Whole-Genome Expression Profiling of *Bradyrhizobium japonicum* in Response to Hydrogen Peroxide

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Bradyrhizobium japonicum, a nitrogen-fixing bacterium in soil, establishes a symbiotic relationship with the leguminous soybean plant. Despite a mutualistic association between the two partners, the host plant produces an oxidative burst to protect itself from the invasion of rhizobial cells. We investigated the effects of H₂O₂-mediated oxidative stress on *B. japonicum* gene expression in both prolonged exposure (PE) and fulminant shock (FS) conditions. In total, 439 and 650 genes were differentially expressed for the PE and FS conditions, respectively, at a twofold cut-off with $q < 0.05$. A number of genes within the transport and binding proteins category were upregulated during PE and a majority of those genes are involved in ABC transporter systems. Many genes encoding σ factors, global stress response proteins, the FixK₂ transcription factor, and its regulatory targets were found to be upregulated in the FS condition. Surprisingly, catalase and peroxidase genes which are typically expressed in other bacteria under oxidative stress were not differentially expressed in either condition. The isocitrate lyase gene (*aceA*) was induced by fulminant H₂O₂ shock, as was evident at both the transcriptional and translational levels. Interestingly, there was no significant effect of H₂O₂ on exopolysaccharide production at the given experimental conditions.

Host plants can protect themselves from invasion of foreign microorganisms by using various defense mechanisms (Soto et al. 2006). A major defense mechanism is to produce an oxidative burst which represents an excessive amount of reactive oxygen species (ROS) (Iannelli et al. 1999). ROS, toxic derivatives of oxygen including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH•), can damage various cellular molecules including lipids, proteins, and DNA (Chang et al. 2006).

In the symbiotic association between legume plants and rhizobia, host plants also produce an oxidative burst during the infection process (Soto et al. 2006). Initially, the two partners exchange signaling molecules such as plant-derived isoflavonoids and the bacterium-derived Nod factors (Loh and Stacey 2001; van Rhijn et al. 1998). The initial signal exchanges acti-

vate a series of events in the nodulation process, including root hair curling and infection, and, thereby, an intercellular tube-like structure called an infection thread is formed. Through the infection thread, rhizobia migrate toward the cortex. As rhizobia reach the cortex and proliferate, cortical cell division is initiated, eventually leading to the formation of root nodules where bacteroids, bacteria in the symbiotic state, reside to fix atmospheric nitrogen into ammonia (Dixon and Kahn 2004; Garg and Geetanjali 2009; Mylona et al. 1995; Pawlowski and Bisseling 1996). Among these events, the production of ROS is modulated at different time points and stages (Levine et al. 1994). Accumulation of H₂O₂ has also been observed in infection threads, but not detected in the nitrogen-fixing zone of the nodules (Rubio et al. 2004; Santos et al. 2001). In addition to intracellular ROS during symbiosis, free-living rhizobia in the rhizosphere might experience ROS constantly produced by plants due to aerobic metabolism during their development. However, the level of ROS could be lower compared with that produced as a consequence of defense mechanisms (Chang et al. 2009).

In order to initiate successful nodule formation in the *Bradyrhizobium*–soybean symbiosis, the rhizobial partner *Bradyrhizobium japonicum* must overcome the ROS-involved host defense mechanism and, thereby, adapt to a different level of oxidative stress. Several strategies by which bacteria cope with ROS include production of antioxidant molecules and ROS-scavenging enzymes such as superoxide dismutase (SOD) and catalase (Barloy-Hubler et al. 2004; Santos et al. 2001). SOD detoxifies O₂⁻ and produces H₂O₂, which can be further detoxified by either catalase or peroxidase (Scandalios 1993). In addition, exopolysaccharides (EPS) have been demonstrated to build a protective slime layer in adverse environmental conditions (D’Haeze and Holsters 2004). For example, EPS-deficient mutants of the plant-pathogenic bacterium *Pseudomonas syringae* were more sensitive to ROS than the wild-type strain (Kiraly et al. 1997), indicating that an EPS matrix is able to protect bacteria from ROS. Furthermore, EPS production and biofilm formation have been known to play an important role in tolerance to other stresses such as osmotic stress, desiccation stress, and immune responses (Cytryn et al. 2007; D’Haeze et al. 2004; Soto et al. 2006).

The effect of H₂O₂ in most bacteria, specifically in rhizobia, has been studied using shock treatments with a high concentration of H₂O₂ (Barloy-Hubler et al. 2004; Mongkolsuk and Helmann 2002). Bacteria were grown until mid- to late exponential phase prior to H₂O₂ treatments (Barloy-Hubler et al.

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2004; Chang et al. 2006; Palma et al. 2004). After a short incubation period, cells were harvested for downstream assays such as gene expression and enzyme activities. Unlike shock experiments, there is little study about rhizobial responses to tolerable, low concentrations of H_2O_2 for a long period of time. It is likely that, in the soil and rhizosphere, lower levels of ROS are occasionally generated by plants and other soil bacteria via the partial reduction of oxygen to water during respiration or exposure to redox reactions with metals and other compounds (Panek and O'Brian 2004; Storz et al. 1990). Thus, free-living rhizobial cells likely experience fluctuations of ROS in their soil and rhizosphere habitats.

Here, we investigate how *B. japonicum* responds to H_2O_2 -mediated oxidative stress when exposed to a lower concentration of H_2O_2 for a relatively long incubation period. We employed microarray technology to reveal a comprehensive gene expression pattern in this condition. We have also examined the effect of H_2O_2 on the gene expression of *B. japonicum* under fulminant shock (FS), in which cells are exposed to high concentrations of H_2O_2 for a short time period. Our transcriptomics studies reveal that *B. japonicum* utilizes a different set of genes to deal with H_2O_2 -mediated oxidative stress generated by shock treatment than those used during prolonged exposure (PE) to H_2O_2 .

RESULTS

Growth and survivability of *B. japonicum* in response to H_2O_2 .

In order to have a basic inference of the bacterial response to H_2O_2 -mediated oxidative stress, the growth and survival of *B. japonicum* was monitored at various concentrations of H_2O_2 . Two different treatments were compared: PE and FS. In the former, low-cell-density cultures were exposed to different H_2O_2 concentrations and the growth was monitored by measuring cell turbidity. There was no difference in the growth rate up to 150 μM , whereas no growth was observed in the culture exposed to 350 μM H_2O_2 (Fig. 1). Interestingly, the culture with 250 μM was in an intermediate situation, exhibiting a slower growth rate compared with the control. Thus, we chose the condition of 250 μM for a transcriptomic study of *B. japonicum* grown until mid-log phase.

Fig. 1. Growth of *Bradyrhizobium japonicum* USDA110 treated with various concentrations of H_2O_2 . Cell turbidity was measured and monitored at the optical density of 600 nm. Each point is the mean of three biological replicates and the error bars represent the standard error of the mean. Symbols: ●, control; ○, 75 μM ; ▼, 150 μM ; △, 250 μM ; ■, 350 μM .

The latter FS experiment was carried out to observe the detrimental effects of H_2O_2 at various concentrations and to determine a sublethal concentration of H_2O_2 for generating genome-wide transcriptional profiles of *B. japonicum*. When cells were exposed to various concentrations of H_2O_2 for 2 h, more than 60% survived at concentrations of up to 10 mM (Fig. 2). However, the survivability significantly decreased within 10 min at concentrations higher than 20 mM H_2O_2 . Our findings are consistent with an H_2O_2 challenge study on *Sinorhizobium meliloti*, where efficient detoxification occurs up to 10 mM H_2O_2 and survival patterns are in a dose-dependent manner (Barloy-Hubler et al. 2004). For transcriptome analysis, we chose the condition of 10 mM H_2O_2 for 10 min of exposure due to its intermediate effects on cell survival.

Genome-wide transcriptional profiles of *B. japonicum* in response to H_2O_2 -mediated oxidative stress.

To address the global gene expression changes of *B. japonicum* in response to H_2O_2 -mediated oxidative stress, genome-wide transcriptional profiles were generated from cells subjected to both PE and FS. In the former condition, RNA was isolated from cells grown to mid-log phase in the presence of 250 μM H_2O_2 while, in the latter case, it was isolated from cells treated with 10 mM H_2O_2 for 10 min.

Although the concentrations selected for the transcriptional analysis were based on growth or survival, we wondered whether H_2O_2 was still present in the culture at the time of cell harvest. There is considerable H_2O_2 (81 μM) remaining in the PE condition (Fig. 3A). We also measured endogenous H_2O_2 in the control culture (no H_2O_2 treatment) but were unable to detect it (data not shown), which rules out the possibility of remaining H_2O_2 coming from free-living cells in the culture medium. Surprisingly, in the FS condition, more than 95% of H_2O_2 was decomposed within 10 min (Fig. 3B), indicating that H_2O_2 detoxification may occur in a culture density-dependent manner.

In total, 439 genes were differentially expressed during PE at a twofold cut-off: 204 upregulated and 235 downregulated (Supplementary Table S1). Substantial numbers of down-regulated genes were observed in the functional categories of

Fig. 2. Survival of *Bradyrhizobium japonicum* USDA110 after challenge with different concentrations of H_2O_2 . Cells were grown in arabinose-glucanate media to an optical density at 600 nm of 0.8. After exposure to H_2O_2 for a given time, cells were plated on agar medium and CFU were counted after 4 days of incubation at 30°C. Each experimental data point is the average of three determinations. Error bars represent the standard error of the mean. Symbols: ●, control; ▲, 100 μM ; ■, 250 μM ; ◆, 500 μM ; ○, 1 mM; △, 10 mM; □, 20 mM; ◇, 100 mM.

amino acid biosynthesis, cellular processes, energy metabolism, and translation (Fig. 4). On the other hand, most of the differentially expressed genes within the transport and binding proteins category were upregulated. A majority of those genes are involved in ABC transporter systems, suggesting that extended exposure of *B. japonicum* to the low concentration of H₂O₂ induces ATP-dependent transporters with high substrate affinity.

Fig. 3. Measurement of the remaining H₂O₂ concentrations during **A**, prolonged exposure and **B**, fulminant shock. Solid lines indicate remaining H₂O₂ in the culture, and dashed lines indicate either **A**, cell growth or **B**, survival. Arrows indicate cell harvest time for RNA isolation.

In the FS condition, 650 genes were differentially expressed: 377 upregulated and 273 downregulated (Supplementary Table S2). Most of the induced genes belong to the functional categories of central intermediary metabolism, regulatory functions, and transport and binding proteins, whereas most of the down-regulated genes are within energy metabolism and translation, with the exception of other categories and hypothetical categories (Fig. 4). The FS condition also induced many nitrogen fixation genes, including *fixK₂* and its regulatory targets, particularly the *fixNOQP* and *fixGHIS* operons, excluding only *fixH*. These genes are known to be induced by the FixLJ-FixK₂ regulatory pathway under micro-oxic conditions (Mesa et al. 2003; Nellen-Anthamatten et al. 1998). Of the 203 known targets of regulation for the *fixK₂* transcription factor (Mesa et al. 2008), 79 genes (approximately 39%) were induced in the FS condition. In addition, approximately 21% of the upregulated genes for the FS condition were part of the FixK₂ regulon. Moreover, a *fixK₂* mutant (*B. japonicum* 9043) was shown to have enhanced sensitivity to a 10-mM H₂O₂ shock treatment. After a 2-h challenge with 10 mM H₂O₂, the percent survival for the wild type was $56.0 \pm 4.78\%$ whereas the *fixK₂* mutant was $25.9 \pm 1.9\%$. In addition, a filter disk assay revealed that the *fixK₂* mutant was also more sensitive to 10 mM H₂O₂ than the wild type. The zone of inhibition for the mutant strain was 15.5 ± 0.17 mm whereas that of the wild type was 11.8 ± 0.44 mm, with a *P* value < 0.05.

Validation of microarray data by quantitative reverse-transcription polymerase chain reaction.

We performed quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to validate our microarray expression data. The expression of 21 genes (11 up- and 10 down-regulated) that were selected based on expression values and functional categories was measured. The comparison of the expression values between the two different methodologies exhibited consistency and reproducibility (Fig. 5).

Decreased ribosomal gene expression is evident in both PE and FS.

The number of differentially expressed genes overlapping between both conditions is shown in a Venn diagram (Fig. 6). In all, 36 and 48 genes were commonly up- and downregulated for both conditions. Among these overlapping genes were 10 ribosomal protein-encoding genes commonly downregulated

Fig. 4. Functional group assignments of genes differentially expressed in response to H₂O₂ in both prolonged exposure (PE) and fulminant shock (FS) at a 2.0-fold cut-off with *q* < 0.05. Black bars represent positive fold-induction values and white bars represent negative fold-induction values. Functional classifications were derived from *Bradyrhizobium japonicum* genome annotations available through Rhizobase.

in both conditions (Table 1). In the PE condition, 64 translation genes, including all 30S and 50S ribosomal proteins (i.e., 54 genes), were downregulated and only 1 was upregulated. In the FS treatment, 22 translation-related genes, including the 10 overlapping ribosomal protein-encoding genes, were downregulated and 13 were upregulated. Interestingly, none of the upregulated genes encodes a ribosomal protein. This is in agreement with the previous transcriptome analysis of the *Pseudomonas aeruginosa* response to H₂O₂ (Palma et al. 2004). The expression of 41 of 54 ribosomal protein-encoding genes was downregulated in *P. aeruginosa* treated with H₂O₂.

Differential expression of oxidative stress-responsive genes between PE and FS.

The FS treatment induced more stress-responsive genes, including chaperones and σ factors, than the PE condition (Table 2), suggesting that fulminant H₂O₂ shock is a more severe stress to *B. japonicum* than prolonged incubation with the lower concentration of H₂O₂. The highest fold (107.8-fold) induction in the FS treatment is shown by bli1028 (*carQ*), which encodes a RNA polymerase σ factor, indicating that *B. japonicum* might mainly utilize the σ factor-related global stress response function to cope with H₂O₂-mediated fulminant oxidative stress. Heat and cold shock proteins and the GroEL chaperonin listed in Table 2 are other examples of global stress responses.

In total, 15 oxidative stress-responsive genes were induced in the FS treatment whereas only 4 genes were induced in the PE condition (Table 2). Among the responsive genes were two organic hydroperoxide resistance (*ohr*) genes (bli4012 and bli0735) which were highly upregulated (98.7- and 30.3-fold, respectively) in FS but not in the PE condition. Similarly, there was 4- to 10-fold induction of *ohr* genes during H₂O₂ exposure in three *P. aeruginosa* strains (Salunkhe et al. 2005). Interestingly, many genes involved in an oxidoreductase function which catalyze electron transfer were upregulated in both PE and FS conditions, although one gene (bli0088) is downregulated in the FS treatment (Table 2). Unexpectedly, catalase (*kat*) and *sod* genes commonly known to be induced by oxida-

tive stresses were not differentially expressed in either condition. Because mRNA levels were measured at 10 min after treatment, we wondered if the expression of genes encoding canonical ROS detoxification enzymes occurs within 10 min. Thus, we employed qRT-PCR to check the expression of the following genes at 2 and 5 min after treatment with 10 mM H₂O₂: blr0778 (*katG*), blr5051 (*sodM*), bli7559 (*Fe/Mn-sod*), bli7774 (*sodF*), and bli0735 (*ohr*). All but bli0735 failed to meet 2.0-fold expression threshold values (data not shown). However, the *ohr* gene was induced 6- and 10-fold at 2 and 5 min, respectively. The possibility does exist that a longer exposure time could be effective in the induction of these genes.

Isocitrate lyase activity is inducible by H₂O₂.

Previously completed transcriptomic studies on several environmental conditions, such as desiccation stress (Cytryn et al. 2007), chemoautotrophy (Franck et al. 2008), and bacteroids (Chang et al. 2007a), have revealed strong induction of the isocitrate lyase gene (*aceA*). Interestingly, this gene was also upregulated by fulminant H₂O₂ shock. Isocitrate lyase functions in the glyoxylate bypass of the tricarboxylic acid (TCA) cycle by catalyzing the conversion of isocitrate into succinate and glyoxylate (Chell et al. 1978; Green et al. 1998). Previously, we constructed the isocitrate lyase (*aceA*; blr2455) mutant of *B. japonicum* (Franck et al. 2008), named WC2455. To restore the wild-type phenotype, its complemented strain, named WC2455-C, was constructed in this study.

The susceptibility of the WC2455 mutant to 10 mM H₂O₂ shock exposure is somewhat greater than the wild-type and WC2455-C strains (Fig. 7A), indicating that the lack of isocitrate lyase affects cell survival under H₂O₂-mediated oxidative stresses. The effects of H₂O₂ shock exposure on isocitrate lyase activity were also investigated across various time points up to 120 min. The enzyme activity of the wild type dramatically increased at 20 min and stayed the same through 120 min (Fig. 7B). Given that the gene expression must occur before protein production, detection of the increased *aceA* mRNA at 10 min makes sense at both transcriptional and translational levels. In contrast, the mutant strain showed very little isocit-

Fig. 5. Correlation between microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) data for both prolonged exposure (PE) and fulminant shock (FS) conditions for the selected genes (slope = 0.98, R^2 = 0.85). These genes were chosen based on fold change and functional categories. Expression differences were log₂ transformed. Symbols: Δ , PE; $\blacktriangle$, FS.

Fig. 6. Venn diagrams of the differentially regulated genes between prolonged exposure (PE) and fulminant shock (FS) conditions. Values shown represent more than twofold changes with $q < 0.05$.

rate lyase activity: <1 U/mg and approximately 2 U/mg before and after 20 min, respectively (Fig. 7B). Interestingly, the factor of induction is greater in the mutant strain when compared with the wild-type strain, even though the magnitude of the activity level is lower.

EPS production is not induced by H₂O₂.

We expected an induction of EPS biosynthesis-related genes during oxidative stress due to the protective nature of

EPS against ROS (Kiraly et al. 1997). However, our transcriptomic data do not support our expectation. Because there is a possibility of post-transcriptional modification on EPS biosynthesis pathways, we quantified the amount of EPS produced by *B. japonicum* exposed to various concentrations of H₂O₂ (75, 150, and 250 µM). Cells and supernatant were separated at mid- and late-log phases for EPS quantification. Contrary to our expectations, the total EPS amount was not significantly affected by these concentra-

Table 1. Partial list of genes commonly regulated during fulminant shock (FS) and prolonged exposure (PE) to H₂O₂^a

Locus (gene name)	Description ^c	Fold change ^b	
		PE	FS
bl11168 (<i>tldD</i>)	TldD protein	2.0	2.7
blr1288	Probable long-chain-fatty-acid-CoA ligase	3.0	3.0
blr1309 (<i>acs</i>)	Acetyl-coenzyme A synthetase	2.2	2.0
blr1404 (<i>clpB</i>)	ATP-dependent protease ATP-binding subunit	2.4	2.4
bl12007 (<i>hemN1</i>)	Coproporphyrinogen III dehydrogenase	2.6	2.2
bl12109 (<i>fixK</i>)	Transcriptional regulatory protein	2.1	2.6
bl12542 (<i>nadA</i>)	Quinolinate synthetase A	4.3	2.4
bsr2765 (<i>fixQ</i>)	cbb3 oxidase subunit IV	2.4	10.7
blr2766 (<i>fixP</i>)	cbb3 oxidase subunit III	2.1	6.3
bl13116	Putative phosphoribosylpyrophosphate synthetase	2.0	2.1
blr3179	Probable alanine dehydrogenase oxidoreductase protein	2.4	2.7
blr3214 (<i>norC</i>)	Nitric oxide reductase subunit C	2.3	8.3
blr4112	Probable cation efflux system protein	2.5	4.2
blr4659	PfkB family carbohydrate kinase	2.7	3.1
bl16063	ABC transporter substrate-binding protein	2.2	2.2
blr6213 (<i>mxhF</i>)	Methanol dehydrogenase large subunit-like protein	3.8	4.7
bl16220	Putative alcohol dehydrogenase precursor	5.9	2.3
blr7961	Probable HspC2 heat shock protein	3.3	2.1
bl17981	Putative dehydrogenase	2.2	7.3
bl17989 (<i>mat</i>)	S-adenosylmethionine synthetase	2.2	2.0
blr0162 (<i>rpmB</i>)	50S ribosomal protein L28	-4.5	-2.0
bl10439 (<i>atpC</i>)	ATP synthase ε chain	-3.4	-2.9
bl10441 (<i>atpG</i>)	ATP synthase γ chain	-2.3	-2.4
bl10442 (<i>atpA</i>)	ATP synthase α chain	-4.5	-3.5
bl10456 (<i>mdh</i>)	Malate dehydrogenase	-2.4	-2.1
blr0481 (<i>ffh</i>)	Signal recognition particle protein	-5.0	-2.3
bl10504 (<i>leuB</i>)	3-Isopropylmalate dehydrogenase	-2.3	-2.3
bl10706 (<i>pheS</i>)	Phenylalanyl-tRNA synthetase α chain	-3.6	-2.0
blr0745 (<i>trpB</i>)	Tryptophan synthase β subunit	-2.0	-2.1
bl11137 (<i>cah</i>)	Probable carbonic anhydrase	-2.5	-2.5
blr1377 (<i>etfS</i>)	Electron transfer flavoprotein β subunit	-2.8	-3.6
bl11440 (<i>ctpC</i>)	Pilus assembly protein	-2.5	-2.8
blr2025 (<i>nodA</i>)	Acyl transferase	-2.1	-2.5
bsl4078 (<i>rpsR</i>)	30S ribosomal protein S18	-6.3	-2.1
bsr4084 (<i>acpP</i>)	Acyl carrier protein	-2.4	-2.0
blr4119 (<i>ndk</i>)	Nucleoside diphosphate kinase	-2.8	-2.9
blr4340	Probable ATP-binding protein	-2.9	-2.2
bl14386 (<i>efp</i>)	Elongation factor P	-2.6	-2.9
bl14734 (<i>secF</i>)	Protein-export membrane protein	-3.1	-2.3
bl14736	Preprotein translocase protein	-3.7	-2.3
blr4838 (<i>glrX</i>)	Glutamyl-tRNA synthetase	-2.1	-2.5
bl14860 (<i>tsf</i>)	Translation elongation factor Ts	-2.6	-2.0
blr4949 (<i>glnA</i>)	Glutamine synthetase I	-2.4	-4.5
bl14962 (<i>rpsI</i>)	30S ribosomal protein S9	-3.4	-2.2
bl15066 (<i>rnpO</i>)	RNA polymerase omega subunit	-3.2	-2.2
bl15378 (<i>rpsM</i>)	30S ribosomal protein S13	-3.7	-2.2
bsl5382 (<i>rpmD</i>)	50S ribosomal protein L30	-16.7	-2.1
bl15383 (<i>rpsE</i>)	30S ribosomal protein S5	-14.3	-2.3
bl15403 (<i>fusA</i>)	Translation elongation factor G	-20.0	-2.9
bl15404 (<i>rpsG</i>)	30S ribosomal protein S7	-20.0	-2.4
blr5706 (<i>rpsD</i>)	30S ribosomal protein S4	-8.3	-2.0
bl16497 (<i>ilvC</i>)	Ketol-acid reductoisomerase	-4.5	-2.5
bl17440 (<i>pth</i>)	Peptidyl-tRNA hydrolase	-3.8	-2.2
bl17441 (<i>rplY</i>)	50S ribosomal protein L25	-12.5	-2.1
bsl7548 (<i>rpmJ</i>)	50S ribosomal protein L36	-3.7	-2.2
bsr8096 (<i>rpmH</i>)	Ribosomal protein L34	-3.3	-2.4
blr8101 (<i>argB</i>)	Acetylglutamate kinase	-2.1	-2.4

^a Differentially expressed genes were selected based on a twofold cut-off with *q* value ≤ 0.05.

^b Positive and negative values indicate up and down regulation in response to H₂O₂, respectively.

^c Gene description (annotations) represents the third level from the three-tiered functional level system of *B. japonicum*.

tions of H₂O₂ (data not shown), which is consistent with the transcriptomic data.

DISCUSSION

In this study, we observed differences in the expression profiles of *B. japonicum* in response to H₂O₂ stress in both PE and FS conditions. In cellular processes and energy metabolism, H₂O₂ appears to suppress aerobic respiration. Most of the respiration-related genes encoding ATP synthase subunits, the Rieske Fe-S protein/cytochrome *bc*₁ complex, and the cytochrome *aa*₃ terminal oxidase that are found in the electron transfer chain, were downregulated in both conditions. The α - and β -subunits of the F₀F₁-ATPase are also reported to be specific proteins significantly damaged by oxidative stress (Zhang et al. 2008). Many genes encoding chaperones and several heat and cold shock protein families were upregulated by the fulminant H₂O₂ shock, whereas only one heat shock protein gene was upregulated in the PE condition (Tables 1 and 2). Specifically, probable HspC2 heat shock protein (blr7961) was upregulated in both conditions (Tables 1

and 2). Upregulation of these heat and cold shock proteins and molecular chaperones is known to play an important role for stress response defense mechanisms (Nollen and Morimoto 2002). Therefore, the induction of the heat and cold shock proteins during H₂O₂-mediated oxidative stress may serve to activate or enhance other bacterial stress response mechanisms.

The microarray data regarding the repression of genes encoding ribosomal proteins are consistent with slower growth and less survival data in the PE and FS conditions, respectively (Figs. 1 and 2), because, in general, faster growing cells highly express their ribosomal proteins and translation factors to make translational machinery available faster (Chang et al. 2007a; Tao et al. 1999). The canonical detoxification enzymes which are conventionally associated with a bacterial response to oxidative stress such as *kat* and *sod* genes were noticeably absent from the list of differentially expressed genes. A previous study with *P. aeruginosa* by Palma and associates (2004) revealed similar results, in that genes such as *katE*, *katN*, *soxR*, and *ahpB* were not induced by the H₂O₂ treatment. This could be explained by the constitutive expression of those genes in

Table 2. List of chaperones, σ factors, and oxidative stress-responsive genes differentially expressed in response to H₂O₂^a

Physiological process	Locus (gene name)	Description ^c	Fold change ^b	
			PE	FS
Chaperones	blI0729 (<i>hasp</i>)	Small heat shock protein	...	8.5
	blr7740	Small heat shock protein	—	8.0
	bsl4595	Cold shock protein	—	7.0
	blr4635	Chaperonin GroEL	—	3.4
	bsr3154 (<i>cspA</i>)	Cold shock protein	—	3.0
	blr2450 (<i>htpx</i>)	Protease heat shock protein	—	2.6
	blr4637	Probable HspC2 heat shock protein	—	2.3
	blr1100 (<i>hslO</i>)	Heat shock protein 33	—	2.0
	blr7961	Probable HspC2 heat shock protein	3.3	2.1
	blI5834 (<i>dksA</i>)	dnaK deletion suppressor protein	—	−2.2
	blr4653 (<i>dnaJ</i>)	Molecular chaperone DnaJ family	3.0	—
	bsl3986 (<i>cspA</i>)	Cold shock protein	−2.1	—
	blr5626 (<i>groEL</i>)	60-kDa chaperonin	−2.3	—
	blr7533 (<i>groEL</i>)	60-kDa chaperonin	−3.3	—
σ Factors	blI1028 (<i>carQ</i>)	RNA polymerase σ factor	—	107.8
	blr7337 (<i>rpoH2</i>)	σ 32-like transcription factor	—	5.9
	blr3038 (<i>SigD</i>)	Extracytoplasmic function family σ factor	—	5.0
	blr7797	RNA polymerase σ -E factor (σ -24) protein	2.6	−4.9
	blr1883 (<i>rpoN1</i>)	RNA polymerase σ -54 subunit	2.3	—
Oxidative stress response	blI4012 (<i>ohr</i>)	Organic hydroperoxide resistance protein	—	98.7
	blI0735 (<i>ohr</i>)	Organic hydroperoxide resistance protein	—	30.3
	blr3215 (<i>norB</i>)	Nitric oxide reductase subunit B	...	12.1
	blr3214 (<i>norC</i>)	Nitric oxide reductase subunit C	...	8.3
	blI3418	Putative epoxide hydrolase 1	—	6.3
	blI7086 (<i>hemN</i>)	Anaerobic coproporphyrinogen III oxidase	—	5.9
	blr7849	Putative glutathione S-transferase	—	5.1
	bsr0210	Glutaredoxin	—	3.6
	blI5249	Oxidoreductase	—	3.0
	blI0751 (<i>trxA</i>)	Thioredoxin C-1	—	2.7
	blI4149	Putative glutathione peroxidase	—	2.7
	blI6540	Putative oxidoreductase	—	2.4
	blI1295	Probable oxidoreductase	—	2.4
	blI0175	Probable oxidoreductase	—	2.1
	blI0593	Putative oxidoreductase protein	—	2.0
	blr1174 (<i>coxG</i>)	Putative cytochrome C oxidase assembly protein	—	−2.0
	blr2503 (<i>gst</i>)	Glutathione S-transferase	—	−2.1
	blI6732	Probable glutathione peroxidase	—	−2.1
	blr1171 (<i>coxA</i>)	Cytochrome C oxidase subunit I	—	−2.3
	blI0088	Oxidoreductase	—	−3.2
	blr2167	Oxidoreductase	6.8	—
	blI3376	Putative oxidoreductase protein	3.0	—
	blr5775	Putative thioredoxin	2.5	—
	blr6218	Putative oxidoreductase protein	2.4	—

^a Differentially expressed genes were selected based on a twofold cut-off with *q* value ≤ 0.05 .

^b Positive and negative values indicate up- and downregulation, respectively, in response to H₂O₂; — = not significantly expressed.

^c Gene description (annotations) represents the third level from the three-tiered functional level system of *B. japonicum*.

response to the endogenous production of H_2O_2 because control cells were growing aerobically.

Cells may also use alternative mechanisms such as the activation of various RNA polymerase σ factors to reduce the toxicity of ROS. For example, bl1028 (*carQ*), blr7337 (*rpoH2*), blr3038 (*sigD*), blr7797, and blr1883 (*rpoN1*) were induced in either PE or FS, although blr7797 was repressed in FS (Table 2). Among them are *CarQ* and *SigD*, which are of particular interest because of their high gene expression and extracytoplasmic function (ECF). ECF σ factors play essential roles in activating transcription of their regulons in adaptation to various environmental changes, including oxidative stress (Helmann 2002). Three ECF σ factors have been known to play a role in response to oxidative stress. In *Caulobacter crescentus*, the ECF σ factor σ^T is necessary for survival under oxidative stress (Alvarez-Martinez et al. 2007). Another example is *Streptomyces coelicolor* σ^R that modulates expression of the thioredoxin system in response to oxidative stress (Paget et al. 1998). The other ECF σ factor, *CarQ*, found in *Myxococcus xanthus*, regulates biosynthesis of carotenoids, which are potent quenchers of singlet oxygen (Gorham et al. 1996).

H_2O_2 -mediated oxidative stress also induced the FixK₂ transcription factor and its regulatory targets *fixNOQP* and *fixGHIS* in the FS condition. FixK₂ is known to control the *fixNOQP* operon, which encodes the high-affinity *cbb3*-type terminal oxidase involved in bacteroid respiration under microaerobic and symbiotic conditions (Mesa et al. 2006, 2009). Other related genes involving the *fixGHIS* operon, the σ factor (*rpoN1*), heme biosynthesis, and nitrate respiration are inducible under oxygen-limiting conditions (Fischer et al. 2001). However, Mesa and associates (2009) reported that oxidative stress repressed expression of these targets of the FixLJ-FixK₂ regulatory pathways, which is inconsistent with our microarray data. These contradictory results may result from different background conditions. In this study, *B. japonicum* subjected to FS was grown under aerobic conditions, whereas cells were grown in a microaerobic condition in the previous study for the transcriptional analysis (Mesa et al. 2009), which makes it difficult to compare the two data sets. Another functional genomics study of the *B. japonicum* response to paraquat-mediated oxidative stress also revealed high expression of FixK₂-related genes by fulminant paraquat shock (Donati et al. 2011), indicating that ROS may create a kind of the transitory micro-oxic condition inside the cells when they are aerobically cultivated.

Among the highly induced genes, blr2455 (*aceA*), which encodes isocitrate lyase, is a principal candidate for functional and physiological analyses because it is involved in the glyoxylate bypass of the TCA cycle and appears to play an important role in adaptation to various environmental stresses such as desiccation (Cytryn et al. 2007), chemoautotrophy (Franck et al. 2008), bacteroid differentiation (Chang et al. 2007a), and now H_2O_2 -mediated oxidative stress. This gene is also induced by high salinity, acid shock stress, and low temperature in *Colwellia maris*, *Mycobacterium tuberculosis*, and *Shewanella* sp. WP3, respectively (Fisher et al. 2002; Li et al. 2006; Watanabe et al. 2002). A previous study (Green et al. 1998) demonstrated that free-living *B. japonicum* possesses negligible isocitrate lyase activity. In that study, multiple carbon sources were compared. In this study, we confirmed the transcriptional data that show isocitrate lyase to be inducible in a condition of oxidative stress in addition to other stressful conditions. The results of the assay for isocitrate lyase activity also revealed low but detectable activity in the mutant strain even though this strain lacks a functional copy of the *aceA* gene. The measured activity cannot be from this protein because it is null. Therefore, we could speculate that isocitrate lyase activity in the *aceA* mutant

is due to genetic heterogeneity, which implies that multiple genes contribute to the phenotype. Alternatively, the indirect enzyme assay that measures the accumulation of the phenylhydrazone derivative of glyoxylate could be a reason for a low level of the isocitrate lyase activity. The induction of aconitase (bl10466), citrate synthase (bl10182), and other pyruvate metabolism-related genes are also noteworthy to be addressed in that these genes link the TCA cycle and glycolytic pathway in *B. japonicum*. Similarly, in *P. aeruginosa*, genes whose products function in the TCA cycle and the terminal aerobic respiratory chain complex are upregulated in response to H_2O_2 (Salunkhe et al. 2005).

We also performed EPS quantification because our a priori hypothesis was that *B. japonicum* would produce more EPS as a defense mechanism against oxidative stress. However, our EPS quantification and transcriptomic data do not provide evidence to satisfy the hypothesis. Therefore, it may be possible that H_2O_2 could be too rapidly detoxified to have a significant effect on EPS production, or constitutive production of EPS could be sufficient to deal with a relatively low concentration of H_2O_2 . In the latter case, *B. japonicum* cells may routinely produce EPS to protect themselves from endogenous ROS derived from the partial reduction of oxygen during aerobic respiration. In contrast, more EPS production has been detected by oxidative stress

Fig. 7. Effect of 10 mM H_2O_2 shock on **A**, survival of the three *Bradyrhizobium japonicum* strains (wild type, WC2455, and WC2455-C) and **B**, their isocitrate lyase activities. Each experimental data point is the average of three determinations. One unit of isocitrate lyase activity is defined as the amount of enzyme required to produce 1 μ mol of glyoxylate per minute at pH 6.8 and 30°C. Symbols: ●, wild type; ○, WC2455; ▼, WC2455-C.

caused by paraquat, a known superoxide radical-inducing agent (Donati et al. 2011), indicating that superoxide radicals, rather than H₂O₂, are the primary ROS which can induce EPS biosynthesis of *B. japonicum* as a defense mechanism.

From the microarray data in this study and others, we speculate that most microorganisms may have overlapping and multiple pathways for detoxifying ROS. A number of bacteria that possess several defense mechanisms, including canonical detoxification enzymes, chaperones, and alternative σ factors, differ in their temporal regulation in response to ROS (Mongkolsuk and Helmann 2002). Moreover, different forms of oxidative stresses and exposure times also disparately affect defense mechanisms. Thus, our findings may provide clues to the potential involvement of several listed *B. japonicum* genes whose mRNA expressions are modulated in response to H₂O₂, one of the ROS. Our continuing investigation will comprise of the mutagenesis of organic hydroperoxide resistance genes (bll0735 & bll4012) and the ECF σ factor (bll1028).

MATERIALS AND METHODS

Bacterial strains and culture medium.

B. japonicum USDA110 was inoculated at a concentration of 0.2% into 20 ml of arabinose-gluconate (AG) medium (Sadowsky et al. 1987) and cultivated at 30°C with shaking at 200 rpm. AG media contain 125 mg of Na₂HPO₄ (Fisher), 250 mg of Na₂SO₄ (Fisher Biotech, Fairlawn, NJ, U.S.A.), 320 mg of NH₄Cl (Fisher), 180 mg of MgSO₄ · 7H₂O (Sigma-Aldrich, St. Louis), 10 mg of CaCl₂ (Fisher), 4 mg of FeCl₃ (Fisher), 1.3 g of HEPES (Sigma-Aldrich), 1.1 g of MES (Sigma-Aldrich), 1.0 g of yeast extract (Difco Laboratories, Detroit), 1.0 g of L-arabinose (Sigma-Aldrich), and 1.0 g of D-gluconic acid sodium sulfate (Sigma-Aldrich) per liter. The *fixK₂* mutant strain (*B. japonicum* 9043) was cultured in AG media supplemented with spectinomycin (200 µg ml⁻¹) and streptomycin (100 µg ml⁻¹). The *aceA* mutant strain (*B. japonicum* WC2455) was cultured in AG media supplemented with kanamycin (150 µg ml⁻¹), and its complemented strain (WC2455-C) was cultured in the same medium supplemented with kanamycin (150 µg ml⁻¹) and tetracycline (100 µg ml⁻¹).

Experimental conditions for PE and FS.

For PE to H₂O₂, a single colony was inoculated into 10 ml of AG, and broth cultures at log phase were inoculated at a concentration of 0.05% into 30 ml of AG containing either H₂O₂ or the same volume of sterilized double-distilled (dd)H₂O and incubated at 30°C with shaking at 200 rpm. These cultures were exposed to different concentrations of H₂O₂ (from 0 to 350 µM) for 2 days, and the optical density at 600 nm (OD₆₀₀) was measured to determine growth rate and cell harvest time for RNA isolation. For the FS treatment, the 300-ml culture was cultivated until an OD₆₀₀ of 0.8, which gives approximately 1 × 10⁹ CFU/ml. Then, the culture was divided into eight 30-ml cultures by pipetting into 125-ml flasks and each culture was treated with various concentrations of H₂O₂ ranging from 100 µM to 100 mM. Survival was monitored at 10, 20, 30, 60, and 120 min after treatment. The control culture was supplemented with the same volume of sterilized ddH₂O. After exposure to H₂O₂, cells were washed with buffer and spread on AG agar plates. CFU were counted after 4 days of incubation at 30°C. The same protocol was used to determine the percent survival for *B. japonicum* 9043 under the same experimental conditions for the wild-type strain.

Filter disk assay.

A comparison of the sensitivity of *B. japonicum* 9043 to 10 mM H₂O₂ was also compared with the wild-type strain by

means of a filter disk assay. Starter cultures for each strain were grown to an OD₆₀₀ of 1.0 and reinoculated to an OD₆₀₀ of 0.05 in 0.9% soft AG agar. A 6-mm filter disk (Becton Dickinson and Co., Franklin Lakes, NJ, U.S.A.) was soaked in 10 mM H₂O₂ and placed at the top of each plate. A disk soaked in ddH₂O was applied to the bottom of each plate to serve as a control. In total, three replications were performed for each strain. Zones of inhibition were measured in millimeters after a 4-day incubation period at 30°C.

Measurement of H₂O₂ in vitro.

The scavenging of H₂O₂ by cells was examined as previously described (Panek and O'Brian 2004) and analysis was completed according to recommendation of the manufacturer's protocol using HRP reagent (Sigma-Aldrich) and Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Molecular Probes, Eugene, OR, U.S.A.). Aerobically grown cells were harvested at various time points when subjected to 250 µM H₂O₂ (for the PE condition) and 10 mM (for the FS treatment). Cells were washed twice in 50 mM NaPO₄ buffer (pH 7.0) and resuspended in the same buffer. After incubation at 30°C, aliquots were assayed immediately for remaining H₂O₂. The culture itself without H₂O₂ treatment was used as a control to check for endogenous H₂O₂ production by *B. japonicum*.

Construction of an *aceA*-complemented strain.

The *B. japonicum aceA* gene (blr2455) was amplified by PCR using the following primers: *Bam*HICATGTTCTGATGT TAC and *Bam*HITCCGCCGTTAGCCGA, forward and reverse respectively. The amplified *aceA* gene was inserted in pGEM-T easy vector (Promega Corp., Madison, WI, U.S.A.). The 1.5-kb fragment of the *Bam*HI-digested *aceA* gene and the *Bam*HI-digested pTE3 (Egelhoff and Long 1985) expression vector (tetracycline resistant) were ligated to construct a 21.8-kb recombinant plasmid. The resulting construct was transferred from *Escherichia coli* to the mutant strain WC2455 (Franck et al. 2008) by triparental mating with the helper strain pRK2073. Transconjugants were selected for kanamycin and tetracycline resistance. The complemented strain, named WC2455-C, possessing the recombinant plasmid was confirmed by colony PCR (data not shown).

Isocitrate lyase enzyme assay.

Cells grown in AG medium were harvested by centrifugation at 8,000 × g for 5 min at 4°C. To remove the medium components, the cell pellets were washed once in 1 ml of 50mM Tris-sodium EDTA (TNE) buffer (pH 7.0) and resuspended in 1 ml of breaking buffer (20 mM TNE buffer [pH7.0], 100 mM NaCl, 5 mM MgCl₂, 0.4 mM EDTA, 1.5 mM dithiothreitol and 2% [wt/vol] glycerol) (Green et al. 1998). The suspension was subsequently homogenized by sonication for 1 min and the cell pellet was removed by centrifugation at 8,000 × g for 5 min.

The isocitrate lyase assay was performed according to recommendation of Sigma-Aldrich's protocol and the method of Chell, with some modifications (Chell et al. 1978). The reaction solution contained 30 mM imidazole buffer (pH 6.8), 5 mM MgCl₂, 1 mM EDTA, 4 mM phenylhydrazine hydrochloride (Sigma-Aldrich), and 1 mM DL-isocitric acid (Sigma-Aldrich). To initiate the enzyme reaction, 0.1 ml of freshly prepared cell-free extract was added to reaction mixture and incubated for 5 min at 30°C. After incubation, the absorbance at 340 nm was measured to determine the enzyme activity. One unit of the isocitrate lyase activity was defined as the amount of substrate consumed to produce 1 µmol of glyoxylate per minute at pH 6.8 and 30°C. The effect of fulminant H₂O₂ shock on the isocitrate lyase activity was determined at various time points up to 120 min.

EPS isolation and quantification.

EPS was isolated and purified from *B. japonicum* USDA110 during growth in PE to H₂O₂. The cultures pretreated with various concentrations of H₂O₂ (0, 75, 150, and 250 μ M) were grown until the mid (OD₆₀₀ $\approx$ 0.8) and late (OD₆₀₀ $\approx$ 1.5) exponential phase. EPS was isolated and purified according to the method of Read and Costerton (1987). Total protein content was measured by Bradford's method (Bradford 1976) using a bovine serum albumin as the standard (Bio-Rad, Hercules, CA, U.S.A.) to normalize the total EPS amount. The total carbohydrate content was determined by the phenol-sulfuric acid method, with glucose as the standard (Chang et al. 2007b).

RNA isolation for PE and FS.

For the PE condition, a 200-ml culture of *B. japonicum* USDA110 initially supplemented with either 250 μ M H₂O₂ or sterilized ddH₂O was grown under aeration at 30°C until the OD₆₀₀ reached the exponential phase (OD₆₀₀ of 0.8). Then, the cultures were harvested and washed with AG media by centrifugation for 20 min at 4°C and 8,000 $\times$ g in a fixed-angle rotor. After decanting the supernatant completely, cell pellets were flash frozen in liquid nitrogen and stored at -80°C until RNA isolation. RNA samples from frozen cells for microarray and qRT-PCR were extracted using a modified hot phenol method as described previously (Bittner et al. 2003). To purify RNA samples, an RNeasy mini kit (Qiagen, Germantown, MD, U.S.A.) was used along with RNase-free DNase (Qiagen) according to the manufacturer's protocol. RNA samples were confirmed on 0.8% agarose gels prior to use to insure RNA integrity, and quantities were measured by the NanoDrop device (Thermo Scientific, Sugarland, TX, U.S.A.). All extractions of RNA were performed with three independent replications and used for both microarray and qRT-PCR experiments.

For the FS treatment, cultures grown at 30°C until the exponential phase (OD₆₀₀ of 0.8) were divided into two cultures and both cultures were immediately treated with either 10 mM H₂O₂ or the same volume of sterilized ddH₂O for 10 min. Cell harvest and RNA isolation were performed as described above.

Microarray experiments and data analysis.

Whole-genome expression profiles of *B. japonicum* in response to PE and FS were created using DNA microarray chips that contain 8,453 open reading frames annotated from the genomic sequence of *B. japonicum* USDA110 (Chang et al. 2007a). Preparation of the microarray, synthesis of cDNA, labeling, hybridization, and scanning were completed as previously described (Chang et al. 2007a). Briefly, after labeling cDNAs with Cy3 and Cy5 monoreactive dyes (GE Healthcare), the cDNA samples were applied to microarray slides following overnight incubation at 42°C. Microarray slides were washed and scanned with the Axon GenePix 4200 scanner, and GenePix Pro 6.0 software was used to quantify intensity values. The signal intensities were normalized using the Lowess algorithm and subsequently analyzed by mixed-effect microarray analysis of variance (MAANOVA) (Kerr et al. 2000). Values obtained from the MAANOVA analysis were subjected to significance analysis of microarray (SAM) using the SAM package (Tusher et al. 2001). Because the spots are printed in duplicates on each array, two technical replicates were combined and averaged. Significantly up- and downregulated genes were identified based on a 2.0-fold cut-off threshold within a false discovery rate lower than 5% (q value $\leq$ 0.05). All experiments were performed with three independent replications.

qRT-PCR analysis.

qRT-PCR was performed to verify the microarray data. qRT-PCR methods used in this study were described previously

(Chang et al. 2007a). Briefly, cDNA was synthesized in a 25- μ l reaction solution containing 1 μ l of M-MLV reverse transcriptase (200 U/ μ l; Promega Corp.), 2 μ l of random hexamers (250 ng/ μ l; Invitrogen, Carlsbad, CA, U.S.A.), 1.25 μ l of 10 mM dNTPs, and 3 μ g of total RNA used in the microarray experiment. Of the reaction solution, 1 μ l of cDNA was used as a template for qRT-PCR. Negative control reactions which lacked reverse transcriptase were used to check for DNA contamination. Gene-specific primers for the selected 21 genes based on expression values and functional categories were designed using Integrated DNA Technologies (Coralville, IA, U.S.A.) company software (Supplementary Table S3). For data normalization, expression values were normalized to the expression of *bll0631 (parA)* which encodes a chromosome-partitioning protein (Cytryn et al. 2007), a gene constitutively expressed under all experimental conditions examined in this study.

Microarray data accession numbers.

The raw microarray data are compiled in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database and are accessible through the GEO series accession numbers GSE26960 and GSE26961 for the PE and FS conditions, respectively.

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AUTHOR RECOMMENDED INTERNET RESOURCES

The Jackson Laboratory, Churchill Group website:
www.jax.org/staff/churchill
 National Center for Biotechnology Information Gene Expression Omnibus (GEO) database: www.ncbi.nlm.nih.gov/geo
 RhizoBase website: genome.kazusa.or.jp/rhizobase