A Functional General Stress Response of *Bradyrhizobium diazoefficiens* Is Required for Early Stages of Host Plant Infection

Raphael Ledermann, Ilka Bartsch, Barbara Müller, Janine Wülser, and Hans-Martin Fischer†

ETH Zurich, Institute of Microbiology, Vladimir-Prelog-Weg 4, CH-8093 Zurich, Switzerland

Accepted 22 December 2017.

Phylogenetically diverse bacteria respond to various stress conditions by mounting a general stress response (GSR) resulting in the induction of protection or damage repair functions. In α -proteobacteria, the GSR is induced by a regulatory cascade consisting of the extracytoplasmic function (ECF) σ factor σ^{EcfG} , its anti- σ factor NepR, and the anti-anti- σ factor PhyR. We have reported previously that σ^{EcfG} and PhyR of *Bradyrhizobium diazoefficiens* (formerly named *Bradyrhizobium japonicum*), the nitrogen-fixing root nodule symbiont of soybean and related legumes, are required for efficient symbiosis; however, the precise role of the GSR remained undefined. Here, we analyze the symbiotic defects of a *B. diazoefficiens* mutant lacking σ^{EcfG} by comparing distinct infection stages of enzymatically or fluorescently tagged wild-type and mutant bacteria. Although root colonization and root hair curling were indistinguishable, the mutant was not competitive, and showed delayed development of emerging nodules and only a few infection threads. Consequently, many of the mutant-induced nodules were aborted, empty, or partially colonized. Congruent with these results, we found that σ^{EcfG} was active in bacteria present in root-hair-entrapped microcolonies and infection threads but not in root-associated bacteria and nitrogen-fixing bacteroids. We conclude that GSR-controlled functions are crucial for synchronization of infection thread formation, colonization, and nodule development.

To cope with stressful environmental conditions, bacteria have evolved genetic circuits which trigger specific or rather unspecific, global cellular responses to different types of stress. The latter process, termed general stress response (GSR), is based on alternative σ factors which enable transcription of genes belonging to stress-inducible regulons (Storz and Hengge 2011). Regulation of the GSR in α -proteobacteria is mediated by a unique regulatory cascade whose core elements are the extracytoplasmic

function (ECF) σ factor σ^{EcfG} , the cognate anti- σ factor NepR, and the NepR antagonist PhyR (Fiebig et al. 2015; Francez-Charlot et al. 2015). The three proteins act together in a partner-switching mechanism based on σ factor mimicry. It is triggered by stress-induced phosphorylation of the C-terminal receiver domain of PhyR and concomitant exposure of its N-terminal σ -factor-like domain, which has a higher affinity for NepR than σ^{EcfG} . Consequently, σ^{EcfG} is released from the NepR- σ^{EcfG} complex and redirects RNA polymerase to transcribe stress response genes (Campagne et al. 2012; Francez-Charlot et al. 2009; Herrou et al. 2012).

A subgroup of α -proteobacteria, symbiotic rhizobia, can fix atmospheric dinitrogen and provide it to their legume host plant in exchange for reduced carbon sources and other nutrients (Clarke et al. 2014; Udvardi and Poole 2013). The process takes place in specialized organs, termed root nodules, which host diazotrophic rhizobia as endosymbionts. Establishment of this mutualistic endosymbiosis involves a molecular cross-talk between both partners. Specifically, rhizobia respond to flavonoids secreted from hosts by the synthesis of species-specific lipo-chitooligosaccharides (D'Haeze and Holsters 2002; Downie 2014; Oldroyd 2013). These so-called Nod factors cause deformation and curling of root hairs, and they stimulate division of cortical root cells. Rhizobia entrapped within curled root hairs form microcolonies from which they initiate invasion of plant roots via infection threads (IT). Within the preformed nodule primordium (NP) in the root cortex, bacteria are released from IT into individual plant cells, which they will densely colonize to eventually differentiate into nitrogen-fixing bacteroids. The transition from free-living soil bacteria to symbiotic bacteroids exposes rhizobia to changing environmental conditions including nutritional, osmotic, and pH alterations which they have to cope with. Moreover, during early stages of infection, host plants transiently induce defense-like responses against rhizobia such as the production of reactive oxygen species or nitric oxide (Damiani et al. 2016; Gibson et al. 2008; Santos et al. 2001) and synthesis of pathogen-related proteins (Gamas et al. 1998; Saeki 2011; Tóth and Stacey 2015).

The stressful conditions to which rhizobia are exposed during early infection may suggest a role of the GSR at this stage; however, studies addressing this point are restricted to only a few selected species. In *Rhizobium etli*, two homologs of σ^{EcfG} are present, the chromosomally encoded σ^{EcfG1} (RpoE4) and the plasmid-borne σ^{EcfG2} (PF00052). Whereas σ^{EcfG1} contributes to tolerance against osmotic stress and heat shock (by regulating the gene for another σ factor, *rpoH2*), σ^{EcfG2} controls genes whose products protect from oxidative stress (e.g., *katG*-encoding catalase). *R. etli* double mutants lacking both σ^{EcfG} factors are particularly sensitive to stress (Jans et al. 2013; Martínez-Salazar et al. 2009).

Current address of I. Bartsch: Division of B Cell Immunology, German Cancer Research Center, Heidelberg, Germany.

†Corresponding author: H.-M. Fischer; E-mail: fischeha@ethz.ch

Funding: This work was funded by grants of the Swiss National Science Foundation (31003A_153446/1 and 31003A_173255/1) and grants from ETH Zurich.

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table and four supplementary figures are published online.

Similarly, *Sinorhizobium meliloti* also possesses two σ^{EcfG} orthologs (RpoE2 and RpoE5, with the former regulating transcription of the latter) but only RpoE2 was characterized functionally, and reports about its role in stress tolerance are controversial (Bastiat et al. 2010; Flechard et al. 2009, 2010; Humann et al. 2009; Sauviac et al. 2007). Most notably, none of the studied σ^{EcfG} mutants of *R. etli* and *S. meliloti* differed from the respective wild type with respect to their symbiotic properties.

The soybean symbiont *Bradyrhizobium diazoefficiens* (formerly known as *B. japonicum*) encodes a single ortholog of σ^{EcfG}

which belongs to a family of 17 different ECF σ factors found in this bacterium, and regulates the GSR in concert with the cognate PhyR and NepR proteins (Gourion et al. 2009; Masloboeva et al. 2012, 2015). In addition to conferring tolerance against heat shock and desiccation, we found that σ^{EcfG} is crucial for the symbiotic interaction between *B. diazoefficiens* and its host plants. Soybean and mung bean plants inoculated with ΔecfG or ΔphyR mutant bacteria showed delayed nitrogen fixation and outgrowth of lateral roots from aberrant nodule-like structures. To generate deeper insights into the peculiar phenotype, we set out to conduct a detailed cellular analysis of the symbiotic defects of a *B. diazoefficiens* ΔecfG mutant. Taking advantage of previously described tagging tools for *B. diazoefficiens* (Ledermann et al. 2015), we followed the fate of mutant bacteria from initial stages of symbiosis formation to mature bacteroids in fully developed nodules. Microscopy-based analysis of early infection stages and emerging nodules (EN) combined with a reporter construct monitoring σ^{EcfG} activity revealed that a functional GSR is required early during the symbiotic interaction between *B. diazoefficiens* and host plants but not in nitrogen-fixing bacteroids.

RESULTS

The ΔecfG mutant efficiently colonizes soybean roots.

Because growth in the rhizosphere markedly differs from growth of pure cultures in liquid media, we first determined whether survival and proliferation of the GSR-deficient ΔecfG mutant strain was affected on soybean roots. *B. diazoefficiens* wild-type and ΔecfG mutant bacteria were inoculated on soybean seedlings and incubated for 4 days under routine plant growth conditions. Roots were washed and CFU in wash fractions were determined. The mutant strain not only survived on plant roots but also multiplied efficiently. When inoculated individually, similar numbers of wild-type and ΔecfG mutant bacteria were found in both loosely root-associated and root-attached fractions (Fig. 1A). However, when the ΔecfG mutant was coinoculated with the wild type, the latter strain out-competed the mutant in both fractions. Though comparable numbers of both strains were inoculated, wild-type bacteria dominated in the wash fractions, indicating that they were more competitive in rhizosphere colonization than the mutant (Fig. 1B).

First physical contacts between *Bradyrhizobium* cells and host plants occur via polar attachment of bacteria to roots, particularly root hairs (Vesper and Bauer 1986; Vesper et al. 1987). We inoculated tagged derivatives of either the wild type or the ΔecfG mutant on soybean roots and inspected epidermal cell layers, including root hairs, for polarly attached bacteria. We found that the mutant is able to attach to root hairs in a polar manner indistinguishable from wild-type cells (Fig. 1C; data for wild-type cells not shown).

Nod-factor signaling is functional in the ΔecfG mutant.

Key steps at the onset of the rhizobia–legume symbiosis are production and secretion of Nod factors by the symbiont and their perception by host plants (D’Haeze and Holsters 2002; Oldroyd 2013). We investigated whether Nod factor signaling is still functional in the symbiotically affected ΔecfG mutant. A typical sign of Nod factor signaling is root hair curling, which is easily observed by light microscopy (Green and Emerich 1999). We inoculated soybean seedlings with either wild-type or ΔecfG mutant bacteria, stained for curled root hairs, and observed epidermal cell layers under the microscope. Both strains caused short, emerging root hairs to curl, which is typical for soybean (Turgeon and Bauer 1985) and occurred at comparable frequency and kinetics (Fig. 2A and B). To further

Fig. 1. Root colonization of *Bradyrhizobium diazoefficiens* wild-type and ΔecfG mutant bacteria (strain 8404). Both strains were inoculated **A**, separately or **B**, together on 2-day-old soybean seedlings which were further incubated for 4 days under plant growth conditions. At 4 days post-inoculation, roots were harvested, loosely associated (wash fraction 1) and root-attached (wash fraction 2) bacteria were washed off, and CFU were determined as described in Materials and Methods. Shown are mean values with standard deviations of four technical replicates (inocula) and six (A) or eight (B) biological replicates (wash fractions). **C**, GusA-tagged ΔecfG mutant cells (04-GusA-1) (white arrowheads) polarly attached to a soybean root hair cell as also observed with wild-type cells (not shown).

show functional Nod factor production by the mutant, we coinoculated an mCherry-tagged $\Delta ecfG$ mutant derivative together with an sYFP2-tagged *nodA* mutant, which is unable to synthesize functional Nod factor and, hence, cannot nodulate host plants (Lamb and Hennecke 1986). When occupancy of nodules was determined, we found that most of the nodules were colonized by the *nodA* strain (Fig. 2C and D), indicating that the Nod factor-deficient mutant was rescued by functional Nod factors produced by the $\Delta ecfG$ mutant.

The $\Delta ecfG$ mutant lacks competitiveness during nodulation.

Intrigued by the fact that the $\Delta ecfG$ mutant was less efficient in nodule colonization when coinoculated with the *nodA*-mutant strain, we also performed competition assays by coinoculation of the $\Delta ecfG$ mutant with the wild type or an *nifH* mutant strain, which is unable to fix nitrogen and, thus, shows a late symbiotic defect (Hahn et al. 1984). In a first round, we coinoculated the $\Delta ecfG$ mutant and either competitor in a 1:1 and 10:1 ratio. We found that all analyzed nodules were inhabited only by the competitor but never with the $\Delta ecfG$ mutant. Therefore, we increased the ratio of $\Delta ecfG$ mutant to wild-type bacteria in subsequent experiments. Even at a 10,000-fold excess of the mutant in the inoculum, all nodules were exclusively occupied by wild-type bacteria (data not shown).

Nodule development is arrested in $\Delta ecfG$ mutant-inoculated plants.

Besides root hair curling, Nod factor signaling also triggers mitotic activity in the root cortex to form NP (Green and Emerich 1999; Oldroyd et al. 2011). When mitotic activity is continuing, these primordia develop into EN. We applied hematoxylin staining to roots inoculated with either wild-type or $\Delta ecfG$ mutant bacteria, which specifically marks mitotic plant cells (i.e., root tips and young nodule structures) (Dudley et al. 1987; Green and Emerich 1999). We counted the occurrence of young developing primordia consisting of only a few dividing cells and of EN which protruded from the root (Fig. 3A) at different time points after inoculation (Fig. 3B). At 48 h post-inoculation (hpi), the first NP were detected on wild-type-inoculated seedlings and their number steadily increased to reach a maximum at 96 hpi. In contrast, first NP were detected only at 72 hpi on $\Delta ecfG$ mutant-inoculated plants, and their number remained smaller than on wild-type-inoculated plants throughout the entire observation period. From 96 hpi on, NP on wild-type-inoculated plants started to develop into EN, and the number of NP did not further increase, because new ones were no longer formed. Though the $\Delta ecfG$ mutant provoked a substantial number of NP, their further development into EN was strongly attenuated, resulting in only few of them detected at 144 hpi.

Formation of IT by the $\Delta ecfG$ mutant is impaired.

To compare the efficiency of IT formation by the wild type and the $\Delta ecfG$ mutant, we inoculated GusA-expressing derivatives of these strains on soybean seedlings and enumerated IT at three different time points (Fig. 4A). In wild-type-inoculated plantlets, IT were observed not later than 5 days postinoculation (dpi). Their number increased until 8 dpi and remained at this level 11 dpi. Notably, at the latter time point, wild-type-inoculated plants had already developed small nodules. In contrast, the $\Delta ecfG$ mutant barely induced IT until 8 dpi and only a few by 11 dpi; and, in agreement with the results described above (Fig. 3B), no nodules had developed at this stage (data not shown). IT induced by the wild type were often associated with mitotically active plant cells forming an NP (Fig. 4B). Consistent with the results of the hematoxylin staining

(Fig. 3A), NP were also detected on $\Delta ecfG$ mutant-inoculated plants when we enumerated IT using GusA staining. However, nearby curled root hairs were mostly devoid of IT and, thus, also of bacteria (Fig. 4C).

Fig. 2. Nod factor signaling by wild-type and $\Delta ecfG$ mutant *Bradyrhizobium diazoefficiens* cells. Wild-type and $\Delta ecfG$ bacteria were inoculated on 2-day-old soybean seedlings. Roots were stained with methylene blue 48 h postinoculation and inspected under the microscope for curled root hairs as a direct sign of Nod factor signaling. Both, **A**, the wild type and **B**, the $\Delta ecfG$ mutant (strain 8404) caused root hair curling. **C**, Tagged derivatives of the $\Delta ecfG$ mutant (04-mCh-4) and *nodA* mutant (*nodA2*-sYFP2-4) were inoculated at a 1:1 ratio and a total cell number of 10^3 bacteria/plant. When analyzed by fluorescent microscopy 21 days postinoculation, most nodules were colonized by the *nodA* mutant strain; few nodules contained only the $\Delta ecfG$ mutant, both strains (mixed), or no visible bacteria (empty). Data are based on the analysis of 10 nodules taken from each of two plants. **D**, Semithin section of a mixed nodule harboring $\Delta ecfG$ (red) and *nodA* (yellow) mutant bacteroids.

Colonization of EN by wild-type and $\Delta ecfG$ mutant bacteria was further analyzed using mCherry-tagged strains. Given the delayed nodule formation by the $\Delta ecfG$ mutant (see above) (Gourion et al. 2009), EN induced by the wild type and $\Delta ecfG$ mutant were compared 8 and 15 to 19 dpi, respectively. Most wild-type-induced EN showed many dispersed, ramified IT populated with fluorescent bacteria all over the inner nodule tissue (Fig. 5A and F). Accordingly, colonized plant cells in mature nodules were evenly distributed and interspersed with a few uninfected cells (Fig. 5B). By contrast, the vast majority of mutant-elicited EN lacked both IT and bacteria, and they consisted of empty, vacuolated plant cells (Fig. 5C, E, and F). When found at all, IT in EN induced by the $\Delta ecfG$ mutant were much less abundant. They were restricted to islands of nonvacuolated cells (characterized by enhanced blue auto-fluorescence), which were separated by large areas of vacuolated cells that never were associated with IT (Fig. 5C) (Newcomb et al. 1979). Consequently, mature $\Delta ecfG$ mutant-elicited nodules showed a patchy colonization, with islands of densely populated plant cells separated by areas of uninfected cells (Fig. 5D).

The GSR is transiently active during formation of symbiosis.

We constructed transcriptional fusions between promoterless *lacZ* or *sYFP2* reporter genes and the σ^{EcfG} -dependent *bll6649* gene, and confirmed that *bll6649* promoter activity is very low in unstressed conditions, and strongly enhanced upon GSR induction by carbon starvation (Supplementary Fig. S1) (Gourion et al. 2009). Using the *sYFP2* fusion, we monitored σ^{EcfG} activity in bacteria at different stages of the symbiotic interaction (i.e., in cells dwelling in the plant growth substrate [vermiculite]; present in the rhizosphere, microcolonies, or IT; or as bacteroids in host cells of mature nodules). Yellow fluorescence indicating GSR activity was detected in a subpopulation of the bacteria present in vermiculite (Supplementary Fig. S2). Bacteria present in the rhizosphere or attached to root hairs showed no *sYFP2* signal, indicating that σ^{EcfG} is not active at this stage (Fig. 6A). By contrast, bacterial cells entrapped as microcolonies in curled root hairs and in emerging IT showed pronounced yellow fluorescence, revealing that σ^{EcfG} was activated between

attachment and microcolony formation (Fig. 6B). The *sYFP2* signal persisted in bacteria present in IT present in young nodules but disappeared in bacteroids of mature nodules (Fig. 6C).

The $\Delta ecfG$ mutant phenotype is similar on various IT-dependent hosts but not on the crack-entry host *Aeschynomene afraspera*.

We have previously shown that the phenotype of the $\Delta ecfG$ mutant is consistent on two different soybean cultivars (Williams 82 and Green Butterbean) and also on mung bean (Gourion et al. 2009). We expanded the phenotypic analysis to include four additional soybean cultivars (Black Jet, Envy, Tohya, and Viking 2265) and two additional hosts of *B. diazoefficiens* (i.e., siratro and cowpea). On all hosts, we observed the characteristic $\Delta ecfG$ mutant phenotype, comprising delayed nitrogen fixation accompanied by chlorotic appearance of plant leaves; heterogeneous, small, and white to almost normally developed nodules; and outgrowth of roots from some nodules (data not shown).

Our finding that the $\Delta ecfG$ mutant is impaired in the induction of IT (see above) prompted us to determine its symbiotic phenotype on a host which is infected via cracks in the root epidermis (crack entry). An example for this type of legume is *Aeschynomene afraspera*, which is nodulated by photosynthetic bradyrhizobia and also *B. diazoefficiens* USDA 110 (Bonaldi et al. 2011; Renier et al. 2011). We analyzed wild-type- and $\Delta ecfG$ -infected *A. afraspera* plants for root colonization (5 dpi) and symbiotic performance (14 and 29 dpi). As on soybean (Fig. 1A and B), the $\Delta ecfG$ mutant was not affected in root colonization when inoculated alone (Supplementary Fig. S3) but lacked competitiveness when coinoculated with the wild type. In contrast to soybean, the root-associated fraction contained similar cell numbers of both strains under competitive conditions. The $\Delta ecfG$ mutant elicited many fewer nodules than the wild type, yet their average dry weight at 29 dpi was more than five times higher than that of wild-type-induced nodules. Though low, nitrogenase activity was detected 14 dpi in young nodules of the $\Delta ecfG$ mutant and, by 29 dpi, it increased to a higher level than that of wild-type nodules. Colonization of both wild-type- and $\Delta ecfG$ mutant-elicited *A. afraspera* nodules was homogenous as deduced from the

Fig. 3. Development of nodule primordia (NP) and emerging nodules (EN) on soybean seedlings infected with either wild-type or $\Delta ecfG$ mutant (strain 8404) *Bradyrhizobium diazoefficiens*. In all, 18 to 20 roots per strain and time point were harvested and stained for mitotic plant cells with hematoxylin. **A**, Stain caused mitotic cells to become dark black. Small dark spots of dividing cells in the outer cortex were classified as NP or EN when bumps became visible on the roots. **B**, Number of NP and EN counted on one half-side of the main root of each plant. The $\Delta ecfG$ mutant produced fewer NP than the wild type, and almost all of these NP failed to develop into EN, whereas the wild type induced increasing numbers of EN from 72 to 120 h postinoculation (hpi). Error bars represent standard deviation. For statistical analysis, a Kruskal-Wallis test (D'Agostino & Pearson omnibus K^2 normality test not passed) with a post hoc Dunn's multiple comparisons test ($\alpha = 0.05$) was performed. Significant differences were detected for EN at 120 hpi (adjusted $P < 0.0001$) and 144 hpi (adjusted $P = 0.0002$); all other differences were not significant (ns; adjusted $P > 0.1$).

uniform red interior color caused by leghemoglobin. This is different from soybean (and all other IT-dependent) nodules, which showed distinct colonization patterns for the wild type and the *ΔecfG* mutant (Fig. 5B and D). Likewise, *A. afraspera* plants inoculated with the *ΔecfG* mutant never formed aborted nodules or nodule-root outgrowths at any time point (data not shown).

Finally, we also investigated GSR activity during infection of *A. afraspera* using the reporter system described above. Unlike our finding with soybean, GSR activity was not induced at any stage of *A. afraspera* infection (i.e., neither in rhizosphere-colonizing bacteria nor in intercellularly invading bacteria nor in microcolonies inside of roots) (Supplementary Fig. S4) (Bonaldi et al. 2011).

DISCUSSION

The symbiotic defects of *B. diazoefficiens* *ΔecfG* and *ΔphyR* mutants which we reported previously (Gourion et al. 2009) indicated that a functional GSR is required for proper development and functioning of nodules; however, at which exact stage of the symbiotic interaction a functional GSR is needed remained undefined. Here, we present a characterization of the symbiotic defects of the GSR-deficient *B. diazoefficiens* *ΔecfG* mutant.

We followed the fate of both wild-type and mutant symbionts and observed host responses from initial interactions until fully developed nodules. The first encounter between the plant and soilborne *B. diazoefficiens* cells takes place in the rhizosphere. Because this environment can be stressful for many bacteria (Matilla et al. 2007), it was tempting to speculate that the GSR of *B. diazoefficiens* was needed at this stage and, hence, a GSR-deficient mutant would be impaired in root colonization. However, our data showed that the *ΔecfG* mutant colonized soybean roots as efficiently as the wild type in monoassociation, and σ^{EcfG} was not active in root hair-associated bacteria. Likewise, we observed root hair curling and formation of numerous NP on mutant-inoculated plants, which are both direct signs of effective Nod factor production and signaling (Crespi and Frugier 2008; Geurts and Bisseling 2002; Green and Emerich 1999). Though it is possible that the decreased rate of NP formation by the mutant was due to reduced levels of Nod factor, it is arguably not the cause for the symbiotic defect because it was observed independently of the inoculum density (data not shown). Moreover, the Nod[−] phenotype of a *B. diazoefficiens* *nodA* mutant (Lamb and Hennecke 1986) was rescued by coinoculation with the *ΔecfG* mutant. Conversely, the *ΔecfG* mutant was not rescued by the coinoculated *nodA* mutant strain, suggesting that the hypothetical, σ^{EcfG} -dependent factors that are crucial for efficient symbiosis are likely not diffusible.

The *ΔecfG* mutant was not competitive, as inferred from the reduced root surface colonization of the mutant when coinoculated with the wild type (Fig. 1B). Unlike bacteria present in the rhizosphere, GSR was induced in a subpopulation of the bacteria dwelling in the plant growth substrate, likely due to limited nutrients, which triggers GSR activation. We hypothesize that GSR induction at this stage primes the wild type for more efficient root colonization under competitive conditions. However, the biased root colonization in favor of the wild type alone can probably not explain why only wild-type nodules were formed because the same result was obtained even when the mutant was present at a 10,000-fold excess in the inoculum.

Although the number of NP induced by the *ΔecfG* mutant 6 dpi was comparable with that of the wild type, most of the mutant-evoked structures lacked nearby IT or bacteria and did not further develop into early nodules. This is typical for pseudoinfections, which solely depend on diffusible Nod factor

and also occur during infection by wild-type rhizobia (Calvert et al. 1984). Thus, in contrast to rhizosphere colonization and Nod factor production, IT formation by the *ΔecfG* mutant was severely impaired, indicating that σ^{EcfG} -controlled functions are crucial for this process. Notably, this is in agreement with our parallel finding that σ^{EcfG} activity was induced in bacterial microcolonies entrapped in curled root hairs. On the other hand, the absence of an sYFP2 signal in nitrogen-fixing bacteroids present in mature nodules indicated that the GSR is no longer activated at this stage, which parallels the efficient (yet delayed) nitrogen fixation ability of *B. diazoefficiens* *ΔecfG* mutant bacteria (Gourion et al. 2009). Remarkably, a similar expression pattern was described for RpoE2 of *S. meliloti*, yet this ortholog of *B. diazoefficiens* σ^{EcfG} was dispensable for symbiosis (Capela et al. 2006; Jamet et al. 2003; Sauviac et al. 2007).

All tested hosts that were infected via IT displayed the characteristic *ΔecfG* mutant phenotype. Because *B. diazoefficiens* also nodulates *A. afraspera*, which uses an IT-independent mode of infection (crack entry) (Bonaldi et al. 2011; Renier et al. 2011), we were interested to learn how the *ΔecfG* mutant performs on this host. The performance of the *ΔecfG* mutant in the rhizosphere of *A. afraspera* and soybean was very similar, except that the root-attached fraction was not biased toward the wild type under competitive conditions. At this point, we only can speculate about the reason for this difference. Notably, plant growth conditions differed for the two plant species. Unlike soybean, *A. afraspera* plants were cultivated under water-logged conditions. Furthermore, *A. afraspera* and soybean belong to different legume clades (Dalbergioids and Millettoids, respectively) and, thus, may differ in colonization-related characteristics (e.g., in

Fig. 4. Infection thread (IT) development on soybean seedlings observed by 5-bromo-4-chloro-3-indolyl- β -D-glucuronide staining of GusA-tagged derivatives of wild-type (strain GusA-1) and *ΔecfG* mutant (04-GusA-1) bacteria. **A**, Number of IT present on one side of the main root. Five individual plants were inspected per strain and time point and standard deviation is indicated. Wild-type bacteria induced many visible IT as early as 5 days postinoculation (dpi), whereas only very few IT were observed on *ΔecfG* mutant-inoculated seedlings even 11 dpi. **B**, IT in wild-type-inoculated plant starting out from a root hair (RH) and progressing into epidermal root cells. Frequently, IT development was associated with anticlinal division of nearby cells, leading to the formation of a nodule primordium (NP). **C**, *ΔecfG* mutant-inoculated seedlings developed NP similarly as the wild type but nearby RH remained largely uninfected.

Fig. 5. Impaired infection of soybean roots by $\Delta ecfG$ mutant bacteria. Emerging soybean nodules (EN) elicited by mCherry-tagged derivatives of the *Bradyrhizobium diazoefficiens* wild type (strain mChe-1) or $\Delta ecfG$ mutant (04-mChe-1) were analyzed by fluorescence microscopy at comparable infection stages (i.e., 8 days postinoculation [dpi] for mChe-1 and 15, 17, and 19 dpi for 04-mChe-1). **A** and **C**, Infection threads (IT) in EN visualized by infecting red fluorescent wild-type and $\Delta ecfG$ bacteria, respectively. In a minor fraction of EN elicited by the $\Delta ecfG$ mutant, few IT were present in regions of nonvacuolated plant cells (nVC) showing pronounced blue autofluorescence separated by regions of vacuolated cells (VC) that largely lack cytoplasmic blue autofluorescence. **B** and **D**, Nodules (28 dpi) colonized by wild-type and $\Delta ecfG$ mutant bacteria showed homogenous and patchy distribution of infected plant cells, respectively. **E**, Most mutant-induced EN lacked IT and, thus, bacteria, and plant cells contained large vacuoles. **F**, Ratio between IT-containing and IT-lacking EN detected on soybean plants infected with wild-type (8 dpi) and $\Delta ecfG$ mutant (15, 17, and 19 dpi) bacteria. Data are based on the analysis of 22 wild-type and 92 mutant nodules.

Fig. 6. Visualization of σ^{EcrG} activity at different stages during symbiosis in *Bradyrhizobium diazoefficiens* cells tagged with a constitutive *mCherry* and a σ^{EcrG} -controlled *sYFP2* gene (strain 9953). Top three rows show inverted gray-value images recorded in red, yellow, and cyan channels, respectively. The fourth row shows false-colored composites of the three images above them. The bottom row displays plots of 16-bit gray values scanned along the thin white lines indicated in the composite images (ends marked by asterisks) with red, blue, and olive lines displaying gray values observed in the red, cyan, and yellow channels, respectively. **A**, Root-attached bacteria (5 days postinoculation [dpi]) lacked σ^{EcrG} activity because red fluorescent cells (e.g., four cells captured by the scan along the white line and labeled with 1, 2 [two cells], and 3) displayed no signals in the yellow channel. **B**, Bacteria in microcolonies and infection threads (ITs) in root hairs (5 dpi) showed σ^{EcrG} activity as deduced from their *sYFP2* fluorescence. The scan along the line which crosses two IT inside the root hair (1 and 2) documents cooccurrence of red and yellow fluorescence. **C**, Absence of *sYFP2* signals and, thus, σ^{EcrG} activity in mature bacteroids (19 dpi). A scan across an infected plant cell documented strong red fluorescence of cytoplasmic bacteroids (2 and 4) and low red signal intensity across plant cell walls (1 and 5) and the (noninfected) nucleus (3). Cell walls showed local maxima in the yellow and cyan channel due to autofluorescence of lignified structures. Cytoplasmic regions of maximal red fluorescence did not show yellow fluorescence higher than background, which indicates that σ^{EcrG} is not active in bacteroids.

the composition of root exudates, including flavonoids) (Renier et al. 2011) or root surface properties. Regardless of the precise reason, we conclude that, overall, the ΔecfG mutant lacks competitiveness in root colonization with both host plants. The symbiotic phenotype of the mutant on *A. afraspera* markedly differed from all other hosts by the absence of aborted or aberrantly colonized nodules. Furthermore, the onset of nitrogenase activity was not delayed when wild-type- and ΔecfG -infected *A. afraspera* plants were compared. This result is compatible with the lack of GSR activation during the intercellular infection process. However, the observation that the mutant induced many fewer, enlarged nodules than the wild type indicates that σ^{EcfG} is indeed involved in formation of symbiosis with *A. afraspera*. It is possible that transient GSR induction during the peculiar crack-entry mode of infection on *A. afraspera* escaped our analysis or that a functional GSR is required at a stage that we have not investigated.

In addition to the crack-entry mode of infection, *A. afraspera* also differs from the other hosts by the fact that no distal NP are initiated before bacteria arrive at the primordium founder cells (Bonaldi et al. 2011). Therefore, it is tempting to speculate that aberrant ΔecfG mutant nodules on IT-dependent hosts resulted from disturbed synchronization of NP development and bacterial delivery. Because the mutant was impaired at the IT initiation stage, invading bacteria could not reach preformed NP or were delayed, which may result in perturbed nodule development or reprogramming of EN leading to root outgrowths.

The molecular determinants causing the symbiotic defects of the *B. diazoefficiens* ΔecfG mutant remain enigmatic, as do the σ^{EcfG} -dependent traits conferring a competitive advantage in the transition from substrate-borne to rhizosphere-colonizing bacteria. The σ^{EcfG} regulon has been determined in transcriptome studies with free-living *B. diazoefficiens* cells (Gourion et al. 2009). It consists of more than 100 genes, of which many are functionally undefined; thus, it is formally possible that the symbiotic phenotype is caused by the simultaneous misregulation of multiple σ^{EcfG} -dependent genes. Although clarification of this aspect requires more work, it is interesting to note that the GSR is involved in interactions of numerous other α -proteobacteria with their hosts. For example, GSR-defective mutants of the phyllosphere-colonizing bacteria *Methylobacterium extorquens* and *Sphingomonas melonis* are either unable to colonize leaves (Gourion et al. 2006) or lack competitiveness (Kaczmarczyk et al. 2011). In the mammalian pathogen *Brucella abortus*, a functional GSR is required not only for tolerance against acidic and oxidative stress but also for survival of the pathogen within spleens of mice (Kim et al. 2013). Likewise, *Brucella melitensis* lacking the σ^{EcfG} -type σ factor RpoE1 are less persistent both in cultured cells and mouse models (Delory et al. 2006); and, in *Bartonella quintana*—the causative agent of trench fever—the GSR is involved in adaptation to high hemin concentrations that prevail in the natural louse vector of this pathogen (Abromaitis and Koehler 2013).

Obviously, our findings raise questions about the nature of the signals acting as GSR inducer at the early infection stage and the involved signal transduction pathways. Although it is known that the σ^{EcfG} -dependent GSR is induced in free-living *B. diazoefficiens* cells exposed to carbon starvation (Gourion et al. 2009) and different other types of stress (osmotic, salt, or pH stress) (unpublished results), we can only speculate at this stage about potential “symbiotic stress signals”. Reactive oxygen species and other products of the plant innate immune response which is activated upon contact with rhizobia are candidate stimuli for triggering the bacterial GSR (Damiani et al. 2016; Gourion et al. 2015; Saeki 2011; Tóth and Stacey 2015). It is also conceivable that maximal GSR induction is brought about by the concerted action of several signals. Support for this view comes

from the observation that, in *S. melonis*, distinct environmental stress signals are perceived and integrated by a set of partially redundant sensory histidine kinases to modulate PhyR activity via phosphorylation (Kaczmarczyk et al. 2014). Notably, *B. diazoefficiens* also encodes several of these sensory histidine kinases, which may be involved in sensing and transducing yet undefined “symbiotic signals” which trigger the GSR at the onset of host infection. Functional characterization of this largely unexplored plant–symbiont interface represents an attractive goal for future studies.

MATERIALS AND METHODS

Bacterial strains and cultivation.

All strains used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown at 37°C in Luria-Bertani medium (Miller 1972), supplemented (if appropriate) with antibiotics at the following concentrations: ampicillin at 200 µg/ml, streptomycin at 50 µg/ml, or tetracycline at 10 µg/ml. *B. diazoefficiens* strains were grown at 30°C in peptone-salts-yeast extract (PSY) medium supplemented with 0.1% L(+)-arabinose (Mesa et al. 2008), and antibiotics were used at the following concentrations: kanamycin at 100 µg/ml, spectinomycin at 100 µg/ml, and tetracycline at 50 µg/ml in solid media and 25 µg/ml in liquid media.

Construction of plasmids and mutants.

Tagged derivatives of *B. diazoefficiens* mutant strains 8404 ($\Delta\text{ecfG}::\text{aphII}$), H1 ($\text{nifH}::\text{Tn5}$), and nodA2 ($\text{nodA}::\text{aphII}$) were constructed following the same strategy described previously for wild-type derivatives (Lederhann et al. 2015).

To monitor σ^{EcfG} activity, we generated reporter strains by integration of promoterless reporter genes (*lacZ*YA and *sYFP2*) downstream of the σ^{EcfG} -dependent bll6649 gene to form synthetic transcriptional fusions. The bll6649 gene encodes a protein of unknown function comprising a PRC-barrel domain and, in previous microarray experiments, it was found to be strongly induced under GSR-activating carbon-starvation conditions (Gourion et al. 2009). To create the bll6649-*lacZ*YA transcriptional fusion, bll6649 was amplified with primers 6649-1/-2 (all oligonucleotides used in this study are shown in Supplementary Table S1). The *lacZ*YA-containing plasmid pRJpaph-*lacZ*YA was digested with *NotI* and *PstI* to release an 817-bp DNA fragment corresponding to the *scoI* downstream region of *B. diazoefficiens*, treated with the Klenow fragment of DNA polymerase I, and religated. In the resulting plasmid pPaph-*lacZ*YA, *PaphII* was excised using *EcoRI* and *SpeI* and replaced with the identically digested bll6649 amplicon to yield pRJ9937. This plasmid was transferred to the *B. diazoefficiens* wild type and ΔecfG mutant by biparental matings. The chromosomal bll6649-*lacZ*YA reporter fusion showed low β -galactosidase background activity in unstressed cells and strong, σ^{EcfG} -dependent induction in carbon-starved cells. To monitor σ^{EcfG} activity during symbiosis by fluorescence, the *lacZ*YA reporter operon was replaced by *sYFP2*, and a constitutively expressed (from *PaphII*) *mCherry* gene was added to the reporter plasmid to facilitate localization of bacteria. To this end, pRJpaph-*sYFP2* was digested with *AarI* and *NotI*, treated with the Klenow fragment of DNA polymerase I, and religated. The *sYFP2* reporter gene was excised from the resulting plasmid pPaph-*sYFP2* using *BglII* (blunted) and *NdeI*, and the fragment comprising *sYFP2* was ligated to *SpeI* (blunted)- and *NdeI*-digested pRJ9937 to yield pRJ9952. This plasmid was further converted to pRJ9953, which included the constitutively expressed *PaphII*-*mCherry* cassette. To do so, a 64-bp fragment was deleted from pRJpaph-*mCherry* by *BglII* digestion and relegation to yield pRJpaph-*mCherry*_a1. From this plasmid, the *PaphII*-*mCherry* fragment was obtained by digestion with *KpnI* (blunted) and *EcoRI* and ligated with *NcoI* (blunted)- and

*Eco*RI-digested pRJ9952, yielding pRJ9953. Plasmid pRJ9953 was transferred into the *B. diazoefficiens* wild type and 8404 Δ *ecfG* mutant strain as plasmid pRJ9937 was. Correct chromosomal insertion of the reporter constructs downstream of *bll6649* was verified by colony polymerase chain reaction using primers 6649-3 in combination with either *lacZ*-1 or *xxFP*-2. The *sYFP2* reporter showed a strong σ^{EcfG} -dependent induction upon stress, as did the *lacZYA* reporter.

Analysis of root colonization and attachment to root hairs.

Strains mChe-4 and 04-mChe-4 or GusA-1 and 04-GusA-1 were inoculated on soybean seedlings and simultaneously spotted as appropriate serial dilutions on PSY agar medium for CFU determination. Inoculated seedlings were incubated under conditions we use for routine plant infection tests (Göttfert et al. 1990). Roots were harvested 4 dpi and washed as described elsewhere (Bulgarelli et al. 2012), with the volume of the wash solution increased to 8 to 10 ml per wash step. Appropriate dilutions of wash fractions were spotted on PSY agar medium for determination of CFU counts and the total number of bacteria washed from whole roots was calculated. For observation of bacterial attachment to root hairs, epidermal cell layers of inoculated roots were peeled off and mounted on glass slides in 50% (vol/vol) glycerol. For mixed inoculations with wild-type

(spectinomycin resistant) and Δ *ecfG* mutant (spectinomycin and kanamycin resistant) bacteria, untagged strains were used and CFU counts of individual strains were deduced from parallel spottings on selective and nonselective plates.

Observation of root hair curling.

To test for Nod factor activity, soybean seedlings were briefly dipped in bacterial suspensions (optical density at 600 nm [OD₆₀₀] = 0.1 to 0.0001) of either the *B. diazoefficiens* wild type or Δ *ecfG* mutant 8404. As controls, seedlings were incubated in sterile PSY medium. Subsequently, seedlings were placed on 0.8% water agar plates and incubated at 28°C in the dark for 48 and 72 h. Three replicates per strain, dilution, and time point were prepared. To observe curled root hairs, a staining procedure was applied based on a combination of previously described methods (Green and Emerich 1999; Stokkermans and Peters 1994). In brief, roots were cut from hypocotyls and placed in 1% (vol/vol) Na-hypochlorite solution for 90 min. Roots were shortly washed in distilled water and stained in 0.01% (wt/vol) methylene blue solution for 20 min, washed in 35% (vol/vol) ethanol for 2 h, and destained in 70% (vol/vol) ethanol for 48 h. Epidermal layers were detached from roots, mounted on glass slides in 50% glycerol, and microscopically examined using bright-field illumination or phase contrast.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype ^a	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> AM15) <i>hsdR17</i> <i>recA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA2</i>	BRL, Gaithersburg, MD, U.S.A.
S17-1 λ pir	Sm ^r Sp ^r <i>hsdR</i> (RP4-2 <i>kan::Tn7</i> <i>tet::Mu</i> ; chromosomally integrated)	de Lorenzo et al. 1993
<i>Bradyrhizobium diazoefficiens</i>		
110spc4	Sp ^r wild type	Regensburger and Hennecke 1983
8404	Sp ^r Km ^r Δ <i>ecfG::aphII</i>	Gourion et al. 2009
H1	Sp ^r Km ^r <i>nifH::Tn5</i>	Hahn et al. 1984
nodA2	Sp ^r Km ^r <i>nodA::aphII</i>	Lamb and Hennecke 1986
mChe-1	Sp ^r Tc ^r 110spc4 with chromosomal integration of pRJpaph-mCherry	Ledermann et al. 2015
sYFP2-4	Sp ^r Tc ^r 110spc4 with chromosomal integration of pRJpaf-sYFP2	Ledermann et al. 2015
mChe-4	Sp ^r Tc ^r 110spc4 with chromosomal integration of pRJpaf-mCherry	Ledermann et al. 2015
GusA-1	Sp ^r Tc ^r 110spc4 with chromosomal integration of pRJpaph-gusA	Ledermann et al. 2015
04-mChe-1	Sp ^r Km ^r Tc ^r Δ <i>ecfG::aphII</i> strain 8404 with chromosomal integration of pRJpaph-mCherry	This work
04-mChe-4	Sp ^r Km ^r Tc ^r Δ <i>ecfG::aphII</i> strain 8404 chromosomal integration of pRJpaf-mCherry	This work
04-GusA-1	Sp ^r Km ^r Tc ^r Δ <i>ecfG::aphII</i> strain 8404 with chromosomal integration of pRJpaph-gusA	This work
H1-sYFP2-4	Sp ^r Km ^r Tc ^r <i>nifH::Tn5</i> strain H1 with chromosomal integration of pRJpaf-sYFP2	This work
nodA2-sYFP2-4	Sp ^r Km ^r Tc ^r <i>nodA::aphII</i> strain nodA2 with chromosomal integration of pRJpaf-sYFP2	This work
9937	Sp ^r Tc ^r 110spc4 with chromosomal integration of pRJ9937 (<i>bll6649-lacZYA</i>)	This work
04-37	Sp ^r Km ^r Tc ^r Δ <i>ecfG::aphII</i> strain 8404 with chromosomal integration of pRJ9937 (<i>bll6649-lacZYA</i>)	This work
9953	Sp ^r Tc ^r 110spc4 with chromosomal integration of pRJ9953 (<i>bll6649-sYFP2</i> <i>P_{aphII}-mCherry</i>)	This work
04-53	Sp ^r Km ^r Tc ^r Δ <i>ecfG::aphII</i> strain 8404 with chromosomal integration of pRJ9953 (<i>bll6649-sYFP2</i> <i>P_{aphII}-mCherry</i>)	This work
Plasmids		
pRJpaph-sYFP2	Tc ^r <i>P_{aphII}-sYFP2</i> for integration downstream of <i>scoI</i>	Ledermann et al. 2015
pRJpaph-mCherry	Tc ^r <i>P_{aphII}-mCherry</i> for integration downstream of <i>scoI</i>	Ledermann et al. 2015
pRJpaf-sYFP2	Tc ^r <i>P_{nut}-sYFP2</i> for integration downstream of <i>scoI</i>	Ledermann et al. 2015
pRJpaf-mCherry	Tc ^r <i>P_{nut}-mCherry</i> for integration downstream of <i>scoI</i>	Ledermann et al. 2015
pRJpaph-gusA	Tc ^r <i>P_{aphII}-gusA</i> for integration downstream of <i>scoI</i>	Ledermann et al. 2015
pRJpaph-lacZYA	Tc ^r <i>P_{aphII}-lacZYA</i> for integration downstream of <i>scoI</i>	Ledermann et al. 2015
pRJpaph-mChe_a1	Tc ^r (pRJpaph-mChe) deleted 64 bp <i>Bgl</i> III fragment between <i>P_{aphII}</i> and <i>mCherry</i>	This work
pPaph-sYFP2	Tc ^r (pRJpaph-sYFP2) <i>scoI</i> downstream region deleted	This work
pPaph-lacZYA	Tc ^r (pRJpaph-lacZYA) <i>scoI</i> downstream region deleted	This work
pRJ9937	Tc ^r (pPaph-lacZYA) <i>bll6649-lacZYA</i>	This work
pRJ9952	Tc ^r (pRJ9937) <i>bll6649-sYFP2</i>	This work
pRJ9953	Tc ^r (pRJ9952) <i>bll6649-sYFP2</i> <i>P_{aphII}-mCherry</i>	This work

^a Abbreviations: Ap^r, Km^r, Sm^r, Sp^r, and Tc^r indicate resistance to ampicillin, kanamycin, streptomycin, spectinomycin, and tetracycline, respectively; P = promoter.

Mixed infections and competition experiments.

For competition experiments, mCherry-tagged *ΔecfG* strain 04-mChe-4 was inoculated together with sYFP2-tagged derivatives of either the *B. diazoefficiens* wild type (sYFP2-4), *nifH::Tn5* (H1-sYFP2-4), or *nodA::aphII* (nodA2-sYFP2-4). Infections, sample preparation, and analysis of nodule occupancy was done as described previously (Ledermann et al. 2015).

Staining of NP and EN.

Soybean seedlings were inoculated with either wild-type or *ΔecfG* strains (OD₆₀₀ = 0.01), or distilled water as a negative control. Plants were grown as described (Göttfert et al. 1990) and harvested at different time points. The method used for selective staining of mitotically active plant cells on roots was based on previously described protocols (Dudley et al. 1987; Green and Emerich 1999). In brief, roots were washed to remove vermiculite and cut from hypocotyls. Main roots were separated from lateral roots, boiled in lactophenol (20% phenol, 16% lactic acid, and 52% glycerol) for 2 min, and subsequently washed in distilled water. Roots were placed in 38 ml of distilled water, and 1 ml of hematoxylin stock solution (2 g in 50 ml 45% [vol/vol] acetic acid) and 1 ml of ferric chloride (32 mM) were added. Roots were stained for 12 h and subsequently washed in distilled water for 2 h. Finally, roots were destained in 20 mM HCl until they became clear. All NP and EN present on one half-side of each main root were counted under the stereomicroscope.

Observations of IT and colonization pattern.

Soybean seedlings were inoculated with *B. diazoefficiens* strains mChe-1, GusA-1, 04-mChe-1, or 04-GusA-1 at OD₆₀₀ = 0.01. Staining and observation of IT in root hairs were done as described previously (Ledermann et al. 2015). For observation of IT in young nodules and patterns of infected plant cells, nodules were treated as described (Ledermann et al. 2015). Fluorescent microscopy was used to visualize mCherry-tagged bacteria (Zeiss filter set 15; BP 546/12 FT580 LP590). For recording plant autofluorescence, Zeiss filter sets 10 (BP 450-490 FT 510 BP 515-565) and 49 (G 365 FT 395 BP 445/50) were used.

Monitoring $\sigma^{\text{Ec}^{\text{G}}}$ activity.

Carbon-starved and unstressed cultures of *B. diazoefficiens* 9937 and 04-37 were grown as described previously (Gourion et al. 2009), and β -galactosidase activity was determined as a proxy for $\sigma^{\text{Ec}^{\text{G}}}$ activity (Miller 1972). To visualize $\sigma^{\text{Ec}^{\text{G}}}$ activity by fluorescence, strains 9953 and 04-53 were constructed, each carrying a constitutively expressed mCherry gene and sYFP2 expressed from the $\sigma^{\text{Ec}^{\text{G}}}$ -dependent promoter of bll6649. Strains exposed to carbon starvation or not (control) were observed under a fluorescent microscope using Zeiss filter sets 15 (BP 546/12 FT580 LP590) and 46 (BP500/20 FT 515 BP535/30). Strains 9953 and 04-37 were inoculated on soybean or *A. afraspera* seedlings and different infection and colonization stages were observed by epifluorescence microscopy. Fluorescence of sYFP2 was recorded with Zeiss filter set 46 which overlaps with filter set 10. Accordingly, for observation of plant autofluorescence, filter sets 10 and 49 were replaced by Zeiss filter set 47 (BP436/20 FT 455 BP 480/40), which records neither mCherry nor sYFP2 fluorescence. Contrast and brightness were adjusted for optimal visibility. Because of the single-copy situation of the reporter fusion and the moderate activity of $\sigma^{\text{Ec}^{\text{G}}}$ -dependent promoters, fluorescent signals were rather low. Therefore, signal intensities were recorded along straight lines in regions of interest using Fiji/ImageJ 1.51g software applied to nonadjusted images.

Plant growth and nodulation experiments.

Seed of host plants—soybean [*Glycine max* (L.) Merr.] cultivars Williams 82 (provided by D.-N. Rodriguez, CIFA, Las

Torres-Tomejil, Seville, Spain), Black Jet, Green Butterbean, Envy, Tohya, and Viking 2265; mung bean [*Vigna radiata* (L.) R. Wilczek]; and cowpea [*Vigna unguiculata* (L) Walp] cultivar Iron & Clay (all purchased from Johnny's Selected Seeds, Albion, ME, U.S.A.); and siratro [*Macropitium atropurpureum* (DC.) Urb.] (provided by W. D. Broughton, University of Geneva, Switzerland)—were sterilized for 5 min in 100% ethanol and 15 min in 35% H₂O₂, intensively washed with sterile water, and germinated for 48 h. Sterilization of *A. afraspera* seed was done as described elsewhere (Renier et al. 2011), and plants were kept under water-logged conditions. Plant growth conditions and evaluation of symbiotic properties of *B. diazoefficiens* strains were described previously (Göttfert et al. 1990).

ACKNOWLEDGMENTS

We thank J. Vorholt for continuous support and valuable comments on the manuscript; M. Furter for help with image analysis; E. Giraud, D. N. Rodríguez-Navarro, and W. Broughton for legume seed; and J.-M. Couzigou for fruitful discussions about nodule organogenesis.

LITERATURE CITED

- Abromaitis, S., and Koehler, J. E. 2013. The *Bartonella quintana* extracytoplasmic function sigma factor RpoE has a role in bacterial adaptation to the arthropod vector environment. *J. Bacteriol.* 195: 2662-2674.
- Bastiat, B., Sauviac, L., and Bruand, C. 2010. Dual control of *Sinorhizobium meliloti* RpoE2 sigma factor activity by two PhyR-type two-component response regulators. *J. Bacteriol.* 192:2255-2265.
- Bonaldi, K., Gargani, D., Prin, Y., Fardoux, J., Gully, D., Nouwen, N., Goormachtig, S., and Giraud, E. 2011. Nodulation of *Aeschynomene afraspera* and *A. indica* by photosynthetic *Bradyrhizobium* Sp. strain ORS285: The nod-dependent versus the nod-independent symbiotic interaction. *Mol. Plant-Microbe Interact* 24:1359-1371.
- Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F. O., Amann, R., Eickhorst, T., and Schulze-Lefert, P. 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488:91-95.
- Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S. A., and Bauer, W. D. 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. *Can. J. Bot.* 62:2375-2384.
- Campagne, S., Damberger, F. F., Kaczmarczyk, A., Francez-Charlot, A., Allain, F. H., and Vorholt, J. A. 2012. Structural basis for sigma factor mimicry in the general stress response of Alphaproteobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 109:E1405-E1414.
- Capela, D., Filipe, C., Bobik, C., Batut, J., and Bruand, C. 2006. *Sinorhizobium meliloti* differentiation during symbiosis with alfalfa: A transcriptomic dissection. *Mol. Plant-Microbe Interact* 19:363-372.
- Clarke, V. C., Loughlin, P. C., Day, D. A., and Smith, P. M. 2014. Transport processes of the legume symbiosome membrane. *Front. Plant Sci.* 5:699.
- Crespi, M., and Frugier, F. 2008. De novo organ formation from differentiated cells: Root nodule organogenesis. *Sci. Signal.* 1:re11.
- Damiani, I., Pauly, N., Puppo, A., Brouquisse, R., and Boscari, A. 2016. Reactive oxygen species and nitric oxide control early steps of the legume - *Rhizobium* symbiotic interaction. *Front. Plant Sci.* 7:454.
- de Lorenzo, V., Cases, I., Herrero, M., and Timmis, K. N. 1993. Early and late responses of TOL promoters to pathway inducers: Identification of postexponential promoters in *Pseudomonas putida* with *lacZ-tet* bicistronic reporters. *J. Bacteriol.* 175:6902-6907.
- Delory, M., Hallez, R., Letesson, J. J., and De Bolle, X. 2006. An RpoH-like heat shock sigma factor is involved in stress response and virulence in *Brucella melitensis* 16M. *J. Bacteriol.* 188:7707-7710.
- D'Haese, W., and Holsters, M. 2002. Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology* 12: 79R-105R.
- Downie, J. A. 2014. Legume nodulation. *Curr. Biol.* 24:R184-R190.
- Dudley, M. E., Jacobs, T. W., and Long, S. R. 1987. Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti*. *Planta* 171: 289-301.
- Fiebig, A., Herrou, J., Willett, J., and Crosson, S. 2015. General stress signaling in the Alphaproteobacteria. *Annu. Rev. Genet.* 49:603-625.
- Flechar, M., Fontenelle, C., Blanco, C., Goude, R., Ermel, G., and Trautwetter, A. 2010. RpoE2 of *Sinorhizobium meliloti* is necessary for

- trehalose synthesis and growth in hyperosmotic media. *Microbiology* 156:1708-1718.
- Flechard, M., Fontenelle, C., Trautwetter, A., Ermel, G., and Blanco, C. 2009. *Sinorhizobium meliloti* *rpoE2* is necessary for H₂O₂ stress resistance during the stationary growth phase. *FEMS Microbiol. Lett.* 290:25-31.
- Francez-Charlot, A., Frunzke, J., Reichen, C., Ebner, J. Z., Gourion, B., and Vorholt, J. A. 2009. Sigma factor mimicry involved in regulation of general stress response. *Proc. Natl. Acad. Sci. U.S.A.* 106:3467-3472.
- Francez-Charlot, A., Kaczmarczyk, A., Fischer, H. M., and Vorholt, J. A. 2015. The general stress response in Alphaproteobacteria. *Trends Microbiol.* 23:164-171.
- Gamas, P., de Billy, F., and Truchet, G. 1998. Symbiosis-specific expression of two *Medicago truncatula* nodulin genes, MtN1 and MtN13, encoding products homologous to plant defense proteins. *Mol. Plant-Microbe Interact* 11:393-403.
- Geurts, R., and Bisseling, T. 2002. *Rhizobium* Nod factor perception and signalling. *Plant Cell* 14:S239-S249.
- Gibson, K. E., Kobayashi, H., and Walker, G. C. 2008. Molecular determinants of a symbiotic chronic infection. *Annu. Rev. Genet.* 42: 413-441.
- Göttfert, M., Hitz, S., and Hennecke, H. 1990. Identification of *nodS* and *nodN*, two inducible genes inserted between the *Bradyrhizobium japonicum* *nodYABC* and *nodIJ* genes. *Mol. Plant-Microbe Interact* 3: 308-316.
- Gourion, B., Berrabah, F., Ratet, P., and Stacey, G. 2015. *Rhizobium*-legume symbioses: The crucial role of plant immunity. *Trends Plant Sci.* 20: 186-194.
- Gourion, B., Rossignol, M., and Vorholt, J. A. 2006. A proteomic study of *Methylobacterium extorquens* reveals a response regulator essential for epiphytic growth. *Proc. Natl. Acad. Sci. U.S.A.* 103:13186-13191.
- Gourion, B., Sulser, S., Frunzke, J., Francez-Charlot, A., Stiefel, P., Pessi, G., Vorholt, J. A., and Fischer, H. M. 2009. The PhyR- σ^{EcfG} signalling cascade is involved in stress response and symbiotic efficiency in *Bradyrhizobium japonicum*. *Mol. Microbiol.* 73:291-305.
- Green, L. S., and Emerich, D. W. 1999. Light microscopy of early stages in the symbiosis of soybean with a delayed-nodulation mutant of *Bradyrhizobium japonicum*. *J. Exp. Bot.* 50:1577-1585.
- Hahn, M., Meyer, L., Studer, D., Regensburger, B., and Hennecke, H. 1984. Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. *Plant Mol. Biol.* 3:159-168.
- Herrou, J., Rotskoff, G., Luo, Y., Roux, B., and Crosson, S. 2012. Structural basis of a protein partner switch that regulates the general stress response of α -proteobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 109:E1415-E1423.
- Humann, J. L., Ziemkiewicz, H. T., Yurgel, S. N., and Kahn, M. L. 2009. Regulatory and DNA repair genes contribute to the desiccation resistance of *Sinorhizobium meliloti* Rm1021. *Appl. Environ. Microbiol.* 75:446-453.
- Jamet, A., Sigaud, S., Van de Sype, G., Puppo, A., and Hérouart, 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.
- Jans, A., Vercruyse, M., Gao, S., Engelen, K., Lambrichts, I., Fauvart, M., and Michiels, J. 2013. Canonical and non-canonical EcfG sigma factors control the general stress response in *Rhizobium etli*. *MicrobiologyOpen* 2:976-987.
- Kaczmarczyk, A., Campagne, S., Danza, F., Metzger, L. C., Vorholt, J. A., and Francez-Charlot, A. 2011. Role of *Sphingomonas* sp. strain Fr1 PhyR-NepR- σ^{EcfG} cascade in general stress response and identification of a negative regulator of PhyR. *J. Bacteriol.* 193:6629-6638.
- Kaczmarczyk, A., Hochstrasser, R., Vorholt, J. A., and Francez-Charlot, A. 2014. Complex two-component signaling regulates the general stress response in Alphaproteobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 111: E5196-E5204.
- Kim, H. S., Caswell, C. C., Foreman, R., Roop, R. M., 2nd, and Crosson, S. 2013. The *Brucella abortus* general stress response system regulates chronic mammalian infection and is controlled by phosphorylation and proteolysis. *J. Biol. Chem.* 288:13906-13916.
- Lamb, J. W., and Hennecke, H. 1986. In *Bradyrhizobium japonicum* the common nodulation genes, *nodABC*, are linked to *nifA* and *fixA*. *Mol. Gen. Genet.* 202:512-517.
- Ledermann, R., Bartsch, I., Remus-Emsermann, M. N., Vorholt, J. A., and Fischer, H. M. 2015. Stable fluorescent and enzymatic tagging of *Bradyrhizobium diazoefficiens* to analyze host-plant infection and colonization. *Mol. Plant-Microbe Interact* 28:959-967.
- Martínez-Salazar, J. M., Salazar, E., Encarnación, S., Ramírez-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.
- Masloboeva, N., Hennecke, H., and Fischer, H. M. 2015. Rhizobial extracytoplasmic function (ECF) σ factors and their role in oxidative stress response of *Bradyrhizobium japonicum*. Pages 307-314 in: *Biological Nitrogen Fixation*. F. de Bruijn, ed. John Wiley & Sons, Hoboken, NJ.
- Masloboeva, N., Reutimann, L., Stiefel, P., Follador, R., Leimer, N., Hennecke, H., Mesa, S., and Fischer, H. M. 2012. Reactive oxygen species-inducible ECF σ factors of *Bradyrhizobium japonicum*. *PLoS One* 7:e43421.
- Matilla, M. A., Espinosa-Urgel, M., Rodríguez-Herva, J. J., Ramos, J. L., and Ramos-González, M. I. 2007. Genomic analysis reveals the major driving forces of bacterial life in the rhizosphere. *Genome Biol.* 8:R179.
- Mesa, S., Hauser, F., Friberg, M., Malaguti, E., Fischer, H. M., and Hennecke, H. 2008. Comprehensive assessment of the regulons controlled by the FixLJ-FixK₂-FixK₁ cascade in *Bradyrhizobium japonicum*. *J. Bacteriol.* 190:6568-6579.
- Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Newcomb, W., Sippell, D., and Peterson, R. L. 1979. The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. *Can. J. Bot.* 57:2603-2616.
- Oldroyd, G. E. 2013. Speak, friend, and enter: Signalling systems that promote beneficial symbiotic associations in plants. *Nat. Rev. Microbiol.* 11:252-263.
- Oldroyd, G. E., Murray, J. D., Poole, P. S., and Downie, J. A. 2011. The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet.* 45:119-144.
- Regensburger, B., and Hennecke, H. 1983. RNA polymerase from *Rhizobium japonicum*. *Arch. Microbiol.* 135:103-109.
- Renier, A., Maillet, F., Fardoux, J., Poinot, V., Giraud, E., and Nouwen, N. 2011. Photosynthetic *Bradyrhizobium* sp. strain ORS285 synthesizes 2-O-methylfucosylated lipochitooligosaccharides for *nod* gene-dependent interaction with *Aeschynomene* plants. *Mol. Plant-Microbe Interact* 24: 1440-1447.
- Saeki, K. 2011. Rhizobial measures to evade host defense strategies and endogenous threats to persistent symbiotic nitrogen fixation: A focus on two legume-rhizobium model systems. *Cell. Mol. Life Sci.* 68: 1327-1339.
- Santos, R., Hérouart, D., Sigaud, S., Touati, D., and Puppo, A. 2001. Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. *Mol. Plant-Microbe Interact* 14:86-89.
- Sauviac, L., Philippe, H., Phok, K., and Bruand, C. 2007. An extracytoplasmic function sigma factor acts as a general stress response regulator in *Sinorhizobium meliloti*. *J. Bacteriol.* 189:4204-4216.
- Stokkermans, T. J., and Peters, N. K. 1994. *Bradyrhizobium elkanii* lipooligosaccharide signals induce complete nodule structures on *Glycine soja* Siebold et Zucc. *Planta* 193:413-420.
- Storz, G., and Hengge, R. 2011. *Bacterial Stress Responses*. ASM Press, Washington, DC.
- Tóth, K., and Stacey, G. 2015. Does plant immunity play a critical role during initiation of the legume-rhizobium symbiosis? *Front. Plant Sci.* 6:401.
- Turgeon, B. G., and Bauer, W. D. 1985. Ultrastructure of infection-thread development during the infection of soybean by *Rhizobium japonicum*. *Planta* 163:328-349.
- Udvardi, M., and Poole, P. S. 2013. Transport and metabolism in legume-rhizobia symbioses. *Annu. Rev. Plant Biol.* 64:781-805.
- Vesper, S. J., and Bauer, W. D. 1986. Role of pili (fimbriae) in attachment of *Bradyrhizobium japonicum* to soybean roots. *Appl. Environ. Microbiol.* 52:134-141.
- Vesper, S. J., Malik, N. S., and Bauer, W. D. 1987. Transposon mutants of *Bradyrhizobium japonicum* altered in attachment to host roots. *Appl. Environ. Microbiol.* 53:1959-1961.