AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

# OxyR-Dependent Transcription Response of *Sinorhizobium meliloti* to Oxidative Stress

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**ABSTRACT** Reactive oxygen species such as peroxides play an important role in plant development, cell wall maturation, and defense responses. During nodulation with the host plant *Medicago sativa*, *Sinorhizobium meliloti* cells are exposed to $H_2O_2$ in infection threads and developing nodules (R. Santos, D. Hérouart, S. Sigaud, D. Touati, and A. Puppo, Mol Plant Microbe Interact 14:86–89, 2001, https://doi.org/10.1094/MPMI.2001.14.1.86). *S. meliloti* cells likely also experience oxidative stress, from both internal and external sources, during life in the soil. Here, we present microarray transcription data for *S. meliloti* wild-type cells compared to a mutant deficient in the key oxidative regulatory protein OxyR, each in response to $H_2O_2$ treatment. Several alternative sigma factor genes are upregulated in the response to $H_2O_2$; the stress sigma gene *rpoE2* shows OxyR-dependent induction by $H_2O_2$, while *rpoH1* expression is induced by $H_2O_2$ irrespective of the *oxyR* genotype. The activity of the RpoE2 sigma factor in turn causes increased expression of two more sigma factor genes, *rpoE5* and *rpoH2*. Strains with deletions of *rpoH1* showed improved survival in $H_2O_2$ as well as increased levels of *oxyR* and total catalase expression. These results imply that Δ*rpoH1* strains are primed to deal with oxidative stress. This work presents a global view of *S. meliloti* gene expression changes, and of regulation of those changes, in response to $H_2O_2$.

**IMPORTANCE** Like all aerobic organisms, the symbiotic nitrogen-fixing bacterium *Sinorhizobium meliloti* experiences oxidative stress throughout its complex life cycle. This report describes the global transcriptional changes that *S. meliloti* makes in response to $H_2O_2$ and the roles of the OxyR transcriptional regulator and the RpoH1 sigma factor in regulating those changes. By understanding the complex regulatory response of *S. meliloti* to oxidative stress, we may further understand the role that reactive oxygen species play as both stressors and potential signals during symbiosis.

**KEYWORDS** OxyR, RpoH, *Sinorhizobium meliloti*, catalase, oxidative stress, sigma factors, transcriptome

Reactive oxygen species (ROS) are omnipresent for aerobic organisms. Enzyme function and the imperfect transfer of electrons to oxygen during aerobic respiration contribute to intracellular production of ROS (reviewed in reference 1). Bacteria also encounter environmental sources of ROS. Soil bacteria might experience ROS from redox-cycling compounds secreted by neighboring cells or from exposure to metals and other compounds in the environment (2).

The majority of the toxicity of ROS such as superoxide and $H_2O_2$ does not appear to arise from direct damage to amino acids, lipids, or nucleic acids. Rather, damage is primarily caused by inactivation of iron cofactors in proteins and, through production of other radicals, by damage to DNA (reviewed in reference 3). For example, $H_2O_2$ reacts with $Fe^{2+}$ via the Fenton reaction to produce hydroxyl radicals, resulting in severe DNA damage (4).

**Received** 25 October 2017 **Accepted** 11 January 2018

**Accepted manuscript posted online** 22 January 2018

**Citation** Lehman AP, Long SR. 2018. OxyR-dependent transcription response of *Sinorhizobium meliloti* to oxidative stress. J Bacteriol 200:e00622-17. https://doi.org/10.1128/JB.00622-17.

**Editor** Thomas J. Silhavy, Princeton University



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As a result of the ubiquity of ROS in aerobic life, numerous cellular defenses against oxidative stress, including those represented by catalases, peroxidases, glutathione, and ascorbate, have developed (2). Replacement and repair of iron-sulfur clusters, increased expression of alternative proteins utilizing different metal cofactors, and downregulation of metabolism are some additional mechanisms used to survive $H_2O_2$-dependent damage (5, 6). Similarly, regulation of iron transport to limit exposure of iron to $H_2O_2$ is a common response to ROS stress. Increased expression of genes that encode DNA repair proteins is also a common response to oxidative stress (2).

The symbiotic nitrogen-fixing bacterium *Sinorhizobium meliloti* exhibits a complex lifestyle: it undergoes free-living growth as a soil saprophyte, but it also elicits and inhabits root nodules of legumes of *Medicago* spp. *S. meliloti* is exposed to ROS in both of these environments (7–11). Plants use $H_2O_2$ as a signal and may transport extracellular $H_2O_2$ into adjacent cells to facilitate signaling (12). ROS play a major role in cell wall formation and in defense reactions in plants (13, 14). Additionally, early events in symbiosis are characterized by suppression followed by enhancement of ROS production in the host plant (11, 15, 16).

As enzymatic defenses against ROS, *S. meliloti* is predicted to have two monofunctional catalases, KatA (encoded on the chromosome) and KatC (encoded on pSymB), a biofunctional catalase-hydroperoxidase, KatB (encoded on pSymA), and a predicted *ahpC*-type alkyl hydroperoxidase, SMb20964 (17). These catalases are expressed in distinct environmental conditions and at specific stages in symbiosis. In culture, the *katA* gene is expressed in exponential growth whereas *katC* is expressed in late stationary phase (18). The genes also respond in various ways to heat shock, osmotic shock, and acid shock and are differentially expressed in nodulation. Individual *katA* or *katC* mutations have no impact on nodulation, but double mutants show lowered nitrogen fixation (18, 19). Among these catalases, KatA is the only one whose expression is increased in response to the presence of $H_2O_2$, and its expression is dependent on the OxyR transcriptional regulator (19, 20).

In *Escherichia coli* and other proteobacteria, OxyR is the primary transcriptional regulator of the $H_2O_2$ stress response (21, 22). OxyR directly senses the redox status in the cell (23, 24). When oxidative stress levels are low, OxyR is in a reduced form that allows it to bind to two adjacent major grooves in DNA, preventing RNA polymerase (RNAP) from recognizing a promoter. When levels of oxidative stress increase, disulfide bridges form within the OxyR protein, leading to a conformational change (23, 24) that allows OxyR to shift its binding upstream to four adjacent major grooves in DNA and to directly interact with RNAP to activate transcription of target genes (25).

In *E. coli*, OxyR regulates the transcription of at least 40 genes (21), including genes involved in reducing levels of ROS in the cell (encoding catalase, alkyl hydroperoxidase, and glutaredoxin 1), genes whose effect is to minimize free iron and diminish DNA damage (such as genes encoding iron-binding proteins and *fur*, encoding a transcription repressor of iron import functions), and genes involved in protein repair (encoding proteins with functions such as Fe-S cluster assembly and chaperone regulation) (5, 21, 22). The sequence of OxyR is well conserved in proteobacterial species (26). *S. meliloti* and *E. coli* share the same genomic context of *oxyR* in that *oxyR* is divergently transcribed from *katA* (27). In *E. coli*, OxyR negatively regulates its own expression and positively regulates the expression of the bifunctional catalase/peroxidase gene *katG*, which is induced by $H_2O_2$ (28). Similarly, *S. meliloti* OxyR negatively regulates its own expression. Unlike *E. coli*, however, *S. meliloti* OxyR both represses and activates *katA* expression depending on its oxidation state (27). *oxyR* is specifically expressed during symbiosis in the nitrogen-fixing zone (27). While ROS appears to play a significant role under free-living and symbiotic conditions, the *S. meliloti* transcriptional response to $H_2O_2$ has not as yet been reported.

One way that bacteria can express large-scale transcriptional change is through the use of alternative sigma factors ($\sigma$s) (29–32). Sigma factors are subunits of RNAP necessary for promoter recognition and transcription initiation. Under normal conditions, a housekeeping $\sigma$ (sigma$^{70}$, encoded by *rpoD*) recognizes and drives

transcription of a large number of genes. Alternative $\sigma$s recognize distinct promoter sequences, generally driving transcription of genes of shared function. To change the cell's transcriptional program, alternative $\sigma$s compete with the housekeeping $\sigma$ for core RNAP. Changing the availability of alternative $\sigma$s to interact with RNAP regulates which promoters are recognized and thus which genes are expressed (reviewed in reference 33).

Bacteria that engage in complex lifestyles tend to have greater numbers of alternative sigma factors than those with simpler lifestyles (34). Fourteen alternative $\sigma$s are encoded in the *S. meliloti* genome; this abundance is consistent with the idea that *S. meliloti* experiences a variety of different environments and stresses in the soil and during nodulation. However, the role of alternative $\sigma$s during different life stages and under different stress conditions in *S. meliloti* is not well known and is an active area of research (35–40).

RpoE2 is an extracytoplasmic function-type (ECF-type) $\sigma$ thought to function as the general stress response sigma factor (36). While RpoE2 is not required for symbiosis, expression of *rpoE2* is increased in response to many different stresses, including stationary-phase growth, heat shock, osmotic stress, and microaerobic stress (36, 37, 41, 42). RpoE2 directly or indirectly controls 346 genes (39), including *katC* and alternative sigma factor genes *rpoE5* and *rpoH2* (36, 43).

The RpoH1 $\sigma$ regulates genes in response to heat shock, stationary phase (37, 40), and pH stress (38) and is required for symbiosis (40, 44, 45). Although both RpoE2 and RpoH1 respond to stress, the two regulons appear to be largely independent, with few overlapping genes (37).

To understand more fully the responses of *S. meliloti* to $H_2O_2$, we performed whole-genome transcriptional analysis of wild-type (WT) and *oxyR* mutant *S. meliloti* strains exposed to two concentrations of $H_2O_2$. We also explored the regulation of the $H_2O_2$ response using *S. meliloti* strains with deletions of alternative $\sigma$ genes. This allowed us to define the *S. meliloti* transcriptional response to oxidative stress induced by $H_2O_2$, to show its dependence on OxyR, and to reveal the role of alternative $\sigma$ RpoH1 in regulating that response.

## RESULTS

**$H_2O_2$ induces a large number of transcriptional changes in *S. meliloti*.** Expression of *katA* is induced by $H_2O_2$ and correlates with oxidative stress levels (20). In order to define the response of *S. meliloti* to $H_2O_2$ challenge, whole-genome transcriptional profiling was performed. All experiments were done in a background of *S. meliloti* CL150, a WT derivative of strain Rm1021, corrected for the function of anti-sigma factor EcfR1 and high-affinity phosphate transporter PstC (39). We chose this strain in case ECF sigma factor RpoE1 played a role in the ROS response. Additionally, we used a *katA*::*uidA* transcriptional fusion to ascertain the conditions with maximal induction of *katA* expression. Comparing expression of *katA* in the CL150 background to that occurring in the WT Rm1021 background, we saw that the basal expression of *katA* in the CL150 background was nearly half that in the WT Rm1021 background. With these lower basal levels of *katA* expression, CL150 showed a nearly 4-fold-larger increase in *katA* expression after $H_2O_2$ exposure (data not shown). The initial conditions used for transcriptome profiling were mid-exponential-phase *S. meliloti* CL150 (optical density at 600 nm [$OD_{600}$] = 0.3 to 0.5) grown in rich medium (TY) treated with 1 mM $H_2O_2$ for 30 min. These were the time and concentration that showed maximum expression of *katA* using the *katA*::*uidA* fusion (data not shown).

In total, 1,370 genes were differentially regulated by $H_2O_2$: 789 genes were upregulated (see Table S1 in the supplemental material), and 581 genes were downregulated (Table S2). Fifteen genes were selected for testing differential expression by reverse transcription-PCR (RT-qPCR) to confirm the Affymetrix GeneChip results (Fig. 1). All of these genes showed similar expression changes in tests using RT-qPCR and Affymetrix GeneChip.

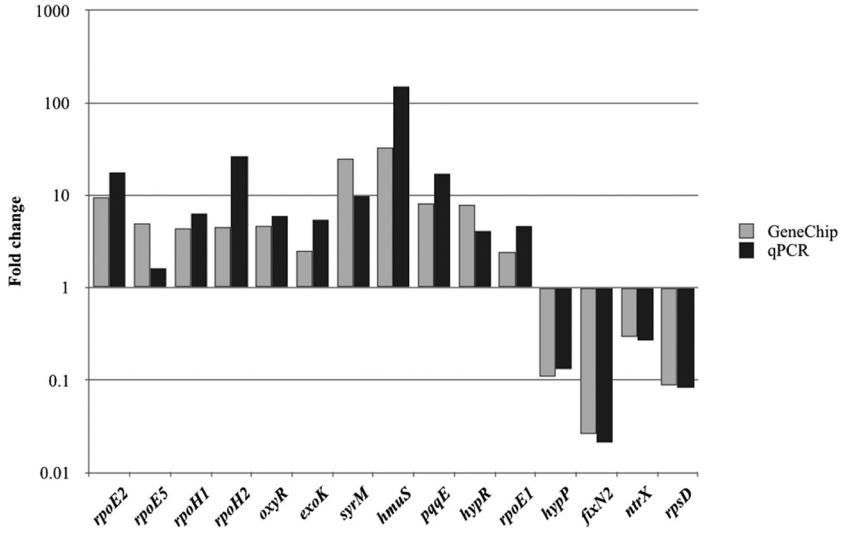


**FIG 1** Comparison qPCR and chip expression data. Data represent fold changes in gene expression of selected genes differentially regulated by the use of 1 mM $H_2O_2$ as determined in three replicates each using Affymetrix GeneChip and qPCR. Raw data and statistical analysis are shown in Table S4.

The tripartite *S. meliloti* genome (6.69 Mb in size) is composed of a 3.65-Mb chromosome (55%), 1.35-Mb pSymA (20%), and 1.68-Mb pSymB (25%). While genes whose expression was increased in response to $H_2O_2$ were distributed fairly evenly (chromosome, 60%; pSymA, 20%; pSymB, 20%), those whose expression was decreased were biased toward the chromosome (93%), with only 1% on pSymA and 6% on pSymB. This strong chromosomal bias of downregulated genes corresponds broadly to decreased expression of genes encoding housekeeping functions. For example, expression of the gene set encoding translation machinery was sharply downregulated in response to $H_2O_2$ (Table 1). As expected, several genes whose expression was upregulated in response to $H_2O_2$ have known antioxidant activities (Table 1).

**The OxyR regulon in *S. meliloti*.** To delineate the part of the *S. meliloti* $H_2O_2$ response that requires OxyR, an insertion mutant in *oxyR* was generated. An *S. meliloti* mutant in *oxyR* (in strain Rm1021) was previously reported to be more sensitive to $H_2O_2$ (27, 46). To determine optimal conditions for transcriptional analysis of the CL150 *oxyR* mutant strain, its survival was tested upon challenge with various concentrations of $H_2O_2$ (data not shown). Since mid-exponential-phase wild-type and *oxyR* mutant cells showed similar levels of survival after a 30-min exposure to 0.5 mM $H_2O_2$, this nonlethal level of $H_2O_2$ was used for direct comparison of these strains via Affymetrix GeneChip analysis.

When WT cells were exposed to 0.5 mM $H_2O_2$, 396 genes showed increased expression and 420 showed decreased expression. Most of these genes (358 whose expression was upregulated and 358 whose expression was downregulated) were also found in the set of genes differentially regulated by exposure to 1 mM $H_2O_2$ (Fig. 2). The *oxyR* mutant, on the other hand, showed increased expression of 236 genes, and the expression of 300 genes was decreased by $H_2O_2$ treatment. The majority of these genes were also differentially regulated by $H_2O_2$ in wild-type *S. meliloti* (Fig. 2); thus, while these genes are differentially expressed in response to $H_2O_2$ exposure, that expression is not dependent on OxyR.

**OxyR is required for part of the response to $H_2O_2$.** OxyR-dependent genes were defined by comparing genes differentially regulated in the wild-type strain (after treatment with both 1 mM and 0.5 mM $H_2O_2$) but not in the *oxyR* mutant (Table S3). In total, transcription of 203 genes (57% of the genes upregulated by both 1 mM and 0.5 mM $H_2O_2$) was increased after $H_2O_2$ addition only in wild-type *S. meliloti*, and expression of 134 (37% of the genes downregulated by both levels of $H_2O_2$) genes was

**TABLE 1** Functional categories of genes differentially regulated by $H_2O_2$[a]

| Gene category | No. of genes with: | |
|---|---|---|
| | Increased expression | Decreased expression |
| Amino acid transport and metabolism | 26 | 44 |
| Carbohydrate transport and metabolism | 5 | 15 |
| Cell cycle control, mitosis, and meiosis | 2 | 2 |
| Cell motility | 0 | 2 |
| Cell wall/membrane biogenesis | 13 | 11 |
| Coenzyme transport and metabolism | 7 | 7 |
| Energy production and conversion | 32 | 32 |
| Function unknown | 3 | 4 |
| Hypothetical protein | 129 | 45 |
| Inorganic ion transport and metabolism | 16 | 5 |
| Lipid transport and metabolism | 14 | 12 |
| Nitrogen regulation | 1 | 0 |
| Nucleotide transport and metabolism | 7 | 28 |
| Posttranslational modification, protein turnover, chaperones | 17 | 13 |
| Replication, recombination, and repair | 26 | 9 |
| RNA | 0 | 15 |
| Secondary metabolite biosynthesis, transport, and catabolism | 9 | 4 |
| Secretion | 0 | 8 |
| Signal transduction mechanisms | 9 | 4 |
| Stress | 3 | 0 |
| Toxin | 0 | 1 |
| Transcription | 36 | 11 |
| Translation | 3 | 86 |

[a]Functional gene categories were determined using clusters of orthologous groups. Categories were refined when possible based on reported gene functions.

decreased. These regulatory changes are presumed to be directly or indirectly dependent on OxyR.

*rpoE2* expression was induced by $H_2O_2$ in an OxyR-dependent manner, but expression of *rpoH1* was increased irrespective of the *oxyR* genotype. Expression levels for several genes encoding antioxidant functions were increased in response to $H_2O_2$, and the changes were dependent on wild-type *oxyR*. The products encoded by those genes included SMb20964, the *ahpC*-type alkyl hydroperoxidase, and three other catalases and peroxidases (Table 2 and Table S3). Increased expression of *katA* and *oxyR* also depended on WT OxyR. This serves as a useful internal control, since OxyR is reported to downregulate its own expression and that of *katA*. In an *oxyR* mutant (27), the repression of *katA* and *oxyR* is relieved. Our interpretation is that expression of these

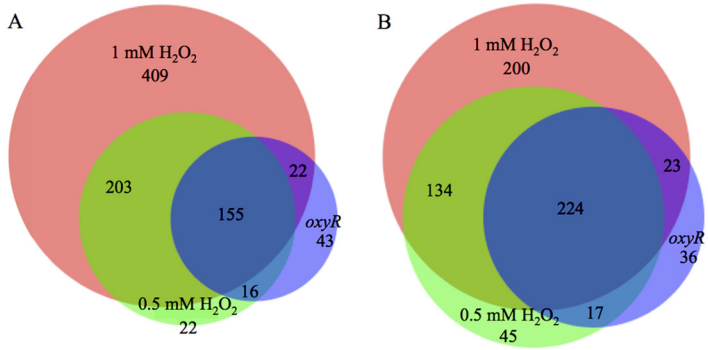


**FIG 2** Overlap of genes differentially regulated in response to two concentrations of $H_2O_2$ and in the *oxyR* mutant. (A) Genes upregulated in response to $H_2O_2$ in wild-type CL150 (treated with 1 mM or 0.5 mM $H_2O_2$) and in the *oxyR* mutant (treated with 0.5 mM $H_2O_2$). (B) Genes downregulated in response to $H_2O_2$ in wild-type CL150 (treated with 1 mM or 0.5 mM $H_2O_2$) and in the *oxyR* mutant (treated with 0.5 mM $H_2O_2$).

**TABLE 2** Representative genes regulated by $H_2O_2$

| Gene | Designation | Description of product | SLR | | |
|---|---|---|---|---|---|
| | | | 1 mM CL150 | 0.5 mM CL150 | 0.5 mM *oxyR* mutant |
| Antioxidants and regulators | | | | | |
| *katA* | SMc00819 | Catalase | 8.7 | 6.16 | |
| *cpo* | SMc01944 | Nonheme chloroperoxidase F (chloride peroxidase; CPO-F) | 4.46 | 2.25 | |
| | SMb20054 | Chloride peroxidase | 4.22 | 1.95 | |
| | SMb20964 | Peroxidase, AhpC family | 2.18 | 1.5 | 0.65 |
| *oxyR* | SMc00818 | Regulator of *kat* genes | 2.23 | 3.38 | |
| Sigma factors | | | | | |
| *rpoH1* | SMc00646 | RNA polymerase sigma factor (sigma-32) | 2.15 | 1.61 | 1.37 |
| *rpoE2* | SMc01506 | RNA polymerase sigma-E factor (sigma-24) | 3.27 | 1.91 | |
| *rpoH2* | SMc03873 | RNA polymerase sigma factor (sigma-32) | 2.19 | | |
| *rpoE5* | SMb21484 | Putative RNA polymerase sigma-E factor (sigma-24) protein | 2.3 | | |
| Degradation | | | | | |
| *paaA* | SMb21640 | Putative phenylacetic acid degradation protein | 3.75 | 4.09 | 2.4 |
| *paaB* | SMb21639 | Putative phenylacetic acid degradation protein | 6.28 | 4.84 | 4.85 |
| Manganese regulation | | | | | |
| *sitA* | SMc02509 | Manganese ABC transporter periplasmic substrate binding protein | 2.01 | 2.31 | 1.62 |
| *sitB* | SMc02508 | Manganese ABC transporter ATPase | 2.56 | 2.64 | 1.81 |
| *sitC* | SMc02507 | Manganese ABC transporter permease | 2.29 | 2.62 | 1.82 |
| *sitD* | SMc02506 | Manganese ABC transporter permease | 2.3 | 2.57 | 1.85 |
| Miscellaneous | | | | | |
| *exoP* | SMb20961 | Protein tyrosine kinase, involved in succinoglycan chain-length determination | 1.97 | | |
| *glgA1* | SMc03924 | Probable glycogen synthase (starch [bacterial glycogen] synthase) protein | 1.09 | | |
| | SMc01113 | Conserved hypothetical protein | 1.01 | | 0.99 |

two genes in the *oxyR* mutant is higher prior to addition of $H_2O_2$ such that any further increase in expression in response to $H_2O_2$ is not significant.

**A Δ*rpoH1* mutant shows increased survival in $H_2O_2$.** Expression levels for the alternative sigma factor genes *rpoH1*, *rpoH2*, *rpoE2*, and *rpoE5* are increased in response to $H_2O_2$ (Table 2). To see if these genes play a functional role in the cell's response to $H_2O_2$, *S. meliloti* mutants with complete deletions of these alternative sigma factor genes were tested for survival after a 30-min exposure to 1 mM $H_2O_2$. To ensure that the anti-sigma factors that regulate RpoE2 and RpoE5 did not interfere with the activity of other ECF sigma factors in the cell, deletion constructs for *rpoE2* and *rpoE5* also included deletion of the putative cognate anti-sigma factor gene. The *rpoE2* and *rpoE5* deletion mutants tended to survive exposure to $H_2O_2$ less well than the wild-type strain, but this difference was not significant in three independent experiments (Fig. 3 and data not shown). However, a mutant carrying a complete deletion of *rpoH1* (Δ*rpoH1* and Δ*rpoH1H2*) showed a slight but significant improvement in $H_2O_2$ survival compared to the wild-type strain (Fig. 3).

**Baseline *oxyR* expression is increased in Δ*rpoH* mutants.** One possible explanation for the improved survival of the Δ*rpoH1* mutant upon $H_2O_2$ treatment is higher expression of genes that protect against oxidative stress. To test if the levels of expression of *oxyR* differed in these mutants, a *uidA* transcriptional fusion to *oxyR* was generated and inserted into the alternative sigma factor deletion strains. Baseline expression of *oxyR* was higher in both *rpoH1* deletion mutants. Figure 4 shows that transcription of *oxyR* increased significantly in the wild-type strain and in the Δ*rpoE2* and Δ*rpoE5* mutants after exposure to $H_2O_2$. In contrast, *oxyR* expression levels did not increase significantly above the already elevated baseline in the Δ*rpoH1* and Δ*rpoH1H2* mutants.

**Total catalase activity is increased in a Δ*rpoH1* mutant.** To check if increased expression of *oxyR* in the sigma factor deletion mutants resulted in increased antioxi-

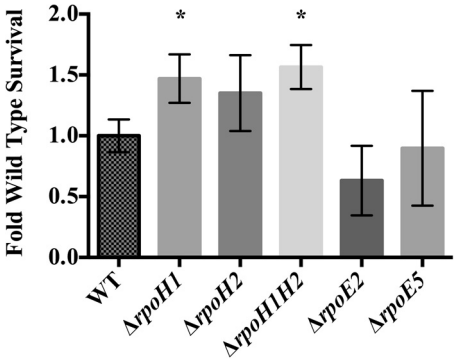


**FIG 3** Effect of $H_2O_2$ on *S. meliloti* mutants with complete deletions of alternative sigma factors. Survival rates were determined after 30 min of exposure to $H_2O_2$ of *S. meliloti* strains deleted for alternative sigma factors implicated in the response to $H_2O_2$. Survival was normalized to wild-type CL150 survival (10.3% ± 1.6%). Error bars indicate standard deviations; *, $P \leq 0.05$. Experiments were performed in triplicate; two other independent experiments were performed with comparable results.

dant activity, total catalase activity was tested using Amplex Red, a stable, highly sensitive probe for $H_2O_2$. As expected, exposure to $H_2O_2$ increased overall catalase activity in all mutants tested. Similarly to the pattern observed with *oxyR* expression, Δ*rpoH1* and Δ*rpoH1H2* mutants had higher catalase activity before exposure to $H_2O_2$ (Fig. 5B). Catalase expression levels were similar whether normalized to $OD_{600}$ or to total cell protein (data not shown).

The *S. meliloti* genome includes three catalase genes (*katA*, *katB*, and *katC*) as well as at least one *ahpC*-type alkyl hydroperoxidase (SMb20964). To determine whether transcription of *katA* was responsible for the increased enzyme activity in the Δ*rpoH1* and Δ*rpoH1H2* mutants, we generated and tested *katA-uidA* transcriptional fusions. We observed no significant difference in *katA* expression levels in any of the sigma factor deletion strains with or without $H_2O_2$ treatment (Fig. 5B). RT-qPCR was used to test expression of the other catalases, using primers specific for *katB*, *katC*, and SMb20964. Expression of these catalase/peroxidase genes was not significantly changed in any strain (data not shown).

## DISCUSSION

*S. meliloti* responds to $H_2O_2$ stress with differential expression of over 600 genes, a set that includes both expected and novel candidates. We defined the relationship of

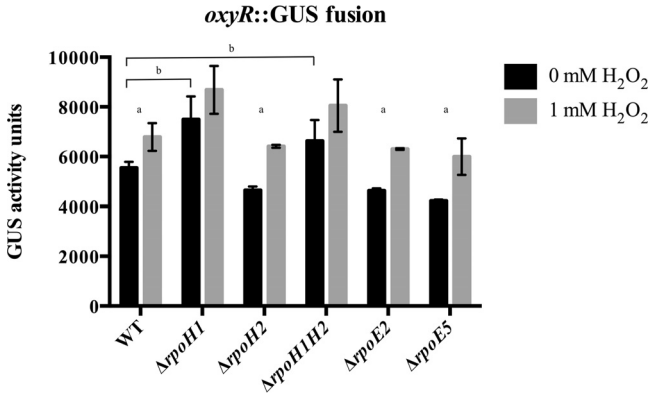


**FIG 4** Transcription of *oxyR* in various *S. meliloti* mutants deleted for alternative sigma factors. Data represent expression of a *oxyR-uidA* transcriptional fusion with and without 30 min of treatment with 1 mM $H_2O_2$ in mutants lacking various *S. meliloti* alternative sigma factors. Error bars indicate standard deviations. "a" indicates a *P* value of ≤0.05 compared to 0 mM $H_2O_2$. "b" indicates a *P* value of ≤0.05 comparing the activity of the mutant strain at 0 mM $H_2O_2$ to the activity of the wild-type strain at 0 mM $H_2O_2$. Experiments were performed in triplicate.

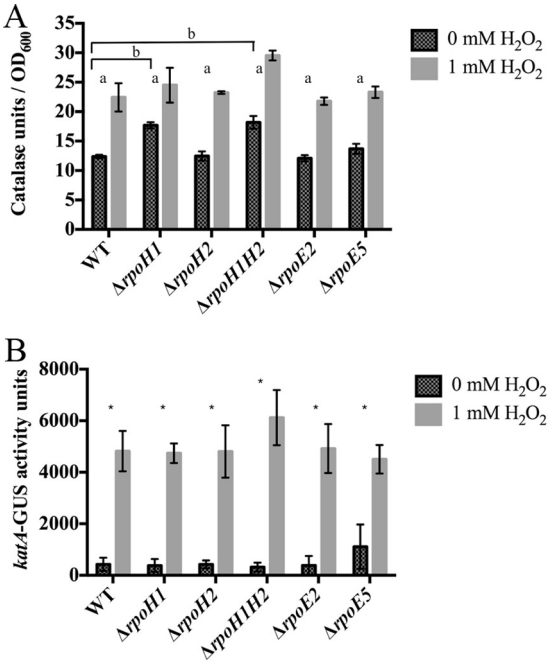


**FIG 5** Catalase activity in *S. meliloti* stains deleted for alternative sigma factors. (A) Total catalase activity of various alternative sigma factor deletions with and without 30 min of treatment with 1 mM $H_2O_2$. Catalase activity was normalized to $OD_{600}$. Error bars indicate standard deviations. "a" indicates a *P* value of ≤0.05 compared to 0 mM $H_2O_2$. "b" indicates a *P* value of ≤0.05 comparing the activity of the mutant strain at 0 mM $H_2O_2$ to the activity of the wild-type strain at 0 mM $H_2O_2$. Experiments were performed in triplicate. (B) Activity of *katA-uidA* transcriptional fusion with and without 30 min of treatment with 1 mM $H_2O_2$. Error bars indicate standard deviations. *, $P \leq 0.05$ (comparing the activity of the mutant strain at 0 mM $H_2O_2$ to the activity of the wild-type strain at 0 mM $H_2O_2$). Experiments were performed in triplicate.

this gene set with transcription control by the LysR-type regulator, OxyR, and discovered an OxyR-independent connection between the RpoH1 sigma factor and response to oxidative stress.

We tested only a limited subset of oxidative stress conditions (using a single oxidant, one time point, and just two concentrations). However, despite these limitations, this work agrees well with other studies characterizing the *S. meliloti* response to ROS. For example, three genes upregulated by $H_2O_2$ in our data set (*exoP*, *glgA1*, and *sitA*) correspond to $H_2O_2$-sensitive mutants discovered by Davies and Walker (47); these three mutants were also defective in symbiosis. Our upregulated gene set also included a gene encoding a secreted peroxidase (SMc01944) and a gene of unknown function (SMc01113) whose expression has been previously shown to be induced by $H_2O_2$ (48, 49).

**Role of OxyR.** Genes that were upregulated in response to $H_2O_2$ in wild-type *S. meliloti* but not in the *oxyR* mutant (57% of the genes generally upregulated by $H_2O_2$) are presumed to be directly or indirectly dependent on the OxyR transcription factor. The OxyR-dependent transcripts are distributed proportionally across all three replicons of the *S. meliloti* genome and include some functions expected as part of an oxidative stress response. The monofunctional catalase gene (*katA*) was previously reported to be divergently transcribed from and dependent on *oxyR* (20, 27). In our data, *katA* was the transcript most strongly upregulated in wild-type cells in response to $H_2O_2$ exposure, and that upregulation was dependent on OxyR. In *E. coli*, AhpC, an alkyl hydroperoxide reductase, is a primary antioxidant expressed in response to $H_2O_2$, and its expression is induced in an OxyR-dependent manner (1, 21). We found that expression of the putative *S. meliloti* ortholog SMb20964 is likewise induced by $H_2O_2$ and dependent on OxyR. Similarly, the $H_2O_2$-induced increase in the levels of transcripts for genes encoding the stress-associated ECF-type sigma factor RpoE2 (36) showed dependence on

OxyR. This study did not address which transcripts are directly or indirectly regulated via OxyR. Future work may be able to elucidate the more direct role of OxyR in the *S. meliloti* transcriptional response to $H_2O_2$.

Numerous genes were differentially regulated by $H_2O_2$ independently of OxyR, including those involved in manganese transport (*sitABCD*) and those predicted to be involved in phenylacetic acid degradation (*paaA* and *paaB* genes). The *S. meliloti sit* genes are among those that have already been found to be involved in ROS resistance (50). Genes in the *paa* operon are differentially expressed during nodulation (51, 52) and are part of the general stress response regulon mediated by RpoE2 (36). Expression of *rpoE2* itself appears to be OxyR dependent. However, $H_2O_2$-induced expression of *rpoH1* and of RpoH1-dependent genes still occurs in the *oxyR* mutant strain. This implies that $H_2O_2$ stress is sensed and transduced to RpoH1 through some mechanism that is independent of OxyR.

**Sigma factors.** Because alternative sigma factors change the recognition of promoters, the consequent changes in gene expression are often numerous. Upregulation of the two alternative sigma factor genes *rpoH1* and *rpoE2* in response to $H_2O_2$ may reflect a need for broad changes in gene expression. Flechard et al. (43) reported that RpoE2 activity was relevant to ROS stress in stationary-phase cells. While 1 mM $H_2O_2$ induced expression of four alternative sigma factor genes (*rpoH1*, *rpoH2*, *rpoE2*, and *rpoE5*), only deletion of *rpoH1* had an effect on survival in 1 mM $H_2O_2$. While deletion of *rpoE2* and *rpoE5* did seem to reduce survival in 1 mM $H_2O_2$, this reduction was not significant. It is possible that these deletion mutants are indeed more sensitive to either other ROS or different concentrations of $H_2O_2$. Investigating the role of RpoE2 and RpoE5 in the *S. meliloti* ROS response may be a fruitful area for future work.

Because of the evidence showing a positive role for RpoH in gene expression related to stress resistance (37) and because Martínez-Salazar et al. (53) showed that *Rhizobium etli rpoH* mutants are more sensitive to $H_2O_2$, we were surprised that deletion of *rpoH1* in *S. meliloti* resulted in increased survival after $H_2O_2$ challenge. The increase in the level of survival was slight, but significant, and was similar to the improved levels of survival seen when wild-type *S. meliloti* cells were pretreated with low concentrations of $H_2O_2$.

Strains lacking *rpoH1* may experience higher levels of oxidative stress than wild-type *S. meliloti* under normal growth conditions because they do not express baseline defenses against endogenous stresses (i.e., under conditions without imposed external stress). Consequently, this postulated higher degree of stress may "prime" the cells to deal more effectively with later increased levels of external $H_2O_2$. The idea of priming is consistent with the observed higher levels of both *oxyR* expression and catalase activity in Δ*rpoH1* strains than in the wild-type strain under normal culture conditions. Perhaps more OxyR is in an oxidized state and *oxyR* expression is derepressed in strains lacking *rpoH1* (46). We have shown that OxyR regulates, directly or indirectly, almost half of the response to $H_2O_2$ in *S. meliloti*, and some of these genes may be responsible for improved survival of Δ*rpoH1* mutants in $H_2O_2$.

**Interaction of RpoH1 with ROS stress responses.** The higher total catalase activity seen in the *rpoH1* deletion mutant correlates with higher *oxyR* expression. Which of the three known *S. meliloti* catalases might be responsible for the increased activity is uncertain. OxyR regulates expression of *katA* and *katB* (46), and *katB* may also be regulated by ActR/ActS. The *katC* gene is primarily expressed during the stationary phase (18), and its expression is also RpoE2 dependent. Disruption of OxyR function in *S. meliloti* leads to higher levels of expression of *katA* and *katB* but lower overall catalase activity (46), indicating that OxyR positively influences the activity of other catalases in the cell.

Expression of SMb20964, the *ahpC*-type alkyl hydroperoxidase, also appears to be unchanged in these sigma factor deletion mutants. We found expression of this gene to be increased by exposure to $H_2O_2$ in an OxyR-dependent manner, while *katA* was the only annotated catalase gene upregulated in response to $H_2O_2$ (also in an OxyR-dependent manner). *katB* and *katC* were not differentially regulated by $H_2O_2$ in either

the wild-type strain or the *oxyR* mutant. RT-qPCR detection of gene expression in *S. meliloti* mutants after exposure to $H_2O_2$ showed that SMb20964 expression does increase in response to $H_2O_2$, and there were similar levels of increased expression after $H_2O_2$ treatment in all strain backgrounds. Perhaps higher levels of OxyR, as found in Δ*rpoH1* and Δ*rpoH1H2* mutants, increase total catalase activity either by acting on other, unknown catalases or peroxidases or by a combination of small increases in the activities of all catalases. Future work will be needed to differentiate between these possibilities (or to identify additional causes of this increased basal catalase activity).

While RpoH1-dependent genes corresponding to heat shock, stationary phase, and acid stress have been defined previously (37, 38, 40), additional RpoH1-dependent genes may be activated during $H_2O_2$ stress. Based on the increased survival of Δ*rpoH1* mutants seen under conditions of $H_2O_2$ challenge, it seems possible that wild-type cells express some unknown RpoH1-dependent genes at low levels prior to encountering any external source of stress. When this low background expression of presumed RpoH1-dependent genes is missing (as in the case of the Δ*rpoH1* mutant), the cells might lack some of the defenses against endogenously produced ROS, leading to a higher basal level of ROS stress even in the absence of environmentally introduced ROS. This, in turn, might lead to activation of ROS responses that normally would be expressed only after exposure to higher levels of ROS. In sum, we infer that when cells are missing RpoH1-dependent responses, both *oxyR* expression and catalase activity increase. Future work will be needed to confirm this hypothesis and to identify which, if any, RpoH1-dependent genes are responsible for the improved survival of the Δ*rpoH1* mutant in $H_2O_2$.

Transcriptional profiling of an *rpoH1H2* double mutant showed some genes with increased expression in the mutant, prominently including predicted transporters, other membrane proteins, and enzymes for small-molecule metabolism. de Lucena et al. (38) reported that *S. meliloti rpoH1* mutants cultured under nonstressed conditions display an increase in rhizobactin synthesis gene expression compared to the wild type, as measured by hybridization to spotted arrays and by rhizobactin production. Our data determined using the Affymetrix GeneChip employed here do not show a rhizobactin expression increase for a double Δ*rpoH1* Δ*rpoH2* mutant under heat shock or nonstressed conditions, similar to results found in other *rpoH1H2* mutants (37; M. J. Barnett and S. R. Long, unpublished data). This difference may arise from the differing characteristics of the two genotypes or from possible differences in conditions. It is not apparent from the transcription profiles alone which might be the genes responsible for improved tolerance of peroxide treatment.

This analysis of the transcriptional changes induced by $H_2O_2$ in *S. meliloti* reveals connections to the major regulator OxyR and points to a role for RpoH1 in maintaining the redox status of the cell. We see these connecting circuits as clues to the important and complex role that oxidative stress plays in the soil and endosymbiotic lives of *S. meliloti*. It is possible that the presence of reactive oxygen species represents both an environmental stress to contend with and also a key signal element that helps determine bacterial fate.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains are described in Table 3. LB or tryptone-yeast extract (TY) medium was used for bacterial growth (58). The following antibiotics were used: streptomycin (Sm; 500 μg/ml), tetracycline (Tet; 10 μg/ml), and hygromycin (Hy; 50 μg/ml). For Affymetrix GeneChip assays, *S. meliloti* bacteria were grown in triplicate at 30°C in liquid TY medium without antibiotics to mid-exponential phase ($OD_{600}$ = 0.3 to 0.4). Cultures were split, and $H_2O_2$ was added to half of the cultures to reach a final concentration of either 1 mM or 0.5 mM. At 30 min after $H_2O_2$ addition, cells were harvested as described previously (51). β-Glucuronidase (GUS) assays were performed in triplicate as described previously (59).

To generate an *oxyR* insertion plasmid, a fragment consisting of bp 303 to 649 of the *oxyR* open reading frame (ORF) was amplified by PCR using primers with SpeI/XhoI sites. The digested PCR fragment was ligated with pDW33, generating pAPL57. To generate an *oxyR-uidA* (encoding β-glucuronidase) transcriptional fusion plasmid, a fragment consisting of 25 bp upstream through the first 296 bp of the *oxyR* ORF was amplified via PCR using primers with SpeI/XhoI sites. pAPL56 was generated by ligating the PCR fragment into pDW33.

**TABLE 3** Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype[a] | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* DH5$\alpha$ | *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* | 54 |
| *E. coli* MT616 | MM294A pRK600 ($Cm^r$) | 55 |
| *S. meliloti* CL150 | Wild type ($Sm^r$, corrected *ecfR1* and *pstC*) | 39 |
| *S. meliloti* RFF155 | Δ*rpoH2* (CL150, $Sm^r$) | R. Fisher |
| *S. meliloti* RFF157 | Δ*rpoH1* (CL150, $Sm^r$) | R. Fisher |
| *S. meliloti* RFF164 | Δ*rpoE2*, *rsiA1* (CL150, $Sm^r$) | R. Fisher |
| *S. meliloti* RFF231 | Δ*rpoH1*, *rpoH2* (CL150, $Sm^r$) | R. Fisher |
| *S. meliloti* RFF272 | Δ*rpoE5*, *Smb21687* (CL150, $Sm^r$) | R. Fisher |
| *S. meliloti* APL91 | CL150 pAPL56 *oxyR-uidA* fusion | This study |
| *S. meliloti* APL92 | RFF155 pAPL56 *oxyR-uidA* fusion | This study |
| *S. meliloti* APL93 | RFF157 pAPL56 *oxyR-uidA* fusion | This study |
| *S. meliloti* APL94 | RFF164 pAPL56 *oxyR-uidA* fusion | This study |
| *S. meliloti* APL95 | RFF231 pAPL56 *oxyR-uidA* fusion | This study |
| *S. meliloti* APL96 | RFF272 pAPL56 *oxyR-uidA* fusion | This study |
| *S. meliloti* APL44 | CL150 pAPL10 *katA-uidA* fusion | This study |
| *S. meliloti* APL81 | RFF155 pAPL10 *katA-uidA* fusion | This study |
| *S. meliloti* APL82 | RFF157 pAPL10 *katA-uidA* fusion | This study |
| *S. meliloti* APL46 | RFF164 pAPL10 *katA-uidA* fusion | This study |
| *S. meliloti* APL83 | RFF231 pAPL10 *katA-uidA* fusion | This study |
| *S. meliloti* APL84 | RFF272 pAPL10 *katA-uidA* fusion | This study |
| *S. meliloti* APL97 | CL150 pAPL57 *oxyR* mutant | This study |
| Plasmids | | |
| pDW33 | Terminator and polylinker preceding *uidA* ($Ap^r$ $Hy^r$) | 56 |
| pAPL10 | $P_{katA}$ in pDW33 ($Ap^r$ $Hy^r$) | 57 |
| pAPL56 | $P_{oxyR}$ in pDW33 ($Ap^r$ $Hy^r$) | This study |
| pAPL57 | Internal fragment of *oxyR* in pDW33 ($Ap^r$ $Hy^r$) | This study |

[a]$Ap^r$, ampicillin resistance; $Cm^r$, chloramphenicol resistance.

pAPL10 (57) and pAPL56 were integrated into the *S. meliloti* genome via single crossover following conjugation by triparental mating to generate *katA* and *oxyR uidA* fusion strains in various *S. meliloti* backgrounds (Table 3). GUS assays were performed in triplicate as described previously (59). pAPL57 was integrated into CL150 via single crossover following conjugation by triparental mating (59), generating *oxyR* insertion mutant APL97.

**RNA purification, cDNA synthesis, labeling, and hybridization.** RNA was purified as described in the supplemental material of reference 51. cDNA first-strand synthesis was performed using Invitrogen SuperScript III and random hexamers as primers. cDNA fragmentation, labeling, and hybridization to custom *S. meliloti* Affymetrix symbiosis chips were performed as described previously (51).

Data analysis was performed as described previously (51). Comparison expression analysis was used where an experimental array was compared to a baseline array: three biological replicates each of control (no $H_2O_2$ exposure) and experimental ($H_2O_2$ exposure) strains yielded nine pairwise comparisons. The level of a given gene's expression was considered increased or decreased if the average signal log ratio (SLR) was $\geq$0.98 with a *P* value of $\leq$0.05 in all nine comparisons. A total of three sets of nine pairwise comparisons were performed as follows: 1 mM $H_2O_2$ exposure compared to no exposure in wild-type CL150, 0.5 mM $H_2O_2$ exposure compared to no exposure in wild-type CL150, and 0.5 mM $H_2O_2$ exposure compared to no exposure in *oxyR* insertion mutant APL97. Functional categories for differentially expressed genes were determined using clusters of orthologous groups (COGs) and annotation from http://iant.toulouse.inra.fr/S.meliloti. Venn diagrams were produced using BioVenn (60).

**RT-qPCR.** Real time RT-qPCR was performed as described previously (61) using a Bio-Rad CFX96 system. A 0.5 $\mu$M concentration of each primer and 10 ng of cDNA were used in each reaction. The internal control was *uppS* (SMc02097) (51). Three technical replicates of each of three biological replicates were performed. Data analysis was performed as described previously (62).

**Hydrogen peroxide sensitivity assay.** $H_2O_2$ sensitivity was determined as described previously (57) with a minimum of 3 replicates per experiment. Briefly, mid-exponential-phase free-living cells were diluted 1:100 in LB and the reaction mixtures were split in half. $H_2O_2$ was added to one half of the cultures to reach a final concentration of 1 mM. After 30 additional minutes at 30°C, cultures were diluted and a 100-$\mu$l aliquot of each dilution was plated on LB plates containing selective antibiotics. Colonies were counted after 3 to 4 days at 30°C, and levels of CFU per milliliter were calculated to determine percent survival of treated versus untreated cultures. Percent survival was then normalized to the survival rate of the wild-type strain.

**Catalase assay.** To determine total catalase activity, overnight cultures of *S. meliloti* were diluted to an $OD_{600}$ of 0.1 in LB medium without antibiotics and grown with shaking at 30°C to mid-exponential phase ($OD_{600}$ = 0.3 to 0.4). Cultures were split in half, $H_2O_2$ was added to one of the halves, and both halves were returned to 30°C for 30 min. A 1-ml volume of treated and untreated cultures was harvested

by centrifugation for 1 min. Cell pellets were resuspended in 1 ml lysis buffer (50 mM sodium phosphate [pH 7], 0.1% Triton X-100) and diluted 5-fold to 10-fold in 1× Amplex Red reaction buffer (Invitrogen catalog no. A22180).

A 25-$\mu$l volume of each diluted cell lysate was incubated with 20 $\mu$M $H_2O_2$ (final concentration) in a 96-well black microtiter plate with a final volume of 50 $\mu$l. Plates were incubated for 30 min at room temperature. Amplex Red reagent (final concentration of 50 $\mu$M) and horseradish peroxidase (final concentration fo 0.2 U/ml) in 1× reaction buffer were added to cell lysates (final volume of 100 $\mu$l), and the reaction mixtures were incubated at 37°C for 30 min in the dark. Plates were read as previously described (15). A standard curve of catalase activity was used to determine equivalent catalase units in each *S. meliloti* sample. Catalase activity was normalized to either the $OD_{600}$ of cultures at the time of lysis or to total cell protein data, as determined by the use of a modified Bradford assay (Bio-Rad protein assay). All assays were performed at least in triplicate.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00622-17.

**SUPPLEMENTAL FILE 1,** XLSX file, 0.2 MB.
**SUPPLEMENTAL FILE 2,** XLSX file, 0.2 MB.
**SUPPLEMENTAL FILE 3,** XLSX file, 0.1 MB.
**SUPPLEMENTAL FILE 4,** XLSX file, 0.1 MB.

## ACKNOWLEDGMENT

This work was supported by NIH (R01 GM093628).

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