Journal of Microbiology (2015) Vol. 53, No. 8, pp. 526–534
DOI 10.1007/s12275-015-5308-9
Copyright ⓒ 2015, The Microbiological Society of Korea

Role of the extracytoplasmic function sigma factor CarQ in oxidative response of *Bradyrhizobium japonicum*[§]

Anchana Thaweethawakorn[1†], Dylan Parks[1†], Jae-Seong So[2], and Woo-Suk Chang[1,3*]

[1]*Department of Biology, University of Texas, Arlington, Texas 76019, USA*
[2]*Department of Biological Engineering, Inha University, Incheon 402-751, Republic of Korea*
[3]*Division of Biotechnology, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan 570-752, Republic of Korea*

(Received Jun 17, 2015 / Revised Jul 17, 2015 / Accepted Jul 21, 2015)

As a nitrogen-fixing bacterium, *Bradyrhizobium japonicum* can establish a symbiotic relationship with the soybean plant (*Glycine max*). To be a successful symbiont, *B. japonicum* must deal with plant defense responses, such as an oxidative burst. Our previous functional genomics study showed that *carQ* (bll1028) encoding extracytoplasmic function (ECF) sigma factor was highly expressed (107.8-fold induction) under oxidative stress. Little is known about the underlying mechanisms of how CarQ responds to oxidative stress. In this study, a *carQ* knock-out mutant was constructed using site-specific mutagenesis to identify the role of *carQ* in the oxidative response of *B. japonicum*. The *carQ* mutant showed a longer generation time than the wild type and exhibited significantly decreased survival at 10 mM H_2O_2 for 10 min of exposure. Surprisingly, there was no significant difference in expression of oxidative stress-responsive genes such as *katG* and *sod* between the wild type and *carQ* mutant. The mutant also showed a significant increase in susceptibility to H_2O_2 compared to the wild type in the zone inhibition assay. Nodulation phenotypes of the *carQ* mutant were distinguishable compared to those of the wild type, including lower numbers of nodules, decreased nodule dry weight, decreased plant dry weight, and a lower nitrogen fixation capability. Moreover, desiccation of mutant cells also resulted in significantly lower percent of survival in both early (after 4 h) and late (after 24 h) desiccation periods. Taken together, this information will provide an insight into the role of the ECF sigma factor in *B. japonicum* to deal with a plant-derived oxidative burst.

***Keywords*:** symbiotic nitrogen fixation, oxidative stress, desiccation, soybean, ECF sigma factor, CarQ

[†]These authors contributed equally to this work.
*For correspondence. E-mail: wschang@uta.edu; Tel.: +1-817-272-3280; Fax: +1-817-272-2855
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Introduction

Bradyrhizobium japonicum is a slow growing, rod-shaped, motile, non-spore forming, Gram-negative soil bacterium that is capable of establishing a symbiosis with soybean and fixing nitrogen in specialized structures known as root nodules (Jordan, 1982). It can change its form from free-living to an endosymbiotic stage, depending upon the oxygen availability and signaling-exchange of soybean-derived isoflavonoids and bacterial Nod factors between the two partners (Kuzma *et al.*, 1993; Soto *et al.*, 2006). Through a series of biochemical reactions within the nodule, atmospheric nitrogen is fixed into ammonia that is transported to other areas in the plant (Day *et al.*, 2001). However, the development of symbiotic stage becomes complicated since *B. japonicum* needs to have a specific mechanism to protect themselves against the plant-defense oxidative burst. Two of the primary reactive oxygen species (ROS) produced during plant defense are superoxide (O_2^-) and hydrogen peroxide (H_2O_2) molecules (Apel and Hirt, 2004), which can be found in the infection thread and nodules during the early plant responses to an exposure of soil bacteria (Santos *et al.*, 2001). H_2O_2 can be generated via the reduction of oxygen generated in photosystem I (PS I), and from the RuBisCo oxygenase reaction in combination with the photorespiratory pathway (Apel and Hirt, 2004). These ROS are severely cytotoxic, causing oxidative damage to membrane-bound proteins, and promoting lipid peroxidation and hydroxyl radical generation (Evans *et al.*, 1999). Interestingly, even after the symbiotic recognition has been established, there is still the presence of ROS during the nodulation process (Levine *et al.*, 1994). In the alfalfa-*Sinorhizobium meliloti* symbiosis, both O_2^- and H_2O_2 were found to accumulate in the infection thread and nodules during early responses to an infection of *S. meliloti*, but neither were found within the nitrogen fixing area (Santos *et al.*, 2001).

To avoid the host defense mechanism and become a successful symbiont with leguminous plants, rhizobia must have effective regulatory methods to mitigate the plant defense response, including the suppression of ROS production by Nod factors, the synthesis of surface polysaccharides, and the antioxidant system (Soto *et al.*, 2006; Aslam *et al.*, 2008; Bulgakov *et al.*, 2012; Liang *et al.*, 2013). Besides the production of Nod factors to plant signal molecules, synthesis of bacterial surface polysaccharides, such as exopolysaccharide (EPS), have been observed other soil bacteria. In *Rhizobium leguminosarum*, an increase in levels of EPS was detected during the invasion process (Skorupska *et al.*, 2006). Several scavenging enzymes, including superoxide dismutase (SOD) and catalase, are key for detoxifying O_2^- and H_2O_2, respec-

tively. These enzymes are produced by bacteria in order to mitigate oxidative stress caused by host defenses during plant-microbe interaction (Loprasert *et al.*, 1996; Jamet *et al.*, 2003).

Another group of molecules that are crucial in dealing with environmental stressors for soil bacteria are sigma factors. Bacterial sigma factors are part of a complex multi-subunit RNA polymerase that plays an important role in the recognition of promoters and the initiation of the transcription process. Among all sigma factor groups of the sigma 70 family, group 4 is well known as extracytoplasmic function (ECF) sigma factors, which specialize in the response to oxidative stress and has been observed in several studies. For instance, in *Rhizobium etli,* rpoE4 has a vital function in saline, osmotic, and H_2O_2 stress responses (Martinez-Salazar *et al.*, 2009). In *Rhodobacter sphaeroides*, RpoE (an ECF sigma factor) controls the activation of $RpoH_{II}$-dependent genes, which encode the oxidative-stress defense system involved in the singlet oxygen dependent response (Nuss *et al.*, 2009). In *Caulobacter crescentus,* SigT is responsible for the survivability of bacteria during osmotic and oxidative stresses (Alvarez-Martinez *et al.*, 2007). Another example is ECF sigma factor CarQ in *Myxococcus xanthus*, which is involved in light-induced carotenogenesis. The end product of the carotenogenesis, caroyinoids, can degrade the triplet-state porphyrin molecule and singlet oxygen (Browning *et al.*, 2003).

The genome of *B. japonicum* USDA110 contains a total of 23 sigma factors, and 17 of which are putative ECF-type sigma factors (Gourion *et al.*, 2009). To date, only 4 of these sigma factors have been characterized. The first ECF sigma factor characterized is PhyR-σ^{ECFG} (bll7795), which is involved in heat and desiccation resistance under carbon starvation conditions (Gourion *et al.*, 2009). A study on EcfS (blr4928) showed that a mutation on the gene caused defects in nodule phenotypes and nitrogen fixation activity (Stockwell *et al.*, 2011). EcfG (blr7797) and EcfQ (bll1028) are highly induced when exposed to the H_2O_2-mediated oxidative stress and singlet oxygen ($^1O_2$) under micro-oxic conditions (Masloboeva *et al.*, 2012). Interestingly, under micro-oxic conditions, there were no significant differences measured for *carQ* mutants in physiological and symbiotic analyses, compared with the wild type (Masloboeva *et al.*, 2012). Recently, a high expression of gene bll1028 under H_2O_2 fulminant shock treatment in oxic conditions has been observed; however, no further study in symbiotic properties have been investigated (Jeon *et al.*, 2011).

In this study, we investigated how a *B. japonicum carQ* knock-out mutant responds to H_2O_2-mediated oxidative stress at high concentrations under shock treatment in oxic-conditions. We also observed the complex role of *carQ* in regard to its symbiotic properties, nitrogen fixation activity, and sensitivity during severe desiccation conditions. Our data suggest that *carQ* is likely to be an important gene involved in many different stress responses for assurance of *B. japonicum* survivability.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The wild type strain of *B. japonicum* USDA110, *carQ* mutant, and *carQ* complementary strains were cultured in arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of Na_2HPO_4, 250 mg of Na_2SO_4, 320 mg of NH_4Cl, 180 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2$, 4 mg of $FeCl_3$, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1 g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate per L (Sadowsky *et al.*, 1987). All *B. japonicum* strains were incubated aerobically at 30°C with vigorously shaking at 200 rpm for 2 days. Appropriate antibiotics were supplied for each strain (μg/ml): chloramphenicol, 30 (all *B. japonicum* strains); kanamycin, 150 (*carQ* mutant strain); tetracycline, 50 (*carQ* complementary strain). *Escherichia coli* strains and plasmids were grown aerobically in Lubia-Bertani (LB) medium at pH 7.0 which contains 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per L of nanopure water (Bertani, 1951). All *E. coli* cultures

Table 1. List of bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
E. coli strain DH5α	supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F′ (traD36, proAB+ lacIq, Δ(lacZ)M15) endA1 recA1 hsdR17 mcrA supE44λ gyrA96 relA1	Bethesda Research Laboratories
B. japonicum strains		
USDA110	Cm^R Wild-type	USDA, Beltsville, MD
carQ mutant	Cm^R Km^R *carQ*::Km	This work
carQ complement	Cm^R Km^R Tc^R *carQ*::Km with pBBR1MCS3 vector with *carQ* insert	This work
Plasmids		
pKD4	Km^R expression vector	Datsenko and Wanner (2000)
pRK2013	Km^R cloning vector	Figurski and Helinski (1979)
pRK2073	Sm^R cloning vector	Leong *et al.* (1982)
pJQ200SK	Gm^R cloning vector	Quandt and Hynes (1993)
pJQ200SK-*carQ*	Gm^R cloning vector containing *carQ* gene	This work
pRK78-pJQ200SK-*carQ*-Km	Gm^R cloning vector with suicide vector and internal *carQ* gene substitute with kanamycin cassette	This work
pBBR1MCS-3	Tc^R cloning vector	Kovach *et al.* (1994)
pBBR1MCS-3-*carQ*	Tc^R cloning vector containing *carQ* gene	This work

Cm, Km, Gm, Sm, and Tc refer to chloramphenicol, kanamycin, gentamicin, streptomycin, and tetracycline, respectively.

were grown at 37°C (except DH5α pRK78 incubated at 30°C), vigorous shaking at 200 rpm overnight where appropriate antibiotics were applied in μg/ml: gentamicin, 15 (pJQ200SK and pRK78); tetracycline, 15 (pBBR1MCS-3); kanamycin, 50 (pRK2013); streptomycin, 50 (pRK2073). Additional 15 g/L of agar was added in the case of making agar plates for every type of medium. In oxidative stress experiments, all *B. japonicum* strains were exposed to 10 mM of H_2O_2 for 10 min, the condition that developed intermediate effects on cell survival of the wild type strain of *B. japonicum* under H_2O_2 stress (Jeon *et al.*, 2011). All bacterial strains and plasmids in this study are listed in Table 1.

Construction of the *B. japonicum carQ* mutant strain

A *carQ* knock-out mutant was constructed using site-specific mutagenesis by means of deletion mutation. The *B. japonicum carQ* gene (bll10128) was amplified by PCR using the following primers: 5′-AATTGGGCCCTGTCCGACGA AACAATTTGGCG-3′ and 5′-TAATGGGCCCTCCGCCT GATGTTGTGGTTTGT-3′, forward and reverse respectively. *Apa*I restriction sites were added to both forward and reverse primers as indicated by the underlined sequence. PCR amplification was performed on C1000 Thermo Cycler (Bio-Rad) and gel electrophoresis, a PowerPac™ Basic (Bio-Rad), was used to confirm the correct size of amplified PCR products. Then, the amplified 0.85 kb *carQ* gene was inserted into a pJQ200SK-Gm^R suicide vector (Quandt and Hynes, 1993) using the heat shock method to construct a 6.0 kb recombinant plasmid. The cloning plasmid was transformed into the Lambda Red recombinase expression plasmid, DH5α pKD78-Cm^R, using electroporation method (Datsenko and Wanner, 2000) to create electrocompetent cells, pRK78-pJQ200SK-*carQ-λ,* using Micro Pulser™ (Bio-Rad), at 1.8 kV. A 1.47 kb kanamycin antibiotic cassette from pKD4-Km^R plasmid was amplified by PCR using the following primers: 5′-GTGTAGGCTGGAGCTGCTTC-3′ and 5′-CATATGA ATATCCTCCTTAG-3′, forward and reverse respectively. Kanamycin antibiotic cassette fragments then were amplified again with 60 bp primers which combined a kanamycin antibiotic cassette primer with an additional 40 bp *carQ* homology sequence: 5′-ATTGCCGACGGCAACCGGACG TCGATGCACATCCTCTATTGGTAGGCTGGAGCTGC TTC-3′ and 5′-CTAAGGAGGATATTCATATGGGCGGA TTTGCTTAAGGCGCGGCGTCGACCGCTTCGCT-3′, forward and reverse respectively. The PCR fragment and the electrocompetent cells were ligated to construct a recombinant plasmid (pRK78-pJQ200SK-*carQ*-Km) by electroporation using Micro Pulser™, at 2.2 kV. The resulting construct was transferred from *E. coli* to the wild type strain USDA110 by tri-parental mating with the helper strain pRK2013-Km^R. Transconjugants were selected for sucrose-induced lethality (SacB^S), and chloramphenicol and kanamycin resistances (Cm^R and Km^R). The mutant strain DNA was extracted and confirmed by PCR using the 2 following primer sets: the 1st set had a forward sequence of 5′-CGAACGTCATCGCCA TCAAC-3′ and reverse of 5′-CCAGTCATAGCCGAATA GCC-3′. The 2nd set of primers had a forward sequence of 5′-AGGATCTCGTCGTGACCCA T-3′ and a reverse sequence of 5′-CCAGTCATAGCCGAATAGCC-3′.

Construction of the *B. japonicum carQ* complementary strain

In order to restore the wild type phenotype, a *carQ* complementary strain was constructed. A 0.823 kb of the *B. japonicum carQ* gene (bll10128), including upstream and downstream regions, was amplified by PCR using the following primers: 5′-AATTGGGCCCTGTCCGACGACGAAACAA TTTGG-3′ and 5′-ATTACTCGAGTGTTTCGTCCGCGGT AACAC-3′, forward and reverse respectively. *Apa*I and *Xho*I restriction sites were added to forward and reverse primers, respectively, as indicated by the underlined sequence. The purified PCR products then were ligated with *Apa*I-*Xho*I digested pBBR1MCS-3-Tc^R plasmid to yield a recombinant plasmid pBBR1MCS-3-*carQ*. The result recombinant plasmid pBBR1MCS-3-*carQ* was transferred back into the *B. japonicum carQ* mutant strain by tri-parental mating using pRK2073-Sm^R plasmid as a helper strain. Transconjugants DNA were extracted and confirmed by PCR using the 2 following primer sets: the 1st set had a forward sequence of 5′-TGATTCTTCTCGCTTCCGGC-3′ and reverse of 5′-CAC GTCTTCCTGGTCGATGT-3′. The 2nd set of primers had a forward sequence of 5′-ACATCGACCAGGAAGACGT G-3′ and a reverse of 5′-GTGCGCTGTTCCAGACTATC-3′.

Growth curve measurements

Initial cultures of wild type, *carQ* mutant, and *carQ* complementary strains were grown in AG medium with appropriate antibiotics until they reached the mid exponential phase at an OD_{600} of 0.8, and then subcultured into 125 ml flasks, 3 replicates of 30 ml for each strain, at an OD_{600} of 0.005. All samples were incubated at 30°C in a shaking incubator. OD_{600} of each culture was monitored every 12 h until each culture reached the stationary phase, and at each time point, each culture was grown on selected antibiotic plates at different serial dilution factors from 1:1000 to 1:1,000,000 for a total of 3 replicates for each sample. All plates were incubated at 30°C for 4 days and the resulting colony forming units (CFUs) were then counted. This experiment was repeated 3 times.

Filter disk assay on agar plates

All three *B. japonicum* strains, including wild type, *carQ* mutant, and *carQ* complementary strains, were compared in regards to their susceptibility toward H_2O_2 at 10 mM concentration by performing a filter disk assay. Each strain was grown in AG medium with appropriate antibiotics and harvested when the optical density at OD_{600} reached the exponential phase (OD_{600}=0.8). Each culture was sub-cultured to an OD_{600} of 0.05 in 0.9% soft AG agar with appropriate antibiotics. After all agar plates were solidified, 2 of 6 mm diameter filter disks (Becton Dickinson and Co.) were placed on each soft agar plate. The first filter disk was soaked with 10 μl of 10 mM H_2O_2 and placed on the top half of the plate. The second filter disk which served as a control was soaked with 10 μl of nanopure water and placed on the bottom half of the plate. The zone of inhibition, a clearing area surround the filter disk, was measured in millimeters after 4-day incubation at 30°C. In this zone of inhibition test, each *B. japonicum* strain was comprised of 6 replicates.

Fulminant shock in liquid culture

A total of 200 ml culture of each *B. japonicum* strain, including wild type, *carQ* mutant, and *carQ* complementary strains in AG medium and selective antibiotics, were incubated aerobically at 30°C with vigorously shaking at 200 rpm until the OD_{600} reached 0.8. Each 200 ml culture was divided into a total of six 30 ml cultures. Three cultures were treated with 10 mM H_2O_2 and the other three were treated with sterilized nanopure water to serve as a control. All cultures were incubated aerobically at 30°C with vigorous shaking at 200 rpm, and survival was monitored in each individual culture, measured at 0, 10, 20, 30, 60, 120 min after treatment. Each culture was washed with fresh AG medium and spread on AG agar plates containing chloramphenicol at 1:1000 to 1:1,000,000 dilution factor. After a 4-day incubation at 30°C, colony forming units were counted in order to determine the percent survival of each strain over time. The experiment was repeated three times for a total of 9 replicates for each strain.

RNA isolation

For fulminant shock treatment, a total of 200 ml culture of *B. japonicum* USDA110 and *carQ* mutant strains were grown in AG media at 30°C with vigorously shaking at 200 rpm until the OD_{600} reached 0.8. Cultures were then divided into six 30 ml cultures and half were treated with 10 mM H_2O_2 for 10 min while the others were treated with sterilized ddH_2O to serve as a control. RNA isolation was performed as described previously (Jeon *et al.*, 2011). Cultures were condensed by centrifugation for 20 min at 4°C and 8,000 × *g* in a fixed angle rotor. The supernatant was decanted and RNA was isolated via a hot phenol method as described previously (Bittner *et al.*, 2003). RNA samples were purified using an RNeasy mini kit (Qiagen) along with RNase-free DNase (Qiagen) according to manufacturer's protocol. Gel electrophoresis was used to confirm the RNA purity, and quantities were measured using NanoDrop device (Thermo Scientific).

Quantitative reverse transcription PCR (qRT-PCR) analysis

The qRT-PCR was performed according to methods previously described (Chang *et al.*, 2007). Primers used for qRT-PCR are listed in Supplementary data Table S1. The expression value of each gene was normalized to the expression of *parA* gene (bll0631), encoding the chromosome-partitioning protein (Cytryn *et al.*, 2007; Donati *et al.*, 2011). All experiments were performed with three biological replicates and fold induction values were calculated in accordance with the method of Pfaffl (2001).

Desiccation experiment

Each *B. japonicum* strain, including wild type, *carQ* mutant, and *carQ* complementary strain, was grown in 5 ml AG medium with selective antibiotics and incubated aerobically at 30°C with vigorous shaking at 200 rpm for 2 days. All strains were subcultured in 200 ml AG media with chloramphenicol and incubated aerobically at 30°C with vigorous shaking at 200 rpm until the strains reached mid exponential phase at OD_{600} of 0.8. For each strain, subcultures were divided into 8 of 20 ml cultures in 50 ml Eppendorf tubes. Each 20 ml culture was transferred to a 0.4 μm sterilized MF^{TM} polycarbonate membrane filter (0.45 mm HA, 47 mm diameter; Millipore) and placed into a 60 × 15 mm sterilized Petri dish. Four of the membrane filters were incubated in the desiccator containing sterilized water to generate the 100% relative humidity (RH) to induce a hydrating condition, whereas another four membrane filters were incubated in the desiccator filled with a saturated potassium acetate solution to give a 27% RH in order to induce a severe desiccating condition (Cytryn *et al.*, 2007). The RH values in desiccators were measured with an Aqua Lab CX-2 water activity meter (Decon Devices). The desiccators were incubated in the dark at 30°C and survival was monitored at 0, 4, 24, 72 h by resuspending cells from membrane filters with Bergersen's minimal salts medium (MM) and spread onto AG agar plates at dilution factors ranging from 1:10,000 to 1:1,000,000. Percent survivals were determined after cells were incubated at 30°C for 4 days. This experiment was repeated three times.

Nodulation assay and symbiotic phenotype observation

Soybean seeds were sterilized by the following steps: seeds were placed into a 250 ml beaker and covered with aluminum foil to protect seeds from all light sources, while approximately 200 of the soybean seeds were washed with 30% Clorox for 10 min with moderate aggravation and immediately rinsed with sterilized ddH_2O three times. The soybean seeds were washed with 10% HCl for 10 min with the moderate aggravation and immediately rinsed with sterilized ddH_2O three times. Seed germination was performed immediately after soybean seeds were sterilized. Approximately 15 soybean seeds were placed on sterilized 3 M paper and placed in a sterile Petri dish. Soybean seeds were selected with the following criteria: light brown color, about 8 × 5 × 5 mm (length, width, and height), have spontaneously wrinkled seed coat around the seed, and no black spot or shape disproportion. To maintain moisture within the Petri dish, a sterilized 3 M paper soaked with sterilized ddH_2O was placed on the top of the soybean seeds before being covered by a Petri dish lid. All Petri dishes were covered with aluminum foil and incubated in the dark at 30°C for 3 days. Germinated seeds that had their roots emerged approximately 1.5 cm were selected and placed into sterilized plastic pouches (Mega International). A total of three seeds were placed in one pouch, and the position of each root tip was marked on the pouch (Halverson and Stacey, 1986). A straw was placed into either end of a pouch where it left the space between its bottom end and the bottom of a pouch approximately 5 cm. Two pouches (with the straw side facing out) were placed into a hanging paper folder using paper clips to attach them together, then this folder was placed into a folder rack. To prepare the inoculum for the pouch experiment, the wild type, *carQ* mutant, and complimentary *B. japonicum* strains were grown in 20 ml of AG medium with the appropriate antibiotics at pH 6.8, and incubated aerobically at 30°C with vigorously shaking at 200 rpm until all cultures reached the mid-log phase at OD_{600} of 0.8. Cells were harvested by centrifugation at 4,000 rpm for 10 min and washed with half-strength nitrogen-free Broughton and Dilworth (B&D) me-

dium (pH 6.8) containing 500 μM $CaCl_2$, 250 μM KH_2PO_4, 250 μM K_2HPO_4, 5 μM Fe-citrate, 125 μM $MgSO_4{\cdot}7H_2O$, 125 μM K_2SO_4, 0.5 μM $MnSO_4{\cdot}H_2O$, 1 μM H_3BO_3, 0.25 μM $ZnSO_4{\cdot}7H_2O$, 1 μM $CuSO_4{\cdot}5H_2O$, 0.05 μM $CoSO_4{\cdot}7H_2O$, and 0.05 μM $Na_2MoO_4{\cdot}2H_2O$ (Lee *et al.*, 2012). Strain suspensions in the half-strength B&D medium were all adjusted to have an OD_{600} of 0.1 (ca. 1×10^8 cells/ml). After pouches and all cultures were prepared, each pouch was nourished with 20 ml of half-strength B&D medium. For each bacterial strain, 1.0 ml of culture (at OD_{600} of 0.1) was inoculated on each seed. All pouches were incubated in a growth chamber with 15 h of day and 9 h of night at 27°C. All pouches were watered with 20 ml of half-strength B&D medium every 2 days. After 30 days incubation, the physical properties of soybean seeds inoculated with different bacterial strains were measured, including average nodule size, nodule number, nodule dry weight, plant dry weight, and the distance of nodules from the root tip. In one pouch experiment, 4 pouches were used for each strain (12 seeds total), and this experiment was repeated three times.

Acetylene reduction assay

An acetylene reduction assay was used to quantify the nitrogen-fixing activity of *B. japonicum* wild type, *carQ* mutant, and *carQ* complementary strains by detecting the ethylene production via gas chromatography (GC). A total of 6 soybean seeds were sterilized, germinated, and inoculated with bacterial culture as described in the pouch experiment section. After 30 days, each soybean-intact root nodule was transferred into a 27 ml sterilized glass jar, and sealed with a serum cap. 10% of the air inside the glass jar was replaced with an equal amount of pure acetylene gas, the air in the glass jar mixed via shaking. 250 μl of gas from the sample was drawn by using a gas-tight syringe (Hamilton Co.) and injected into the GC (GC-2014; Shimadzu) equipped with a hydrogen flame detector. Glass jars with no root served as a control for this experiment. The area of ethylene peak (3.6 min) and acetylene peak (4.75 min) were recorded at the end of the run (8 min). After acetylene reduction assay measurement, the nodules of each sample were dried in the oven (Chicago Surgical & Electrical Co.) at 65°C for 3 days. The nodule dry weight was measured by using an analytical balance (Denver Instruments APX-60). This experiment was repeated three times for each *B. japonicum* strain.

Results

The *carQ* mutant has a longer generation time

Our previous functional genomics study showed that *carQ* (bll1028) encoding ECF sigma factor was highly expressed (107.8-fold induction) under oxidative stress (Jeon *et al.*, 2011). To identify the role of *carQ* in the oxidative response of *B. japonicum*, we constructed *carQ* mutant and its complemented strains. Firstly, growth of *B. japonicum* wild type, *carQ* mutant, and complementary strains was monitored via OD_{600} until all cultures reached the stationary phase. The generation times of the wild type, mutant, and complementary strains were 8.5 ± 0.2, 9.6 ± 0.1, and 8.7 ± 0.1 h, respectively. From this result, generation time of the mutant strain is statistically different from that of the wild type and complementary strains ($P < 0.05$). The mutant strain also reached stationary phase faster with a lower maximum OD when compared to the other two strains (Fig. 1).

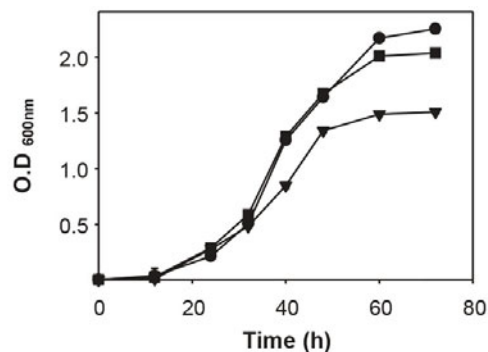

Fig. 1. Growth of *B. japonicum* USDA110 (wild type), *carQ* mutant, and complementary strain in AG media. Each time point represents an average OD of three replicates with standard error. Symbols: (●) wild type; (▼) *carQ* mutant; (■) *carQ* complementary (*carQ*-C) strain.

The *carQ* mutant is more susceptible to H_2O_2-mediated oxidative stress

In order to demonstrate how the *carQ* gene is involved in the bacterial oxidative stress response, two different experiments were conducted: filter disk assay on solid agar plates and fulminant shock treatment in liquid culture. In the former, the susceptibility of bacterial response to the H_2O_2 exposure was indicated by the zone of inhibition, a clear zone around the disk. Under this treatment, a *carQ* mutant showed a significant increase in susceptibility to H_2O_2, compared with the wild type USDA110 and complementary strains ($P < 0.05$). The average diameter of the zone of inhibition for the *carQ* mutant was 25.6 ± 0.3 mm, whereas the average diameter of the zone of inhibition for the wild type and complementary strains were 17.7 ± 0.2 and 16.7 ± 0.2 mm, respectively (Fig. 2).

For the latter experiment, cell survival of the wild type, *carQ* mutant, and complementary strains was monitored under 10 mM H_2O_2 at various time points up to 120 min. As shown in Fig. 3, more than 73% of the wild type and complementary strains survived at 10 mM H_2O_2 shock exposure throughout the treatment periods. However, the average percent cell survival of the *carQ* mutant was approximately 30% lower compared to that of the wild type ($P < 0.05$), suggesting the

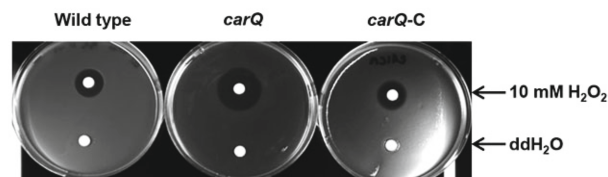

Fig. 2. Inhibition zones of hydrogen peroxide on three *B. japonicum* strains. Hydrogen peroxide was applied onto top filters, while sterilized ddH_2O as a control was applied onto bottom filters.

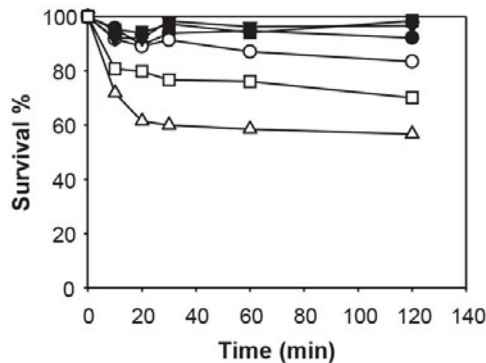

Fig. 3. Survival of *B. japonicum* USDA110, *carQ* mutant, and *carQ*-C strains after challenge with 10 mM of H_2O_2. Symbols: (●) wild-type control; (○) wild-type treatment (10 mM H_2O_2); (▼) *carQ* control; (△) *carQ* treatment (10 mM H_2O_2); (■) *carQ*-C control; (□) *carQ*-C treatment (10 mM H_2O_2).

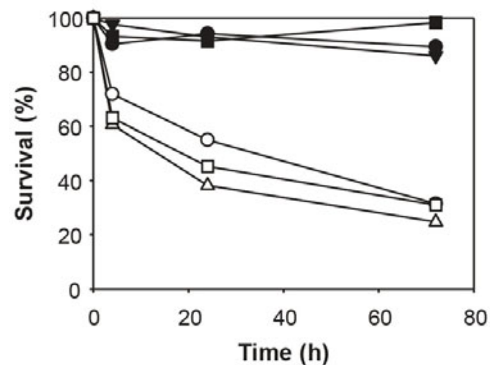

Fig. 4. Survival of *B. japonicum* USDA110, *carQ* mutant, and *carQ*-C strains, following incubation under desiccating (27% RH) and hydrated (100% RH) conditions. Symbols: (●) wild-type control (100% RH); (○) wild-type treatment (27% RH); (▼) *carQ* control (100% RH); (△) *carQ* treatment (27% RH); (■) *carQ*-C control (100% RH); (□) *carQ*-C treatment (27% RH).

importance of the *carQ* gene and its involvement in response to oxidative stress.

We also checked expression of oxidative stress-related genes such as *katG* encoding catalase and *sod* genes encoding SOD, respectively, under oxidative stress. As shown in Table 2, one *katG* and four *sod* genes were selected based on *B. japonicum* annotation in Rhizobase (http://genome.microbedb.jp/rhizobase/Bradyrhizobium). Surprisingly, there was no differential expression in *katG* and *sod* genes between the wild type and *carQ* mutant (Table 2). In the micro-oxic condition, similar results were observed for these genes between the wild type and the *ecfQ* mutant (Masloboeva *et al.*, 2012). This indicates that catalase and SOD are not regulated by the ECF sigma factor CarQ in response to oxidative stress in both oxic- and micro-oxic conditions.

Cell viability of the *carQ* mutant under desiccation conditions

Rhizobia have been known for their sensitivity to drought (i.e., desiccation), one of the major environmental stresses that reduce efficiency in development of symbiotic relationships between rhizobia and their host plants. In addition, it has been known that desiccation also influences endogenous oxidative stress in soil bacteria (Chang *et al.*, 2009). Previous studies of genome-wide transcriptional analyses of *B. japonicum* under desiccation conditions revealed that genes involved in oxidative stress and sigma factors were highly expressed during desiccation (Cytryn *et al.*, 2007). This result suggests i) the possible involvement of drought and oxidative stress and ii) co-regulation of those genes by sigma factors. Therefore, it is likely that ECF sigma factor CarQ is also involved in response to desiccation. To observe the role of the *carQ* gene in a desiccation stress response, the cell survival of the wild type, *carQ* mutant, and complementary strains was compared under hydrated (100% RH) and desiccating conditions (27% RH) at 4, 24, and 72 h of the incubation periods. Under the desiccating condition, survivability of the mutant strain was significantly decreased compared to that of the wild type in all 3 incubation periods ($P < 0.05$), and showed the most significant decrease of percent cell survival after 24 h of incubation (Fig. 4).

The *carQ* mutant displays different symbiotic phenotypes

Plant tests including nodulation assay were conducted to identify the role of the *carQ* gene on *B. japonicum*-soybean symbiotic development, especially in nodule development and its phenotypes. Soybean seeds were inoculated with the wild type, *carQ* mutant, or complementary strains at OD_{600} of 0.1 (ca. 1×10^8 cells/ml) and the plants were grown for 4 weeks with 15 h of day and 9 h of night. Each individual soybean plant was collected and the nodule development and phenotypes were observed, including the average distance of a nodule from the initial root tip (mm), dry weight of nodules (mg), nodule numbers, nodule sizes (mm), and dry weight of plant (mg). The results showed that phenotypic parame-

Table 2. Comparison of gene expression level changes between wild type and *carQ* mutant strains under oxidative stress by qRT-PCR analysis

Locus (Gene)	Gene description[a]	10 mM H_2O_2[b]	
		Wild type	*carQ*
blr0778 (*katG*)	catalase	0.8 ± 0.2	1.8 ± 0.5
blr5051 (*sodM*)	superoxide dismutase SodM-like protein	0.6 ± 0.2	0.9 ± 0.1
bll7559 (*chrC*)	probable Fe/Mn superoxide dismutase	0.6 ± 0.2	0.9 ± 0.3
bll7774 (*sodF*)	superoxide dismutase	1.9 ± 2.0	2.4 ± 0.3
bll8073	probable Mn superoxide dismutase	1.0 ± 0.1	1.0 ± 0.8

[a] Gene descriptions represent third level annotations from the three-tiered functional level system of *B. japonicum* (www.kazusa.or.jp/rhizobase/Bradyrhizobium/cgi-bin/category_brady.cgi).
[b] Values are not significantly different for each gene between wild type and *carQ* mutant strains using student's *t*-test ($P > 0.05$).

Table 3. Comparison of symbiotic phenotypes among three strains

Strains	Root tip distance[a] (mm)	Nodule dry weight[b] (mg)	Nodule number per plant[c]	Plant weight[d] (mg)
USDA110	+1.6 ± 2.4 A	4.7 ± 0.5 A	5.3 ± 0.4 A	223.2 ± 9.6 A
carQ mutant	-18.9 ± 2.7 B	2.1 ± 0.2 B	3.6 ± 0.3 B	147.8 ± 6.0 B
carQ complement	-8.8 ± 2.2 C	3.9 ± 0.4 A	4.7 ± 0.3 A	185.6 ± 9.9 A

Values followed by different capital letters are statistically significant ($P < 0.05$) based on Student's *t*-test.
[a] For root tip distance measurements, the positive and negative values indicate the position of nodules formed from to the root tip mark line at time zero: positive (+) nodules formed. above the root tip, while negative (-) nodules below the root tip.
[b] Average nodule dry weight (mg) per plant.
[c] Average number of nodules per plant.
[d] Average plant weight.

ters were significantly different ($P < 0.05$) between the wild type and the mutant strains (Table 3). The average distance of nodules from the root tip per plant formed by each strain were different. The wild type's average nodule distance was slightly above the root tip, the *carQ* mutant's distance was far below the root tip, and the complementary strain's distance was moderately below the root tip (Table 3). The average nodule dry weight per plant inoculated with the wild type, *carQ* mutant, and complementary strain differed as well. The mutant's average nodule dry weight was less than that of the wild type, and the complementary strain appeared to almost completely restore the wild type nodule weight phenotype (Table 3). Soybeans inoculated with the mutant strain showed an approximately 50% lower dry weight of nodules per plant compared to the wild type and complementary strains. Moreover, in samples inoculated with the wild type and complementary strains, there were more nodules per plant compared to samples inoculated with the mutant strain (Table 3). The average of nodule sizes formed by the mutant strain was also lower compared to the wild type and complementary strains. Interestingly, mutation on the *carQ* gene also affected plant growth as indicated by the significantly lower plant dry weight compared to the wild type and complementary strains after 4 weeks (Table 3).

The *carQ* mutant shows reduced nitrogen-fixing capabilities

Due to a lower number of nodules and smaller nodule size in plants inoculated with the *carQ* mutant strain, an acetylene reduction assay was conducted to measure the nitrogenase activity by means of indirect measurement of ethylene production, converted by nitrogenase from acetylene. The result from this experiment showed that mutation on the *carQ* gene affected the nitrogenase activity inside the nodule compared to the wild type and complementary strains (Fig. 5). The average amount of ethylene production of soybeans inoculated with the *carQ* mutant was significantly decreased, with 0.28 ± 0.06 μmole per min per g of dry weight nodules ($P < 0.05$) of ethylene being produced, whereas the ethylene production of soybean samples inoculated with the wild type and complementary strain was 0.44 ± 0.04 and 0.43 ± 0.05 μmole per min per g of dry weight nodules, respectively.

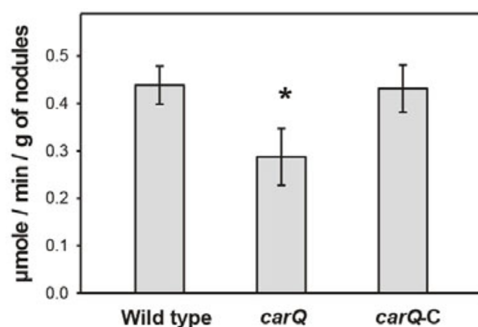

Fig. 5. Comparison of nitrogenase activities measured by ethylene (C_2H_2) production among the wild type USDA110, *carQ* mutant, and *carQ*-C strains after growth for 4 weeks. Nodules were completely dried in the oven before weight measurement. Error bars represent the standard error of the mean. The asterisk indicates statistical significance (Student's *t*-test with $P < 0.05$).

Discussion

Soybean plants are able to produce an oxidative burst, which is a rapid increase in the concentration of ROS as a defense mechanism against foreign bacteria including both pathogenic and beneficial partners. During the oxidative burst state, H_2O_2 is the primary ROS source released by the plant at the invasion site (Klotz, 2002). Therefore, bacterial symbiont *B. japonicum* needs to apply an effective mechanism, not only to protect themselves from H_2O_2 present in the infection thread, but also to successfully establish a symbiotic relationship with their host plant. Rhizobial ECF sigma factors are a notable group of proteins well known for their roles in response to environmental stresses (Gruber and Gross, 2003). The results of this study reveal that the *carQ* gene, which encodes an ECF sigma factor, plays an important role in i) the oxidative stress response, ii) the desiccation response, iii) symbiotic development, and iv) nitrogenase activity.

Disruption of the *carQ* gene causes a change in growth behavior in *B. japonicum*. The mutant strain had a longer generation time and a lower maximum OD compared to the wild type. The difference in growth patterns may be caused by a lack of the gene itself, resulting in the deferment to a different metabolic pathway or more likely by regulation of genes involved in central metabolisms controlled by the ECF sigma factor. In addition, the filter disk assay and fulminant shock experiments on liquid culture revealed that lack of *carQ* gene activity showed a significant change in cell susceptibility and survival under the H_2O_2 shock treatment. These results indicate that this gene primarily responds to H_2O_2-mediated oxidative stress in oxic conditions. However, there was no significant difference of *B. japonicum* survivability in response to H_2O_2 shock treatment in the filter disk assay performed under micro-oxic conditions (Masloboeva

et al., 2012). In shock experiments on liquid culture, even though the mutation of the gene caused a significant decrease in cell survival after 20 min incubation, percent survival was only slightly decreased throughout the treatment, suggesting that *B. japonicum* is still able to maintain an effective detoxification process (Fig. 3). This also indicates that the expression of *carQ* is a part of the regulatory network of stress response genes, which in the absence of *carQ*, the oxidative stress response is suspected to be compensated by other regulatory genes within the stress response gene family. For instance, the result from previous genome-wide transcriptional analysis indicated a relatively high amount of expression in a group of oxidative stress response genes, particularly bll4012 and bll0753 (*ohr*) genes, as well as other *B. japonicum* sigma factor genes, such as blr7337 (*rpoH2*) and blr3038 (*SigD*) (Jeon *et al.*, 2011).

Drought (i.e., desiccation) is an inevitable problem in the practice of agriculture, mainly causing degradation of soil quality. This severe environmental cue can endanger plants and influence the viability of rhizobia in soil, affecting the symbiotic relationship between plants and their symbionts (Vriezen *et al.*, 2007). Although several soybean cultivars have been engineered to become more resistant to drought, a corresponding bacterial symbiont *B. japonicum* has yet to show improvement in this stress condition (Cytryn *et al.*, 2007). Previous research also indicates that desiccation can interfere with the interaction between a plant and their symbiotic microorganisms by limiting nitrogen fixation (Zahran, 1999). In addition, desiccating conditions could induce ROS generation in rhizobial species (Vriezen *et al.*, 2007). Interestingly, our study also showed that cell viability of the *carQ* mutant significantly decreased when the mutant was exposed to a severe desiccating condition at 27% RH, compared to the wild type. The previous work on genome-wide transcriptional analysis of *B. japonicum* under severe desiccation conditions corroborates our results by illustrating the upregulation of several oxidative stress-related genes and sigma factor genes, including *carQ* (Cytryn *et al.*, 2007). The study related with drought is of particular importance, since perturbation of cell membranes by desiccation might subvert electron flow in the electron transfer chain, thereby increasing endogenous superoxide radicals, which could be a source for ROS generation.

All results in the phenotypic determination of the mutant showed a significant difference compared to the wild type strain. The *carQ* mutant showed lower numbers of nodules and the decrease in nodule size, nodule dry weight, and plant dry weight. The inoculation of root with the mutant strain caused delayed nodulation, as indicated by a longer distance of nodule formation from the root tip when compared with the wild type. Interestingly, soybeans inoculated with the complementary strain did not show the exact same results as the wild type, as compensation of this gene was supposed to restore the complete wild type phenotypes. This incomplete restoration might be explained by i) weak activity of the promoter, and/or ii) the natural loss of the recombinant plasmid containing *carQ* gene during the treatment periods due to the absence of selection force (e.g., antibiotic absence). The results from the acetylene reduction assay showed that the *carQ* gene may play a significant role in nitrogen fixation, since the *carQ* mutant showed a significant decrease in ethylene production.

Previous studies on *B. japonicum* genome-wide transcriptional analysis in response to H_2O_2-mediated oxidative stress revealed that the highest level of expression of the *carQ* sigma factor gene can be found in both oxic (108 fold) and micro-oxic conditions (35 fold) (Jeon *et al.*, 2011; Masloboeva *et al.*, 2012). However, under micro-oxic conditions, there were no significant differences between the carQ mutant and wild type in both physiological and symbiotic analysis, even though *carQ* showed the highest expression levels in response to H_2O_2-mediated oxidative stress (Masloboeva *et al.*, 2012). This suggested that *carQ* gene might play different roles when regulated under micro-oxic conditions. It could be possible that a concentration of oxygen is a key factor that triggers expression of the *carQ* gene in response to H_2O_2-mediated oxidative stress, as well as indicating a crucial role of *carQ* gene in the early stages (i.e., aerobic conditions) of symbiotic development. This also suggests another possible role of *carQ* gene in response to the change of oxygen concentrations, and triggers the transition of the bacterial cells development from free-living stage to endosymbiotic stage.

These findings will benefit and serve as an important piece of information to improve bacterial-plant symbiosis and therefore increase plant productivity, as well as reduce the world's dependence on nitrogen fertilizers. Future studies on the functionality of the ECF sigma factor CarQ are necessary to better understand how the organism adapts and responds to a variety of stresses from the environment, and to gain insight into how this gene regulates and contributes to the survival of the bacteria.

Acknowledgements

This work was supported, in part, by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0016797) and an Inha University Research grant.

References

Alvarez-Martinez, C.E., Lourenco, R.F., Baldini, R.L., Laub, M.T., and Gomes, S.L. 2007. The ECF sigma factor sigma(T) is involved in osmotic and oxidative stress responses in *Caulobacter crescentus*. *Mol. Microbiol.* **66**, 1240–1255.

Apel, K. and Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55**, 373–399.

Aslam, S.N., Newman, M.A., Erbs, G., Morrissey, K.L., Chinchilla, D., Boller, T., Jensen, T.T., De Castro, C., Ierano, T., Molinaro, A., *et al.* 2008. Bacterial polysaccharides suppress induced innate immunity by calcium chelation. *Curr. Biol.* **18**, 1078–1083.

Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* **62**, 293–300.

Bittner, M., Butow, R., DeRisi, J., Diehn, M., Eberwine, J., Epstein, C.B., Glynne, R., Grimmond, S., Ideker, T., Kacharmina, J.E., *et al.* 2003. Expression analysis of RNA. pp. 101–288. *In* Bowtell, D. and Sambrook, J. (eds.), DNA Microarrays: A Molecular Cloning Manual. Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, NY, USA.

Browning, D.F., Whitworth, D.E., and Hodgson, D.A. 2003. Light-induced carotenogenesis in *Myxococcus xanthus*: functional characterization of the ECF sigma factor CarQ and antisigma factor CarR. *Mol. Microbiol.* **48**, 237–251.

Bulgakov, V.P., Gorpenchenko, T.Y., Veremeichik, G.N., Shkryl, Y.N., Tchernoded, G.K., Bulgakov, D.V., Aminin, D.L., and Zhuravlev, Y.N. 2012. The *rolB* gene suppresses reactive oxygen species in transformed plant cells through the sustained activation of antioxidant defense. *Plant Physiol.* **158**, 1371–1381.

Chang, W.S., Franck, W.L., Cytryn, E., Jeong, S., Joshi, T., Emerich, D.W., Sadowsky, M.J., Xu, D., and Stacey, G. 2007. An oligonucleotide microarray resource for transcriptional profiling of *Bradyrhizobium japonicum*. *Mol. Plant-Microbe Interact.* **20**, 1298–1307.

Chang, W.S., Li, X., and Halverson, L.J. 2009. Influence of water limitation on endogenous oxidative stress and cell death within unsaturated *Pseudomonas putida* biofilms. *Environ. Microbiol.* **11**, 1482–1492.

Cytryn, E.J., Sangurdekar, D.P., Streeter, J.G., Franck, W.L., Chang, W.S., Stacey, G., Emerich, D.W., Joshi, T., Xu, D., and Sadowsky, M.J. 2007. Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. *J. Bacteriol.* **189**, 6751–6762.

Datsenko, K.A. and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**, 6640–6645.

Day, D.A., Poole, P.S., Tyerman, S.D., and Rosendahl, L. 2001. Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. *Cell Mol. Life Sci.* **58**, 61–71.

Donati, A.J., Jeon, J.M., Sangurdekar, D., So, J.S., and Chang, W.S. 2011. Genome-wide transcriptional and physiological responses of *Bradyrhizobium japonicum* to paraquat-mediated oxidative stress. *Appl. Environ. Microbiol.* **77**, 3633–3643.

Evans, P.J., Gallesi, D., Mathieu, C., Hernandez, M.J., de Felipe, M., Halliwell, B., and Puppo, A. 1999. Oxidative stress occurs during soybean nodule senescence. *Planta* **208**, 73–79.

Figurski, D.H. and Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc. Natl. Acad. Sci. USA* **76**, 1648–1652.

Gourion, B., Sulser, S., Frunzke, J., Francez-Charlot, A., Stiefel, P., Pessi, G., Vorholt, J.A., and Fischer, H.M. 2009. The PhyR-sigma(EcfG) signalling cascade is involved in stress response and symbiotic efficiency in *Bradyrhizobium japonicum*. *Mol. Microbiol.* **73**, 291–305.

Gruber, T.M. and Gross, C.A. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu. Rev. Microbiol.* **57**, 441–466.

Halverson, L.J. and Stacey, G. 1986. Effect of lectin on nodulation by wild-type *Bradyrhizobium japonicum* and a nodulation-defective mutant. *Appl. Environ. Microbiol.* **51**, 753–760.

Jamet, A., Sigaud, S., Van de Sype, G., Puppo, A., and Herouart, D. 2003. Expression of the bacterial catalase genes during *Sinorhizobium meliloti-Medicago sativa* symbiosis and their crucial role during the infection process. *Mol. Plant-Microbe Interact.* **16**, 217–225.

Jeon, J.M., Lee, H.I., Donati, A.J., So, J.S., Emerich, D.W., and Chang, W.S. 2011. Whole-genome expression profiling of *Bradyrhizobium japonicum* in response to hydrogen peroxide. *Mol. Plant-Microbe Interact.* **24**, 1472–1481.

Jordan, D.C. 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *Int. J. Syst. Bacteriol.* **32**, 136–139.

Klotz, L.O. 2002. Oxidant-induced signaling: effects of peroxynitrite and singlet oxygen. *Biol. Chem.* **383**, 443–456.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M.2nd, and Peterson, K.M. 1994. pBBR1MCS: a broad-host-range cloning vector. *BioTechniques* **16**, 800–802.

Kuzma, M.M., Hunt, S., and Layzell, D.B. 1993. Role of oxygen in the limitation and inhibition of nitrogenase activity and respiration rate in individual soybean nodules. *Plant Physiol.* **101**, 161–169.

Lee, H.I., Lee, J.H., Park, K.H., Sangurdekar, D., and Chang, W.S. 2012. Effect of soybean coumestrol on *Bradyrhizobium japonicum* nodulation ability, biofilm formation, and transcriptional profile. *Appl. Environ. Microbiol.* **78**, 2896–2903.

Leong, S.A., Ditta, G.S., and Helinski, D.R. 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from *Rhizobium meliloti*. *J. Biol. Chem.* **257**, 8724–8730.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**, 583–593.

Liang, Y., Cao, Y., Tanaka, K., Thibivilliers, S., Wan, J., Choi, J., Kang, C., Qiu, J., and Stacey, G. 2013. Nonlegumes respond to rhizobial Nod factors by suppressing the innate immune response. *Science* **341**, 1384–1387.

Loprasert, S., Vattanaviboon, P., Praituan, W., Chamnongpol, S., and Mongkolsuk, S. 1996. Regulation of the oxidative stress protective enzymes, catalase and superoxide dismutase in *Xanthomonas*-a review. *Gene* **179**, 33–37.

Masloboeva, N., Reutimann, L., Stiefel, P., Follador, R., Leimer, N., Hennecke, H., Mesa, S., and Fischer, H.M. 2012. Reactive oxygen species-inducible ECF sigma factors of *Bradyrhizobium japonicum*. *PLoS One* **7**, e43421.

Martinez-Salazar, J.M., Salazar, E., Encarnacion, S., Ramirez-Romero, M.A., and Rivera, J. 2009. Role of the extracytoplasmic function sigma factor RpoE4 in oxidative and osmotic stress responses in *Rhizobium etli*. *J. Bacteriol.* **191**, 4122–4132.

Nuss, A.M., Glaeser, J., and Klug, G. 2009. RpoH(II) activates oxidative-stress defense systems and is controlled by RpoE in the singlet oxygen-dependent response in *Rhodobacter sphaeroides*. *J. Bacteriol.* **191**, 220–230.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 2002–2007.

Quandt, J. and Hynes, M.F. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* **127**, 15–21.

Sadowsky, M.J., Tully, R.E., Cregan, P.B., and Keyser, H.H. 1987. Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybean. *Appl. Environ. Microbiol.* **53**, 2624–2630.

Santos, R., Herouart, D., Sigaud, S., Touati, D., and Puppo, A. 2001. Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. *Mol. Plant-Microbe Interact.* **14**, 86–89.

Skorupska, A., Janczarek, M., Marczak, M., Mazur, A., and Krol, J. 2006. Rhizobial exopolysaccharides: genetic control and symbiotic functions. *Microb. Cell Fact.* **5**, 7.

Soto, M.J., Sanjuan, J., and Olivares, J. 2006. Rhizobia and plant-pathogenic bacteria: common infection weapons. *Microbiology* **152**, 3167–3174.

Stockwell, S.B., Reutimann, L., and Guerinot, M.L. 2011. A role for *Bradyrhizobium japonicum* ECF16 sigma factor EcfS in the formation of a functional symbiosis with soybean. *Mol. Plant-Microbe Interact.* **25**, 119–128.

Vriezen, J.A., de Bruijn, F.J., and Nusslein, K. 2007. Responses of rhizobia to desiccation in relation to osmotic stress, oxygen, and temperature. *Appl. Environ. Microbiol.* **73**, 3451–3459.

Zahran, H.H. 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* **63**, 968–989.