by ROS. Enzymes like superoxide dismutase, catalase, and peroxidase remove ROS. The glutathione/glutaredoxin system and thioredoxins reduce cytoplasmic proteins that are oxidized by ROS. Other proteins have functions involved in protecting DNA against ROS or in DNA repair (1, 3, 35).

As it has in many other respects, *Escherichia coli* has served as a model organism to study the oxidative stress response. Two main regulatory systems control the *E. coli* response to ROS, the OxyR protein and the SoxRS system (26). OxyR regulates gene expression in response to H_2O_2. In the presence of H_2O_2, OxyR forms an intramolecular disulfide bond (26). Oxidized OxyR is in turn reduced by glutaredoxin 1, which is accompanied by the consumption of glutathione (26). As a transcription factor belonging to the LysR family, OxyR binds to its target sites (23, 29) in its oxidized form and, in most cases, activates gene expression by contacting the alpha subunit of DNA polymerase (10, 36). In some cases, however, repression of gene expression by OxyR has been observed (36). The following genes belonging to the *E. coli* OxyR regulon were identified in classical gene expression studies: *katG* (encoding hydroperoxidase I), *ahpCF* (alkyl hydroperoxide reductase), *gorA* (glutathione reductase), *grxA* (glutaredoxin 1), *trxC* (thioredoxin 2), *fur* (ferric uptake repressor), *fhuF* (ferric reductase), *dps* (nonspecific DNA binding protein), *oxyS* (regulatory RNA), and *agn43* (outer membrane protein). Several new OxyR-activated genes, including *hemH* (ferrochelatase) and the *suf* operon (Fe-S cluster assembly or repair), were identified by transcriptional profiling using DNA microarrays (36). These studies revealed that OxyR activates most genes that are highly induced by H_2O_2. However, some genes are repressed by OxyR, and other genes are induced by H_2O_2 independent of OxyR. In contrast to OxyR, the SoxRS system of *E. coli* mediates responses to the superoxide anion. The SoxR protein is activated by direct oxidation of its [2Fe-2S] cluster. This results in increased transcription of *soxS*, whose product in turn activates expression of a number of genes involved in prevention and/or repair of oxidative damage (26).

Rhodobacter sphaeroides is a facultatively photosynthetic bacterium belonging to the alpha subdivision of the *Proteobacteria* that can effectively adapt to different environmental conditions and choose from various metabolic pathways for energy conversion. This bacterium can perform aerobic respiration for generation of ATP at various oxygen tensions. However, when the oxygen tension decreases, synthesis of photosynthetic complexes is induced, which allows the generation of ATP by anoxygenic photosynthesis when the concentration of oxygen is very low or there is no oxygen, in the presence of light. Under

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anaerobic conditions in the dark in the presence of an alternative electron acceptor, anaerobic respiration is carried out. The free-living organism *Rhodobacter* has to cope with great variations in the oxygen concentration in its environment, which can be high due to oxygenic photosynthesis by other organisms.

Studies of the defense against oxidative stress in *R. sphaeroides* and its close relative *Rhodobacter capsulatus* have been initiated recently (12, 13, 14, 34, 35). Evidence that the oxidative stress response in *Rhodobacter* differs significantly from the oxidative stress response in *Escherichia coli* is accumulating (12, 13, 14). Both *Rhodobacter* species contain a homolog of the *E. coli* OxyR protein (34). *oxyR* mutants of both species are more sensitive to H_2O_2 than wild-type strains are, indicating that in *Rhodobacter* OxyR has a role in the oxidative stress response similar to the role of its *E. coli* counterpart (34). However, our previous expression studies suggested that the composition of the OxyR regulon in *R. capsulatus* is different from the composition of the OxyR regulon in *E. coli* (14).

To obtain a comprehensive view of the H_2O_2-dependent responses in a facultatively photosynthetic bacterium, we performed transcriptome profiling of *R. sphaeroides* wild-type cells that were grown with an intermediate oxygen tension and exposed to H_2O_2 for 7 min (primary responses) and 30 min (adapted cells) (35). In this paper, we describe regulatory mechanisms involved in the H_2O_2 response per se and focus on the role of the *R. sphaeroides* OxyR protein in this process.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* cultures were grown in Luria-Bertani medium at 37 or 20°C with continuous shaking at 180 rpm. *R. sphaeroides* cultures were cultivated at 32°C in malate minimal salt medium (4) under semiaerobic conditions (approximately 0.85 mg liter^{-1} dissolved oxygen) with continuous shaking at 140 rpm. Ampicillin (200 μg ml^{-1}), spectinomycin (10 μg ml^{-1}), kanamycin (25 μg ml^{-1}), and trimethoprim (50 mg ml^{-1}) were added to the medium as needed. *R. sphaeroides* wild-type strain 2.4.1 (30), *oxyR* mutant 2.4.1*oxyR* (34), *rpoE chrR* mutant TF18 (24), and *fnrL* mutant JZ1678 (33) were treated with 1 mM (final concentration) H_2O_2 for 7 min at an optical density at 660 nm of 0.5. After 0, 7, and 30 min, cells were harvested and used for viable cell counting or RNA extraction. H_2O_2 was purchased from Sigma.

Determination of survival rates. At 0, 7, and 30 min, aliquots were removed, and appropriate dilutions were plated on agar medium. After 2 days of incubation at 32°C, colonies were counted. A level of survival of 100% corresponded to the viable cell count at time zero. The viable cell counts obtained after exposure to oxidative stress agents were expressed as percentages based on the counts at time zero.

RNA extraction and quantitative RT-PCR. After no exposure to H_2O_2 (no stress) and after 7 min of exposure to H_2O_2, *R. sphaeroides* cells were collected by centrifugation. Total RNA was isolated by the hot phenol method (31) and quantified by spectrophotometric analysis (A_{260}). For microarray experiments, contaminating genomic DNA was removed from RNA samples by DNase treatment, followed by purification on RNeasy minicolumns (QIAGEN, Chatsworth, CA). cDNA synthesis, fragmentation, labeling, genechip hybridization, and scanning were performed using the specifications of Affymetrix (Santa Clara, CA) exactly as described previously (20). RNA and cDNA quality was tested at each step using capillary electrophoresis (Bioanalizer; Agilent Technologies, California). For semiquantitative reverse transcription (RT)-PCR, 60 ng of total RNA was used. The *rpoZ* gene (encoding the ω-subunit of RNA polymerase) was used to normalize expression values. For RT-PCR a One-step RT-PCR kit (QIAGEN, Hilden, Germany) and a *Revers*e-iT one step-RT-PCR kit (ABgene, Hamburg, Germany) were used according to the manufacturers' instructions, except that the total volumes used were 15 and 10 μl, respectively. SYBR green was used to monitor gene amplification and to determine the amounts of PCR products using a Rotor-Gene 3000 real-time PCR cycler (LTF, Wasserburg, Germany). The relative level of expression of the mRNA was calculated using the method of Pfaffl (21). Oligonucleotides used for gene amplification are shown in Table S2 in the supplemental material.

DNA microarray experiments and data analysis. A high-density oligonucleotide *R. sphaeroides* microarray, Affymetrix genechips, corresponding to the whole genome was used for transcriptome profiling. All experimental procedures were performed as described previously (20). Robust Multi-Array Analysis with quantile normalization (9; http://www.stat.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html), Microsoft Excel, and the GeneSpring 7.2 software package (Silicon Genetics, California) were used for genechip data analysis and representation. We recorded and analyzed transcriptome profiles for three independent cultures. The experimental reproducibility values (*r* values) for replicates grown under the same conditions ranged from 0.96 to 0.99. Fold changes were calculated based on the average expression values for replicate experiments for every condition, using time zero as a baseline. Average expression values are reported below. We defined a "meaningful change" as a change when the average value after exposure to H_2O_2 was ≥2-fold higher or lower than the average value at time zero. To account for experimental variability, we included only genes whose levels of expression for each replicate for H_2O_2-treated cells differed by a factor of at least 1.15 from the levels of expression for each replicate at time zero (no stress), provided that the directions of all the changes observed in comparisons of values for individual replicates matched the direction of the change observed for mean values for each condition compared. The fold changes in levels of expression (rounded to 0.5-fold) are shown below along with the RSP designations of the corresponding genes; e.g., twofold upregulation compared with the original level is reported as "+2," and downregulation to a level that is one-half of the original level is reported as "−2." The range of changes for a group of genes is reported as follows: +2.+5, indicating two- to fivefold upregulation.

Expression and isolation of the *R. sphaeroides* OxyR protein. Oligonucleotides 2.4.1oxyRstartARGBam and 2.4.1oxyRdownKpn (see Table S2 in the supplemental material), which hybridize to the 5′ and 3′ regions of the *oxyR* gene, respectively, were used to amplify the *oxyR* coding region. 2.4.1oxyRstartARGBam changes the second codon in the *oxyR* sequence, AGA (arginine), to a CGT codon (arginine), since AGA is rarely used by *E. coli* (18). The 985-bp PCR product was digested with BamHI and KpnI and was ligated into pQE30 to generate pQE2.4.1*oxy*R, which was transformed into *E. coli* JM109. The correct construct, as confirmed by sequencing using a Genetic Analyzer 310 sequencer (ABI), was transformed into *E. coli* M15(pREP4) for overexpression of His-tagged OxyR. For this purpose M15(pREP4/pQE2.4.1*oxy*R) was grown in 500 ml of Luria-Bertani medium to an optical density at 600 nm of 0.5 at 37°C and was then shifted to 20°C. After 2 h of incubation at 20°C cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 15 h at 20°C. Following harvest, cells were resuspended in lysis buffer (20 mM Tris [pH 7.5], 500 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride, 1 μg μl^{-1} lysozyme) and disrupted by brief sonication. The lysate was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was loaded onto Ni-nitrilotriacetic acid agarose and incubated at 4°C for 5 to 6 h. Proteins were washed with washing buffer (0.05 M Tris [pH 7.5], 0.25 M NaCl) containing 20 to 50 mM imidazole and were eluted with imidazole at concentrations between 90 and 150 mM. The purified OxyR protein in a 2-ml mixture was applied to a Superdex 200 HR 16/60 gel filtration column (Pharmacia Biotech) equilibrated with Z-buffer (50 mM HEPES [pH 8.0], 0.5 mM EDTA [pH 8.0], 10 mM $MgCl_2$, 100 mM KCl). Aliquots of the fractions were analyzed on 12% sodium dodecyl sulfate-polyacrylamide gels, and only fractions containing pure OxyR protein were used for the experiments described below.

Protein-DNA binding studies. (i) Gel mobility shift assays. Binding of the OxyR protein to the upstream regions of RSP0434, RSP0437, RSP0557, RSP0920, RSP0922, RSP1408, RSP1543, RSP2229, RSP2723, RSP2779, RSP1090, RSP1091, RSP1547, RSP2199, RSP2354, RSP2638, RSP3003, RSP3751, and RSP4242 was determined by a gel mobility shift assay as described previously (34). Briefly, DNA fragments containing between 250 and 350 bp of the upstream region of a gene were generated by PCR. Oligonucleotides used for the PCRs are listed in Table S2 in the supplemental material. Binding reactions were carried out in 20-μl (final volume) mixtures containing an appropriate amount of protein, a [γ-^{32}P]ATP-labeled DNA probe, and binding buffer (10 mM Tris-Cl [pH 8.0] 50 mM NaCl, 1 mM EDTA, 5% glycerol). Since purified OxyR protein is predominantly in the oxidized form (34, 36), we added 200 mM DTT to the binding reaction mixture to obtain the reduced form of OxyR (34). The reaction mixtures were incubated for 30 min at room temperature (25°C) before the samples were loaded onto a 4% polyacrylamide gel in 0.5×Tris-borate-EDTA buffer and electrophoresed at 130 V for 2 h.

(ii) DNase I footprinting assay. A BamHI-StuI DNA fragment containing approximately 350 bp of the RSP3751 upstream region was amplified using

oligonucleotides shown in Table S2 in the supplemental material and was cloned into the pDrive cloning vector (QIAGEN, Hilden, Germany) to generate plasmid pRSP3751up. Plasmid pRSP3751up was linearized with BamHI, dephosphorylated using Antarctic phosphatase (NEB, Massachusetts), and labeled with polynucleotide kinase (NEB) and [γ-^{32}P]ATP. The DNA was then digested with StuI, and the BamHI-StuI fragment was purified from a 6% nondenaturing polyacrylamide gel. The binding reaction mixtures (final volume, 20 μl) contained appropriate amounts of the purified *R. sphaeroides* OxyR protein and the ^{32}P-labeled DNA probe (approximately 10^5 cpm/mixture) in the binding buffer (10 mM Tris-Cl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 5% glycerol). DTT (200 mM) was added to the binding reaction mixture to obtain the reduced form of OxyR (34). After 30 min of incubation at room temperature, 5 μl of buffer containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ and 1 μl of DNase I (0.1 U μl^{-1}; Promega, Wisconsin) were added, and the mixture was incubated for 2 min. The reaction was stopped by addition of 1 μl of 250 mM EDTA. After phenol-chloroform extraction, purified DNA was dissolved in the formamide-containing dye and analyzed by 6% polyacrylamide gel electrophoresis. The plasmid containing the upstream region was sequenced with an oligonucleotide shown in Table S2 in the supplemental material (oligonucleotide designation with the suffix SEQ) by the dideoxy chain termination method (22), using a T7 sequencing kit (USB).

Microarray data accession number. The raw expression data described here have been deposited in the Gene Expression Omnibus (GEO) database of NCBI (www.ncbi.nlm.nih.gov/geo), platform GPL162, under the "oxidative stress" series (accession no. GSE2829).

RESULTS AND DISCUSSION

Previously, we showed that long-term survival of the *R. sphaeroides oxyR* mutant strain 2.4.1*oxyR* is compromised in the presence of H_2O_2 (34). To characterize the OxyR regulon of *R. sphaeroides* by means of DNA microarrays, we used H_2O_2 stress conditions that do not significantly affect cell survival. We determined that after exposure of strain 2.4.1*oxyR* to 1 mM H_2O_2 for 7 and 30 min the level of survival was 97% ± 3%, compared to 100% at time zero, which is similar to the level of survival of wild-type strain 2.4.1 (35).

By using DNA microarrays with *R. sphaeroides* wild-type strain 2.4.1, we have shown previously that a primary response of the *R. sphaeroides* transcriptome to H_2O_2 was established after 7 min of exposure to H_2O_2 (35). Approximately 9% of all genes present on the chip showed significant changes (≥2-fold) in expression after 7 min of exposure to H_2O_2. After 30 min cells were adapted to H_2O_2 stress, as significantly fewer genes were affected by H_2O_2 (approximately 2%) (35). Therefore, to characterize the OxyR regulon, we compared the transcriptome profiles of strains 2.4.1*oxyR* and 2.4.1 after 7 min of exposure to H_2O_2, as well as prior to stress (see Table S1 in the supplemental material).

Transcriptome profile of the *R. sphaeroides oxyR* mutant in the absence of exogenous H_2O_2. The levels of expression of only 48 open reading frames differed by ≥2-fold when strain 2.4.1*oxyR* was compared to wild-type strain 2.4.1 prior to H_2O_2 addition (the level of expression of 25 open reading frames was lower, and the level of expression of 23 open reading frames was higher). The magnitude of the differences in the levels of expression between strains 2.4.1*oxyR* and 2.4.1 under no-stress conditions showed that *R. sphaeroides* OxyR plays a role in gene expression even in the absence of exogenous H_2O_2.

Transcriptome profile of the *R. sphaeroides oxyR* mutant in the presence of exogenous H_2O_2. After 7 min of exposure of strain 2.4.1*oxyR* to H_2O_2, the levels of the transcripts of 544 genes changed ≥2-fold (the levels of 325 genes decreased, and the levels of 219 genes increased) compared to the levels of transcripts in strain 2.4.1*oxyR* at time zero. This number is higher than the number of genes whose expression changed in wild-type strain 2.4.1 after 7 min of exposure to H_2O_2 (a total of 394 genes; expression of 179 genes decreased, and expression of 215 genes increased [35]). The higher number of genes that were repressed by H_2O_2 in strain 2.4.1*oxyR* than in the wild type indicates that the lack of regulation by OxyR may result in greater H_2O_2 stress in general, which leads to stronger OxyR-independent gene repression. It was shown previously that OxyR is involved in upregulation of *katE* and that the KatE catalase is the major enzyme for H_2O_2 detoxification in *R. sphaeroides* (34, 35). The high number of H_2O_2-regulated genes in 2.4.1*oxyR* shows that, besides the OxyR pathway, additional regulatory pathways mediate the H_2O_2 stress response and that the number of genes directly dependent on OxyR is limited. Below, we focus on the OxyR-dependent pathway and discuss additional H_2O_2-responsive pathways discovered in our experiments.

OxyR regulon. Based on the expression profile on DNA microarrays, OxyR-regulated genes were grouped into three classes (Table 1). Class I contains all genes whose expression is induced by OxyR only under H_2O_2 stress conditions, indicating that OxyR has an activating effect under these conditions. All these genes had to meet the following criteria: (i) a higher level of expression (≥2-fold) in strain 2.4.1 after 7 min of exposure to H_2O_2 than at time zero; and (ii) a higher level of expression (≥2-fold) in strain 2.4.1 at 7 min than in strain 2.4.1*oxyR* at 7 min. All these genes exhibited similar basal levels of expression (<2-fold difference) before addition of H_2O_2 in the two strains. This suggests that OxyR, most of which is reduced in the absence of H_2O_2, does not affect the basal level of expression of class I genes, while oxidation of OxyR after addition of H_2O_2 leads to transcriptional activation.

The second class of OxyR-regulated genes consists of genes that are also induced by H_2O_2 but show significantly lower basal levels of expression in strain 2.4.1 than in strain 2.4.1*oxyR*. This expression pattern indicates that OxyR has a repressor function under no-stress conditions. The criteria used for the transcriptome data for the class II genes were as follows: (i) a higher level of expression (≥2-fold) in strain 2.4.1 after 7 min of exposure to H_2O_2 than at zero time; and (ii) a lower level of expression (≥2-fold) in strain 2.4.1 at time zero than in strain 2.4.1*oxyR* at time zero.

Genes which exhibited reduced levels of expression under H_2O_2 stress conditions but significantly higher basal levels of expression in strain 2.4.1 than in strain 2.4.1*oxyR*, suggesting that OxyR had an activating effect under no-stress conditions, were grouped into class III. The following criteria were used for this group of OxyR-regulated genes: (i) a lower level of expression (≥2-fold) in strain 2.4.1 after 7 min of exposure to H_2O_2 than at time zero; and (ii) a higher level of expression (≥2-fold) in strain 2.4.1 at time zero than in strain 2.4.1*oxyR* at time zero.

None of the genes had an expression pattern that suggested that OxyR had a repressing effect under H_2O_2 stress conditions (i.e., a higher level of expression in strain 2.4.1 at time zero than after 7 min of exposure to H_2O_2).

Table 1 shows all genes that meet the criteria for OxyR regulation. We identified 10 class I genes. The most significant differences in the levels of class I gene transcripts at 7 min

TABLE 1. Three classes of OxyR-regulated genes

Gene	Description	Fold change in expression[a]		2.4.1*oxyR*/2.4.1 ratio at time zero[b]
		2.4.1, 7 min of exposure to H_2O_2	2.4.1*oxyR*, 7 min of exposure to H_2O_2	
Class I genes				
RSP0434	*sufD*, iron-regulated ABC transporter	+3.4	(−1.4)[c]	1.4
RSP0437	*sufC*, iron-regulated ABC transporter	+8.1	(+1.9)	1.7
RSP0557	Unknown function	+8.5	+3.7	1.9
RSP0922	*tonB*, involved in iron transport	+8.7	+3.1	1.2
RSP1408	ATPase with chaperone activity	+3.8	(+1.5)	1.7
RSP1543	Hypothetical protein	+5.9	+2.1	1.3
RSP2229	*cheB*, chemotaxis methyl transferase protein	+2.8	(+1.1)	1.2
RSP2723	*leuA2*, 2-isopropylmalate synthase protein	+3.5	(+1.3)	1.3
RSP2779	*katE*, catalase	+61.5	(+1.2)	1.3
RSP3162	Unknown function	+3.5	(+1.1)	1.0
Class II genes				
RSP1090	Hypothetical protein	+15.1	+5.6	2.1
RSP1547	Bacterioferritin-associated ferredoxin	+69.8	+17.5	2.6
RSP1573	Hypothetical protein	+2.9	+1.5	2.1
RSP2199	Acyl coenzyme A dehydrogenase	+3.2	(+1.2)	2.9
RSP2354	ATP synthase B subunit	+4.0	(+1.5)	2.8
RSP2638	Ca^{2+}/Na^{+} antiporter	+2.9	(+1.0)	2.7
Class III genes				
RSP3003	Unknown function	−2.5	(−1.3)	−2.3
RSP3751	Unknown function	−21.7	−12.9	−2.5
RSP4242	Calcium-binding protein	−7.8	−3.4	−2.3

[a] Fold change compared to the expression at time zero.
[b] Ratio of gene expression in 2.4.1*oxyR* to gene expression in the wild type 2.4.1 at time zero.
[c] The values in parentheses are insignificant changes according to the criteria used.

between strains 2.4.1 and 2.4.1*oxyR* were observed for *katE* (RSP2779; +61 in 2.4.1) encoding catalase, *sufCD* (RSP0434 to RSP0437; +3.+8 in 2.4.1) involved in iron-sulfur cluster synthesis and repair, *tonB* (RSP0922; +9 in 2.4.1) involved in energizing Fe^{3+}-siderophore transporters, and a gene with an unknown function (RSP0557; +9 in 2.4.1). The RSP0434 to RSP0437 genes most likely belong to the same operon, and RSP0922 is most likely cotranscribed with RSP0920 and RSP0921 encoding the ExbB and ExbD proteins, respectively. TonB, ExbB, and ExbD form an inner membrane complex that transduces energy to outer membrane transporters of ferric siderophores, hemin, or hemoglobin. RSP0920 and RSP0921 are also strongly regulated by H_2O_2. While H_2O_2-dependent activation of RSP0921 is greater in strain 2.4.1 (+18) than in strain 2.4.1*oxyR* (+10), this is not the case for RSP0920 (+14 in 2.4.1 and +19 in 2.4.1*oxyR*). In addition, a gene encoding an ATPase with chaperone activity (RSP1408; +4 in 2.4.1), a gene encoding a CheB homologue (RSP2229; +3 in 2.4.1), and some genes with unknown or poorly characterized functions, including genes encoding a putative cytochrome *c* (RSP3162; +4 in 2.4.1), a hypothetical protein (RSP1543; +6 in 2.4.1), and a putative isopropylmalate synthase (RSP2723; +4 in

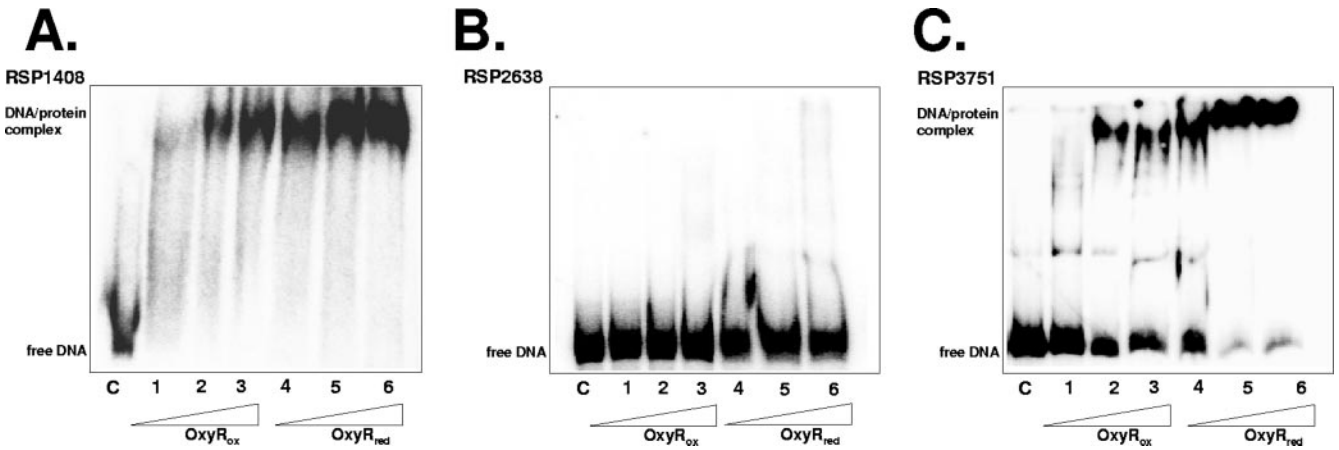

FIG. 1. Binding of oxidized OxyR ($OxyR_{ox}$) and reduced OxyR ($OxyR_{red}$) to the upstream regions of RSP1408 (A), RSP2638 (B), and RSP3751 (C), as determined by gel mobility shift assays. To generate reduced conditions, 200 mM DTT was added to the binding reaction mixtures. The following amounts of OxyR were added to the reaction mixture: 50 ng (lanes 1 and 4), 100 ng (lanes 2 and 5), and 150 ng (lanes 3 and 6). Lane C contained the control without OxyR.

2.4.1), fall into this class. We also included RSP0557, which did not meet the second criterion but exhibited clearly stronger H_2O_2-dependent expression in strain 2.4.1 than in strain 2.4.1*oxyR*. Most of these genes showed no response to H_2O_2 in 2.4.1*oxyR*, suggesting that OxyR is the only regulator involved in this response. However, for RSP0557, RSP0922, and RSP1543 we observed some H_2O_2-dependent increase in expression in strain 2.4.1*oxyR*,, suggesting that additional regulators besides OxyR may be involved in the H_2O_2-dependent regulation of these genes.

Six genes that are induced by H_2O_2 exhibited significantly lower basal levels of expression (<2-fold) in strain 2.4.1 than in strain 2.4.1*oxyR*. Because of these differences in the basal levels of expression, these genes are grouped into class II, although they are induced by H_2O_2. This expression pattern indicates that OxyR has a repressing effect on gene expression under no-stress conditions. However, under oxidizing conditions (addition of H_2O_2) this repressing effect is diminished, and gene expression is induced. Since the levels of expression of some of the class II genes (RSP1573, RSP2199, RSP2354, and RSP2638) were similar in strain 2.4.1*oxyR* under no-stress conditions and in strain 2.4.1 after 7 min of exposure to H_2O_2, OxyR seems to be the main regulator. This is not true for RSP1090 and RSP1547. These two genes also exhibited strong H_2O_2-dependent induction in strain 2.4.1*oxyR*, suggesting that an additional factor besides OxyR participates in gene regulation.

The expression pattern of class II genes suggests that OxyR functions as a repressor under no-stress conditions, as reported previously for the *E. coli* OxyR protein (27, 32, 36, 37). However, in contrast to *E. coli*, DNA binding studies revealed that OxyR does not bind to upstream regions of class II genes and thus that its effect on the expression of these genes is indirect (see below).

Three genes that are repressed by H_2O_2 meet our criteria for class III OxyR-regulated genes. These genes exhibit significantly higher basal levels of expression (≥2-fold) in strain 2.4.1 than in strain 2.4.1*oxyR*. The expression pattern of these genes suggests that reduced OxyR can function as an activator of transcription and loses its activating effect upon oxidation. To our knowledge, such a function of OxyR has not been described previously. To exclude the possibility that the classification of these three genes was a consequence of experimental artifacts of the microarray analysis, we determined their basal levels of expression in the wild type and mutant by real-time RT-PCR. We confirmed that the basal levels of expression of these three genes were significantly lower in strain 2.4.1*oxyR* (compared to the level of expression in the wild type, the levels of expression were 2.3-fold lower for RSP3751, 2.6-fold lower for RSP4242, and 1.5-fold lower for RSP3003). Because the levels of expression of RSP3751, whose function is unknown, and RSP4242, which encodes a putative calcium binding protein, were significantly lower in strain 2.4.1*oxyR* after 7 min of exposure to H_2O_2 than at time zero (Table 1), we predicted that these genes are affected not only by OxyR but also by an additional factor(s).

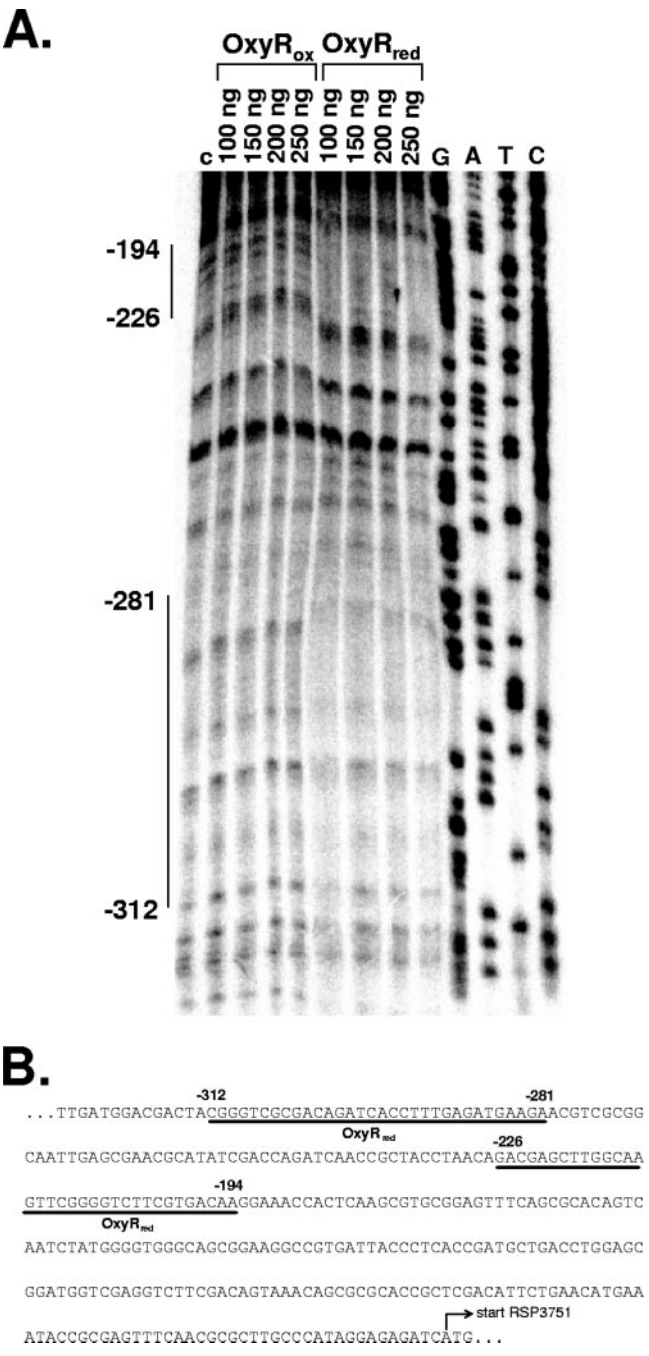

FIG. 2. (A) Footprint assay of oxidized OxyR (OxyR$_{ox}$) and reduced OxyR (OxyR$_{red}$) for the upstream region of the *R. sphaeroides* class III gene RSP3751. To generate reduced conditions, 200 mM DTT was added to the binding reaction mixtures. The regions protected by OxyR are indicated by vertical lines on the left; the numbers indicate the binding positions with respect to the translational start site. Lane c contained the control without OxyR. Lanes G, A, T, and C show the sequencing reaction. (B) Sequence of the upstream region of RSP3751. The start of the RSP3715 coding sequence is indicated by an arrow. Binding sites of reduced OxyR are underlined.

Binding of OxyR to upstream regions of OxyR-regulated genes. Our transcriptome analysis suggested that OxyR can have very different effects on gene expression. OxyR is known to directly affect gene expression by binding to promoter regions of regulated genes (23, 26, 28, 29, 34, 36). However, it is also conceivable that OxyR has an indirect effect on the expression of some genes by affecting expression of other regulators and/or by influencing intracellular levels of ROS. Since

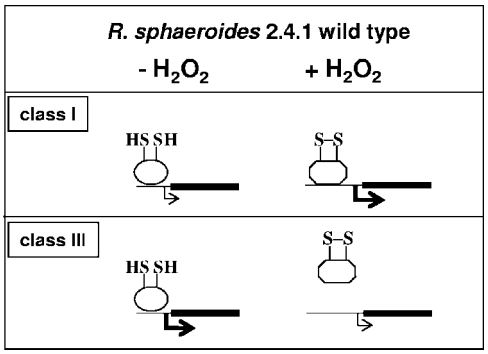

FIG. 3. Model describing the OxyR mode of action for class I and class III genes. Based on microarray data and DNA-protein binding studies, we concluded that oxidized OxyR (in the presence of H_2O_2) and reduced OxyR (no-stress conditions) have the following direct effects on the expression of OxyR-dependent genes. For class I genes, OxyR activates gene expression in the presence of H_2O_2. Class I genes are activated by binding of oxidized OxyR to the upstream region, whereas under no-stress conditions reduced OxyR binds to the DNA but has no regulatory effect on the expression of these genes. For class III genes, reduced OxyR binds to the upstream regions and activates class III genes under no-stress conditions. Oxidized OxyR does not bind or only weakly binds to the upstream region of these genes, and gene expression decreases in the presence of H_2O_2.

catalase is under the control of OxyR, the lack of larger amounts of this protein during exposure to H_2O_2 significantly reduces the H_2O_2-detoxifying capacity of cells (34). Increased levels of ROS may be sensed by other (regulatory) proteins, which mediate H_2O_2-dependent gene expression.

In order to determine which of these possibilities is correct, we tested for binding of purified *R. sphaeroides* OxyR to the upstream regions of most genes listed in Table 1. Binding of OxyR was detected for all class I genes except RSP0434, RSP0922, RSP1543, and RSP2723, which are most likely part of polycistronic operons, as described above. Consistent with this, OxyR bound to the upstream regions of the first genes in these operons, RSP0437 and RSP0920. In general, the affinity of the reduced OxyR protein for upstream sequences of class I genes was somewhat higher than that of the oxidized protein, as shown in Fig. 1A for RSP1408.

We did not detect binding of OxyR to class II genes (shown for RSP2638 in Fig. 1B; all class II genes but RSP1573 were tested), confirming that class II is indeed distinct from class I. RSP1090 is most likely cotranscribed with RSP1091, and the latter gene is the first gene of the operon. We also did not detect binding of OxyR to the RSP1091 upstream region. Apparently, class II genes are indirectly controlled by OxyR. This indirect effect could be explained by elevated levels of ROS in strain 2.4.1*oxyR*, which might lead to effects on, e.g., iron-sulfur clusters of other regulatory proteins that in turn are involved in the regulation of these genes (as below).

While the levels of expression of class II genes are significantly higher in strain 2.4.1*oxyR* than in strain 2.4.1 in the absence of H_2O_2, the opposite is true for class III genes (Table 1). Gel shift analyses indicated that there was binding of OxyR to the upstream regions of all three genes in class III and that there was significantly higher affinity of the reduced protein (shown for RSP3751 in Fig. 1C). This was confirmed for RSP3751 by footprint analysis (Fig. 2). The binding regions of reduced OxyR were located between nucleotides −194 and −226 and between nucleotides −281 and −312 relative to the translational start site of RSP3751. For most of the known *E. coli* OxyR-regulated genes, the transcriptional regulator binds at a position that overlaps or is directly upstream of the −35 sequence of the promoter (36). However, footprint assays carried out for the *E. coli sufA* upstream region showed that oxidized OxyR bound exclusively to a region 253 bp upstream of the *sufA* start codon, although this binding site was far upstream of the putative promoter sequence (11, 36). OxyR might exert its regulatory effect on gene expression (although binding sites are far upstream of the putative promoter) via

TABLE 2. Expression of known or predicted regulators of gene expression in response to exposure to H_2O_2 for 7 min

Gene	Description	Fold change in expression after exposure to H_2O_2 for 7 min[a]	
		2.4.1	2.4.1*oxyR*
RSP0032	Putative σ^{28} factor	−2.4	(−1.3)[b]
RSP0049	Histidine kinase and related kinases	−2.6	(−1.9)
RSP0051	Transcriptional regulator of the NtrC family	−3.6	(−1.1)
RSP0085	Putative histidine kinase	−3.4	−3.2
RSP1565	AppA, antirepressor of PpsR, *appA*	−6.5	−6.1
RSP2423	Putative small RNA binding protein	−3.1	−2.8
RSP2888	Transcriptional regulator, *rrf2* family	−3.6	−4.5
RSP2950	Transcriptional regulator, LysR family	−3.6	−3.6
RSP3044	Putative histidine kinase	−3.7	−3.6
RSP3642	Putative transcriptional regulator	−6.4	−5.4
RSP3857	Transcriptional regulator, Lrp family	−3.4	−3.3
RSP4195	Putative response regulator	−2.0	−3.2
RSP4201	Transcriptional regulator, *arsR* family	−11.3	−11.0
RSP0601	σ^{38} factor, *rpoH2*	+10.3	+12.3
RSP0611	Putative transcriptional regulator	+2.2	+2.8
RSP0698	*fnrL*, anaerobic activator of gene expression	+3.1	+3.0
RSP0847	Putative transcriptional regulator	+16.1	+11.8
RSP1092	σ^{E} factor, *rpoE*	+8.0	+6.6
RSP1093	Anti-σ^{E} factor, *chrR*	+10.0	+9.5
RSP1306	Putative histidine kinase	+2.2	(+1.1)
RSP1890	Transcriptional regulator, LysR family	+3.3	+3.6
RSP1997	SOS response transcriptional repressor	+3.6	+3.1
RSP2236	Transcriptional regulator, MarR family	+2.3	+3.4
RSP2410	σ^{37} factor, *rpoH1*	+9.2	+8.4
RSP3324	Bacterial regulatory protein, GerE	+4.1	+5.0
RSP4275	σ^{70} factor	+3.0	+2.4
RSP4277	Putative transcriptional regulator	+2.1	+2.2

[a] Fold change compared to the expression at time zero.
[b] The values in parentheses are insignificant changes according to the criteria used.

TABLE 3. Induction of expression of genes of the σ^E regulon by H_2O_2 and blue light under semiaerobic conditions

Gene	Description	Fold change in expression[a]		
		2.4.1, 7 min of exposure to H_2O_2	2.4.1*oxyR*, 7 min of exposure to H_2O_2	2.4.1, 5 min of exposure to blue light
RSP0600[b]	Hypothetical protein	+3.4	+2.6	+2.6
RSP0601[c]	σ^{38} factor, *rpoH2*	+10.3	+12.3	+4.8
RSP1088[b]	Hypothetical protein	+2.1	(−1.2)[d]	+4.0
RSP1089[b]	Unknown function	+2.8	(+1.2)	+4.9
RSP1090[b]	Hypothetical protein	+15.1	+5.6	+7.8
RSP1091[c]	Flavin-containing amine oxidoreductase	+14.3	+8.0	+4.9
RSP1092[c]	σ^E factor, *rpoE*	+8.0	+6.6	+4.0
RSP1093[b]	Anti-σ^E factor, *chrR*	+10.0	+9.5	+4.4
RSP1409[c]	Unknown function	+17.2	+14.4	+3.2
RSP1852[c]	Putative UV endonuclease	+12.1	+11.0	+4.3
RSP2143[c]	DNA photolyase, *phrA*	+3.7	+2.3	+3.9
RSP2144[b]	Acylphospholipid synthase	+2.4	+2.0	+3.3

[a] Fold change compared to the expression at time zero.
[b] Gene that is proposed (based on gene arrangement) to be cotranscribed with the genes of the σ^E regulon.
[c] Gene of the σ^E regulon.
[d] The values in parentheses are insignificant changes according to the criteria used.

looping of the whole DNA-protein complex (by IHF) near the promoter region (11). For the upstream region of *R. sphaeroides* RSP3751 no DNA sequences with significant homology to known promoter consensus sequences are present. It is noteworthy that the gene organization in the genome of *R. sphaeroides* 2.4.1 suggested that RSP3751 might be cotranscribed with its neighboring gene, RSP3750, but we were not able to detect any binding of OxyR to the RSP3750 upstream region.

Expression of class III genes is repressed by H_2O_2. Reduced OxyR must therefore function as an activator of transcription of these genes in the absence of exogenous H_2O_2. Oxidation of OxyR apparently reduces DNA binding (Fig. 1C and 2). There is evidence that oxidized OxyR from *E. coli* acts as an activator as well as a repressor (27, 32, 36, 37), whereas so far reduced OxyR has only been shown to act only as a repressor. This is the first experimental evidence that reduced OxyR can function as a transcriptional activator.

In summary, the DNA binding studies confirmed that our classification of differentially expressed genes is biologically meaningful. A model describing the direct OxyR mode of action with class I and class III genes is shown in Fig. 3.

Consensus *Rhodobacter* OxyR binding sequence. OxyR belongs to the group containing the LysR-type regulators, which show little conservation of their DNA target sequences (23). However, despite weak sequence conservation among the OxyR binding sites in *E. coli*, it was possible to define the *E. coli* OxyR consensus sequence as $ATAGN_7CTAT$ (where N is a nucleotide [19]). *R. sphaeroides* DNA sequences of the OxyR binding regions were not enriched with the *E. coli* OxyR consensus, even if mismatches were allowed. Furthermore, no statistically significant consensus sequence could be identified in the upstream regions. The pattern $TN_{11}A$, defining the minimum binding site for LysR-type regulators (5), was present in the majority of OxyR binding regions (not shown). However, the predictive value of this pattern is too low to be useful for identification of putative OxyR-dependent genes in the candidate genes described above.

Additional regulatory mechanisms of H_2O_2-dependent gene expression. For some of the OxyR-regulated genes the expression pattern suggests that OxyR is not the only factor involved in gene expression. Using the microarray data, we searched for putative additional regulatory factors.

The expression of 27 genes encoding proteins known or predicted to control transcript abundance changed in strain 2.4.1 after 7 min of exposure to H_2O_2 compared to the expression in untreated cells (Table 2). Because of the magnitude of the changes, it was not possible to determine all the regulatory pathways corresponding to these regulators. The majority of these genes encoding putative transcriptional regulators showed similar responses to H_2O_2 in strains 2.4.1 and 2.4.1*oxyR*, implying that they are not regulated by OxyR. Below we comment on regulatory factors characterized previously.

Several sigma factor genes were significantly upregulated after 7 min of exposure to H_2O_2. One of these genes is *rpoE* (RSP1092; +8 in 2.4.1) encoding the stress response σ factor σ^E. The *rpoE* gene is transcribed by RNA polymerase containing its own product, σ^E. The activity of σ^E depends on the anti-σ^E factor designated ChrR (RSP1093; +10 in 2.4.1) (16, 17). The fast upregulation of *rpoE* suggests that H_2O_2 is capable of inactivating ChrR-σ^E interactions, leading to upregulation of the σ^E regulon. Previously, we observed induction of the σ^E regulon after blue light irradiation of cells grown with an intermediate oxygen concentration (2). Apparently, H_2O_2 can also be sensed by these genes. All of the genes of the σ^E regulon identified by us previously (2) were upregulated after 7 min of H_2O_2 treatment, thus verifying that their assignments were correct. While the responses of some of these genes to H_2O_2 and to blue light were similar, other genes exhibited a significantly stronger response to H_2O_2 (Table 3). The *rpoH2* gene encoding the stress response σ^{38} factor belongs to the σ^E regulon (2). The observed upregulation of *rpoH2* (RSP0601; +10 in 2.4.1) and of its regulon deduced by us previously (2) is in agreement with this suggestion. Also upregulated was the *rpoH1* gene (RSP2410; +9 in 2.4.1) encoding another stress response sigma factor, σ^{37}.

To confirm that the level of expression of *rpoE* (RSP1092) is increased by H_2O_2, we performed a semiquantitative RT-PCR analysis using wild-type strain 2.4.1. A 4.3-fold increase in the level of *rpoE* expression in strain 2.4.1 after 7 min of exposure to H_2O_2 was observed, confirming the microarray data. To analyze the possible involvement of σ^E in the regulation of at least some of the OxyR-regulated genes, a semiquantitative RT-PCR analysis of wild-type 2.4.1 and the *rpoE chrR* mutant TF18 (24) was performed for the RSP0557, RSP1547, RSP1090, and RSP3751 genes after 7 min of H_2O_2 treatment. These OxyR-regulated genes were chosen since they still showed changes in gene expression after H_2O_2 treatment in 2.4.1*oxyR*, indicating that other regulators besides OxyR may also be involved in their regulation (Table 1). For RSP0557 and RSP1547 higher levels of expression were observed in strain TF18 than in the wild type after 7 min of H_2O_2 treatment (>2-fold increase). This indicates that σ^E had a negative effect on the expression of these genes. For RSP1090 we observed no change in gene expression in strain TF18 after exposure to H_2O_2. Compared to the wild-type strain, in which there was a significant increase in the level of expression, this result suggests that σ^E has an activating effect on RSP1090 expression under H_2O_2 stress conditions.

For the class III gene RSP3751 there was no difference between expression in the wild-type strain and expression in TF18, suggesting that σ^E is not involved in the regulation of this gene.

Together, these data revealed that σ^E is involved in the expression of at least some OxyR-regulated genes.

Previously, we discussed involvement of the *R. sphaeroides* FnrL protein (RSP0698) in directing the shift toward high-oxygen metabolism during exposure to H_2O_2 of semiaerobically grown *R. sphaeroides* (35). *R. sphaeroides* FnrL is a homologue of a regulator of anaerobic metabolism, FNR (10, 33). Apparently, the role of FnrL extends to H_2O_2 stress defense per se. In all the H_2O_2-regulated genes, we identified a putative FnrL consensus site, $TTGN_8CAA$, upstream of RSP1547 encoding bacterioferritin-associated ferredoxin (position "−73" relative to the first codon; http://genome.ornl.gov/microbial/rsph). To test whether FnrL is involved in the regulation of these genes under H_2O_2 stress conditions, we performed a semiquantitative RT-PCR analysis for the RSP1547 gene in wild-type strain 2.4.1 and *fnrL* mutant JZ1678 (33). After 7 min of exposure to H_2O_2 the level of expression of RSP1547 was more than fourfold higher in the *fnrL* mutant than in strain 2.4.1. This indicates that FnrL had a repressing effect on RSP1547 expression under H_2O_2 stress conditions. Most likely, RSP1547 is under direct control of FnrL. The indirect effect of OxyR on RSP1547 expression (Fig. 1 B) may therefore be due to an effect of elevated levels of ROS in strain 2.4.1*oxyR* on the iron-sulfur center of FnrL. As mentioned above, RSP1547 seems also to be regulated by the sigma factor σ^E. This shows that the response to H_2O_2 in *R. sphaeroides* not only is mediated by OxyR but also involves several other regulators, which, in some cases, even regulate identical genes (e.g., σ^E and FnrL for RSP1547).

Concluding remarks. Multiple regulatory pathways are involved in the response to H_2O_2 in *R. sphaeroides*. Most of these pathways are transient and operate during the first few minutes after oxidative stress is imposed. The OxyR-dependent pathway plays a critical role in long-term survival during H_2O_2 stress because it is required for induction of the catalase gene, *katE*, which encodes the main H_2O_2-detoxifying enzyme in *R. sphaeroides*. Several members of the OxyR regulon have been identified, and OxyR binding to class I and III genes was verified. OxyR can function as an activator of gene expression in its oxidized form, as well as in the reduced form. Based on verified OxyR targets, it appears that the *R. sphaeroides* OxyR regulon contains only a few members characteristic of the OxyR regulons in other bacteria, including *E. coli*. These genes are genes of the *suf* operon and *katE* (36). However, the *R. sphaeroides* OxyR regulon also includes unique genes, most of which have unknown or poorly characterized functions. These genes represent targets for subsequent functional analysis. The OxyR pathway appears to be one of several regulatory pathways involved in the oxidative stress response in *R. sphaeroides*.

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