A Role for *Bradyrhizobium japonicum* ECF16 Sigma Factor EcfS in the Formation of a Functional Symbiosis with Soybean

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Alternative sigma (σ) factors, proteins that recruit RNA polymerase core enzyme to target promoters, are one mechanism by which bacteria transcriptionally regulate groups of genes in response to environmental stimuli. A class of σ^{70} proteins, termed extracytoplasmic function (ECF) σ factors, are involved in cellular processes such as bacterial stress responses and virulence. Here, we describe an ECF16 σ factor, EcfS (Blr4928) from the gram-negative soil bacterium *Bradyrhizobium japonicum* USDA110, that plays a critical role in the establishment of a functional symbiosis with soybean. Nonpolar insertional mutants of *ecfS* form immature nodules that do not fix nitrogen, a defect that can be successfully complemented by expression of *ecfS*. Overexpression of the cocistronic gene, *tmrS* (*blr4929*), phenocopies the *ecfS* mutant in planta and, therefore, we propose that TmrS is a negative regulator of EcfS, a determination consistent with the prediction that it encodes an anti- σ factor. Microarray analysis of the *ecfS* mutant and *tmrS* overexpressor was used to identify 40 transcripts misregulated in both strains. These transcripts primarily encode proteins of unknown and transport-related functions and may provide insights into the symbiotic defect in these strains.

The gram-negative soil bacterium *Bradyrhizobium japonicum* occupies two distinct niches: free-living in the soil and within specialized structures on soybean roots called nodules that form as part of an endosymbiotic relationship. *B. japonicum* must be able to sense and transition between these two environments, each with its own challenges: differing in the availability of nutrients, pH, and oxygen concentration. One generalized way in which bacteria respond to changing environmental conditions is by altering their transcriptional profile through the use of alternative σ factors. These proteins fall into two structurally distinct families (σ^{54} and σ^{70}), each with the ability to interact with and recruit RNA polymerase core enzyme to consensus sequences within the promoters of target genes (Brooks and Buchanan 2008; Gruber and Gross 2003).

This study is focused on the characterization of 1 of 15 putative *B. japonicum* strain USDA110 group IV σ^{70} proteins, also

known as extracytoplasmic function (ECF) σ factors (Kaneko et al. 2002b; Staron et al. 2009). Bacterial ECF σ factors, comprising 43 distinct groups, represent the most diverse and abundant class of σ^{70} proteins (Staron et al. 2009). Although first appreciated for their role in cell envelope stress responses, the diversity of function and complexity of the protein networks controlled by these proteins has been expanding as the number of characterized ECF σ factors has grown. What unifies ECF σ factors is the presence of only two (regions 2 and 4) of the four conserved functional domains found in groups 1, 2, and 3 of the σ^{70} protein family (Gruber and Gross 2003; Kazmierczak et al. 2005). Genes encoding ECF σ factors are often clustered with those encoding cognate negative regulators, collectively termed anti- σ factors (Staron et al. 2009). The majority of ECF anti- σ factor proteins span the cytoplasmic membrane, allowing for outside-in signal transduction by way of periplasmic sensory and cytoplasmic response domains (Staron et al. 2009). However, a collection of ECF σ factors (subgroups ECF11–15) are regulated by anti- σ factors located in the cytoplasm (Bastiat et al. 2010; Gourion et al. 2009; Staron et al. 2009). ECF15 (also called EcfG-like) σ factors are additionally indirectly regulated by anti-anti- σ factor-like membrane-spanning response regulator proteins of the PhyR family (Bastiat et al. 2010; Gourion et al. 2009; Staron et al. 2009). The sole characterized *B. japonicum* ECF σ factor, EcfG, belongs to this subgroup (Gourion et al. 2009).

EcfG-like (group ECF15) σ factors have been shown to function in the general stress response of α -proteobacteria (Staron et al. 2009). EcfG-like RpoE2 plays this role in *Sinorhizobium meliloti*, in which it is required for a variety of stress responses (i.e., stationary phase survival in the presence of high levels of hydrogen peroxide, desiccation, and growth in hyperosmotic conditions), presumably due to the transcriptional control of a large regulon of stress-associated genes (Flecharde et al. 2009, 2010; Humann et al. 2009; Sauviac et al. 2007; Staron et al. 2009). Likewise, mutants of *B. japonicum* *ecfG* demonstrate an increased sensitivity to desiccation and thermal stress following carbon starvation (Gourion et al. 2009). Unlike *S. meliloti* *rpoE2*, which is dispensable for symbiosis, *B. japonicum* *ecfG* is involved in forming an efficient symbiosis with mungbean and soybean (Gourion et al. 2009). Plants inoculated with *ecfG* mutants are slow to develop nodules and those that do form are often aberrantly shaped and produce ectopically emerging roots (Gourion et al. 2009). Soybean plants nodulated by the *ecfG* mutant recover a functional symbiosis by 4 weeks postinoculation, suggesting a role for EcfG in early but not necessarily late stages of the symbiosis (Gourion et al. 2009).

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With a well-documented role for ECF σ factors in virulence and the recent link between ECF σ factor EcfG and the *B. japonicum*–soybean symbiosis, we hypothesized that there may be other ECF σ factors present in *B. japonicum* that are dedicated to adaptation to the symbiotic niche. Furthermore, we predicted that such a symbiosis-specific ECF σ factor would not be required for general stress responses but, rather, may respond to a signal unique to the in planta environment. Here, we describe the initial characterization of the extracytoplasmic function σ factor for symbiosis (*ecfS*) (*blr4928*) and its cocistronic gene transmembrane regulator of symbiosis (*tmrS*)

Fig. 1. *ecfS* and *tmrS* cocistronic genomic organization, mutant and plasmid construction, and end-point reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. **A**, (a) Extracytoplasmic function (ECF) σ factor deletion/insertion (i.e., Δ *ecfS*:*aacC1*) mutant construction compared with the (b) wild-type chromosomal allele as well as the (c) complementation plasmid. **A** and **B**, Scale bar represents approximately 500 bp of DNA. The region containing the presumptive *ecfS* promoter (based on predicted -10/-35 sites at approximately 370 and 391 bp upstream of the *ecfS* translational start site; BPROM online promoter prediction tool) is indicated by small black arrowheads labeled with “p”. The *ecfS* promoter was predicted to be located within 500 bp upstream of the *ecfS* start codon. The black box at the end of *aacC1* in construct (a) represents the 40 bp from the end of *ecfS* that remains in this construct to allow for a ribosome binding site for *tmrS*. **B**, End-point RT-PCR was used to amplify a 500-bp region spanning the *ecfS*–*tmrS* open reading frames from wild-type USDA110-derived cDNA. Products are shown (white arrowhead). Lanes from left to right: +RT = PCR product resulting from wild-type cDNA made with RT = –RT and Neg., minus RT and no template control reactions yielding no products; Chr., positive chromosomal DNA control reaction; MW, molecular weight ladder with relevant band size indicated.

(*blr4929*) by way of mutational and transcriptomic analysis. In other bacteria, ECF σ factors have been shown to control nearby genes (e.g., FecI regulation of downstream *fecABCDE* in *Escherichia coli*) (Braun et al. 2006); thus, *blr4928* and *blr4929* were of particular interest due to their close genomic proximity (i.e., within eight open reading frames [ORF]; approximately 7.5 kbp) to the USDA110 ferrichrome receptor gene (i.e., *blr4920*) (Small et al. 2009) whose homolog in strain 61A152 (i.e. *fegA*) has been implicated in the symbiosis (Benson et al. 2005). Results show that other than a slight growth defect characterized by an extended lag phase, cultured cells of *ecfS* mutants have no discernible in vitro phenotypes under the conditions tested. However, either loss of *ecfS* or overexpression of *tmrS* blocks early stages of the symbiosis, suggesting that the proper balance of EcfS and TmrS is critical for in planta bacterial adaptation.

RESULTS

ecfS and *tmrS* are contained within one transcriptional unit.

The *ecfS* and *tmrS* coding regions are on the same strand and overlap by 4 bp within the *B. japonicum* USDA110 genome (Kaneko et al. 2002b); thus, we hypothesized that these genes make up one transcriptional unit. Using end-point reverse-transcriptase polymerase chain reaction (RT-PCR), we amplified a 500-bp DNA fragment spanning the *ecfS*–*tmrS* ORF junction (Fig. 1B. +RT lane) from wild-type USDA110-derived cDNA. No product was observed in either the –RT or no-template control reactions, although a robust 500-bp band was observed in the positive control reaction containing purified USDA110 chromosomal DNA (Fig. 1B; –RT, Neg., and Chr. lanes).

Δ *ecfS* mutants have a severe symbiotic defect.

ECF σ factors are known to be required for the virulence of a variety of bacterial pathogens (Kazmierczak et al. 2005; Llamas et al. 2009; Rowley et al. 2006). Because *B. japonicum* is able to persist in association with its plant host, in a manner similar to that of intracellular bacterial pathogens, we tested an *ecfS* deletion mutant for its ability to form a functional symbiosis with soybean. A dramatic phenotype is observed when

Fig. 2. Nonfunctional symbiosis, characterized by the formation of small nodules resulting in chlorotic plants, occurs upon deletion of *ecfS* or overexpression of *tmrS*. **A** to **F**, Leaf and **G** to **L**, root nodule photographs of 5-week-old soybean plants inoculated with **A** and **G**, USDA110; **B** and **H**, *tmrS* overexpressor (OE); **C** and **I**, Δ *ecfS*; **D** and **J**, Δ *ecfS*/pLAFR3; **E** and **K**, Δ *ecfS*/*pectS*; or **F** and **L**, no bacteria are shown. The entire root crown of a representative mock inoculated plant is shown. The *ecfS* mutant defect can be complemented by expression of *ecfS* in trans.

soybean plants are inoculated with $\Delta ecfS$. These plants form many (34 ± 5 $\Delta ecfS$ versus 5 ± 1 wild-type nodules per gram fresh shoot weight), small nodules (1.0 ± 0.2 mg of $\Delta ecfS$ versus 19.2 ± 1.0 mg of wild-type nodules) (Figs. 2 and 3). $\Delta ecfS$ mutant nodules do not contain leghemoglobin, consistent with an inability to fix nitrogen. Plants inoculated with $\Delta ecfS$ are extremely chlorotic, presumably because these plants are starved for nitrogen (Figs. 2C and I and 3B). Nitrogenase activity of the inoculated roots was not measured due to the obvious nature of the nitrogen starvation. $\Delta ecfS$ symbiotic phenotypes are fully complemented by the introduction of a wild-type copy of *ecfS* in trans, expressed from 500 bp of upstream sequence (Figs. 2E and K and 3). The mutant carrying the empty vector is indistinguishable from the $\Delta ecfS$ strain in plant assays (Figs. 2D and J and 3). When plants were grown in nutrient-rich soil with sufficient levels of nitrogen, the presence or absence of bacteria (i.e., wild-type USDA110 or $\Delta ecfS$) had no impact on soybean germination or growth.

To further characterize the $\Delta ecfS$ symbiosis defect, nodules formed on plants inoculated with $\Delta ecfS$, $\Delta ecfS/pLAFR3$, or $\Delta ecfS/pecfS$ were harvested at 5 weeks postinoculation and viewed by transmission electron microscopy (TEM). Although nodules infected with the complemented strain contain many symbiosome-filled cells (indistinguishable from nodules infected with wild-type USDA110), there were no bacteria observed in nodules from plants inoculated with either the $\Delta ecfS$ or the $\Delta ecfS/pLAFR3$ strain (Fig. 4). Plant cells from nodules induced by $\Delta ecfS$ or $\Delta ecfS/pLAFR3$ appeared empty, with little or no cytoplasmic material, unlike what is typically observed for uninfected cells (Fig. 4D and E, cells labeled "UN"). Additionally, nodule cell shape (Fig. 4A to C) and cell wall thickness is irregular (Fig. 4A). Unlike cells inoculated with bacteria capable of eliciting a normal symbiosis, recognizable mitochondria and nuclei are rarely observed in *ecfS* mutant-induced nodules (Fig. 4A to C). Only one discernable

nucleus (Fig. 4C) was observed in all of the mutant nodule sections. Additionally, because no bacteria or infection threads were observed in these nodules, we believe that the mutant bacteria are defective in nodule invasion. This is supported by our inability to recover mutant cells from inoculated plants.

Overexpression of *tmrS* results in a severe symbiotic defect, indistinguishable from that of $\Delta ecfS$.

tmrS (*blr4929*) is predicted to encode a typical ECF16-associated protein with a conserved domain (DUF1109; six transmembrane spanning regions placing both the N and C termini in the cytoplasm) of unknown function (Staron et al. 2009). To assess TmrS's possible role as an anti- σ factor protein and, thus, a negative regulator of the symbiosis, an overexpressor (OE) strain was created in lieu of a deletion. In doing so, an additional copy of the *tmrS* ORF was introduced into the chromosome of wild-type strain USDA110, resulting in a merodiploid strain. The *tmrS* gene was cloned downstream of and, therefore, expressed from the predicted endogenous *ecfS* promoter. As expected, the introduction of an additional copy of *tmrS* in the merodiploid strain resulted in a 3.8-fold overexpression of *tmrS* over wild-type USDA110 levels (as shown by microarray analysis; data not shown) and, thus, the strain was termed the *tmrS* OE.

Indistinguishable from $\Delta ecfS$, the *tmrS* OE has a severe symbiotic defect compared with USDA110 (Figs. 2B and H and 3). Plants inoculated with the *tmrS* OE produce large numbers (27 ± 3 nodules/g of fresh shoot) of tiny nodules (0.6 ± 0.1 mg/nodule) at levels similar to those formed on plants infected with $\Delta ecfS$ (34 ± 5 nodules/g of fresh shoot; 1.0 ± 0.2 mg/nodule) (Figs. 2 and 3).

Stress sensitivity of $\Delta ecfS$ is similar to the wild type.

Due to the well-established role of some ECF σ factors in environmental stress response and survival, $\Delta ecfS$ was tested

Fig. 3. Quantification of the symbiosis formed by various strains infecting soybean plants. Seeds were inoculated and plants were grown and harvested at 5 weeks postinoculation with planting. **A**, Shoots were separated from roots and weighed. **B**, Chlorophyll was extracted from plant leaves and quantified. **C**, Nodules were removed from roots, counted, and normalized per gram fresh shoot weight. **D**, Picked nodules were weighed. Standard error of the mean is shown ($n = 7$ to 39). Asterisks indicate Student's *t* test *P* values of <0.04 (A) or <0.001 (B to D) compared with wild-type levels.

for increased sensitivity to oxidative stress, detergents, and extreme pH, as assessed by measuring growth inhibition zones on plates containing point-deposited hydrogen peroxide, sodium dodecyl sulfate (SDS), NaOH, or HCl. The growth inhibition zone diameters of the wild type and the *ecfS* mutant strain are similar upon the addition of hydrogen peroxide (wild type, 34.1 ± 0.6 mm; $\Delta ecfS$, 36.2 ± 0.9 mm), SDS (approximately 22 mm in diameter), NaOH (approximately 19 mm), or HCl (approximately 10.5 mm) (Fig. 5). Similar results were observed when experiments were performed on cells grown to late log phase and resuspended and incubated for 20 h in minimal media $\pm 0.2\%$ xylose (data not shown).

Transcriptome analysis reveals similarly misregulated genes in the *ecfS* mutant and *tmrS* OE.

Microarray analysis was used to identify $\Delta ecfS$ /pLAFR3 and *tmrS* OE misregulated transcripts in hopes of elucidating a cause for the common symbiotic defect of these strains. The complemented mutant ($\Delta ecfS$ /*pecfS*) and wild-type USDA110 strains were used as functional symbiont controls. The transcriptome experiment was performed on cultured cells to circumvent technical difficulties associated with the in planta environment. That is, the inability to recover *ecfS* mutant cells from inoculated plants makes it impossible to isolate enough in-planta-derived bacterial mRNA for microarray analysis. Genes with at least a twofold change in expression above or below wild-type levels when grown in peptone salts–yeast extract (PSY) broth under standard laboratory conditions were included in the analysis. Using this criterion, 791, 487, and 743 misregulated transcripts were identified in $\Delta ecfS$ /pLAFR3, *tmrS* OE, and $\Delta ecfS$ /*pecfS* cultures, respectively, compared with the wild type (Fig. 6A). It was found that there was a 2.6-fold increase in *tmrS* expression over wild-type levels in the *ecfS* mutant, confirming that the in-frame *aacC1* ORF insertion in this strain is not polar on downstream gene *tmrS* (data not shown). A 3.8-fold overexpression of *tmrS* was confirmed in the merodiploid *tmrS* OE compared with the wild type (data not shown). *ecfS* expression was below threshold detection levels in all strains (data not shown), most likely due to inherently low levels of expression from the endogenous promoter. Phenotypic changes in the complemented mutant suggest that *ecfS*

levels in this strain, although undetectable, are sufficient for the symbiosis.

Because transcripts similarly misregulated in $\Delta ecfS$ /*pecfS* and the *ecfS* mutant or *tmrS* OE strain are most likely not responsible for the symbiosis phenotypic differences observed, these genes were removed from further analyses. Thus, 419 (i.e., 222 and 197) and 316 (i.e., 222 and 94) were extracted from the *ecfS* mutant and *tmrS* OE lists, respectively (Fig. 6A). Of the remaining transcripts, there were found to be 332 (179 below and 153 above wild-type levels; Supplementary Table S2) and 131 (57 below and 74 above wild-type levels; Supplementary Table S3) transcripts uniquely misregulated in the

Fig. 5. The *ecfS* mutant is not hypersensitive to oxidative (hydrogen peroxide)-, detergent (sodium dodecyl sulfate [SDS])- or extreme pH (NaOH or HCl)-induced cell stress compared with the wild type. Plate sensitivity assays were performed on USDA110 (black bars) and $\Delta ecfS$ (white bars). Bacterial lawns were plated and various solutions were point deposited into the center of each plate. The diameter of growth inhibition zones around each compound was quantified. Standard error of the mean is shown (*n* values of 14 to 21).

Fig. 4. Representative transmission electron micrographs of nodules formed on plants inoculated with the *ecfS* mutant and complemented strains. Nodules were harvested from 5-week-old soybean plants inoculated with **A** and **B**, $\Delta ecfS$ or **C**, $\Delta ecfS$ /pLAFR3 or **D**, **E**, and **F**, $\Delta ecfS$ /*pecfS*. Scale bars represent 2 μ m. $\Delta ecfS$ mutant-infected nodule cells are irregularly shaped and have altered cell wall thickness and few identifiable organelles. No infection threads or bacterial cells were observed in $\Delta ecfS$ - or $\Delta ecfS$ /pLAFR3-infected nodules. $\Delta ecfS$ /*pecfS*-infected nodules contain many symbiosomes, visible organelles, and few uninfected cells (UN). These nodules are indistinguishable from those infected with wild-type *Bradyrhizobium japonicum* (wild-type images not shown) (Benson et al. 2005; Libault et al. 2011).

ecfS mutant or *tmrS* OE strain, respectively (Fig. 6A). In all, 40 transcripts (13 below and 27 above wild-type levels) were misregulated in both the *ecfS* mutant and *tmrS* OE strain and, thus, represent a subset of particularly promising genes for determining the cause of the symbiotic defect of these strains (Fig. 6). Of note, only one of these transcripts (*bll3387*, hypothetical protein) was differentially regulated in the *ecfS* mutant (i.e., down, 0.312) and *tmrS* OE (i.e., up, 2.388) strains. A complete list of the 40 $\Delta ecfS$ /pLAFR3 and *tmrS* OE overlapping misregulated transcripts is provided in Table 1.

The general functional groups represented in the *ecfS* mutant, *tmrS* OE, and overlapping gene lists were derived from the genome annotation put forth by Kaneko and associates (2002a and b) and are summarized in Figure 6B. In all lists, there was a bias toward transcripts predicted to encode proteins of hypothetical or unknown functions (38.5%, *ecfS* mutant; 57.3%, *tmrS* OE; 40%, overlap; 30.1%, full genome) (Kaneko et al. 2002a). In the *ecfS* mutant and $\Delta ecfS$ -*tmrS* OE overlap lists, there was also a bias toward transcripts predicted to encode proteins with transport-related functions (19.9%, *ecfS* mutant; 20%, overlap; 9%, full genome) (Kaneko et al. 2002a).

DISCUSSION

B. japonicum USDA110 is predicted to contain 15 ECF σ factors with representatives of nine subgroups (i.e., ECF15, 16, 18, 20, 26, 29, 33, 41, 42) (Staron et al. 2009) (Table 2). Although there is a generalized understanding of subgroups ECF01 to -10 due to previously characterized founding members, little is known about subgroups 11 to 43 (Staron et al. 2009). Thus, the characterization of the *B. japonicum* ECF σ factors, all of which are members of relatively novel subgroups, is sure to lead to a more thorough understanding of the role and mechanism of ECF σ factors in bacterial adaptation and survival.

Because the transition between the free-living state and symbiosis with host legumes involves a series of adaptive steps, we hypothesized that one or more *B. japonicum* ECF σ factor is involved in altering gene expression for this adaptation. Here, as an extension of this hypothesis, we describe the identification and initial characterization of ECF16 subgroup σ factor, EcfS (Blr4928). We have confirmed that *ecfS* is in an operon with an overlapping downstream gene, *tmrS* (Fig. 1), and have demonstrated a specific and dramatic in planta phenotype of the nonpolar (confirmed by microarray analysis) *ecfS* mutant we constructed. No $\Delta ecfS$ stress-related in vitro phenotypes have been identified under the culture conditions tested; thus, we conclude that EcfS is not likely to be required for general stress responses. This is in agreement with the classification of EcfS as an Ecf16 (i.e., not an Ecf15- or EcfG-like, general stress-response-associated) σ factor protein (Staron et al. 2009). Rather, due to the severe symbiotic defect of the *ecfS* mutant, we believe that EcfS functions primarily in the bacterial adaptation to the in planta environment.

Because *tmrS* overexpression (3.79-fold over wild-type levels) phenocopies the *ecfS* mutant in planta, we propose that TmrS functions as a negative regulator in the symbiosis. Thus, EcfS and TmrS perform opposing functions in planta, and perhaps it is the appropriate functional balance between these proteins that allows the bacteria to establish themselves in the symbiosis. This is supported by the results of our transcriptomic analysis. Specifically, loss of EcfS in the *ecfS* mutant leads to a 2.6-fold increase in *tmrS* expression compared with the wild type (data not shown). This may be due to compensatory enhanced transcriptional activity at the shared *ecfStmrS* promoter in response to the loss of EcfS function in the *ecfS* mutant. Although it has been proposed that *tmrS* encodes a

typical ECF16-associated anti- σ factor containing a DUF1109 domain and six transmembrane regions (Staron et al. 2009), future studies are needed to examine possible TmrS-EcfS protein interactions typical of σ factor-anti- σ factor partners.

Based on the immature nodules and lack of observable infection threads that formed on plants inoculated with $\Delta ecfS$, we conclude that active EcfS is required for the early stages of nodule formation and plant invasion. However, the initial stages of infection (i.e., root hair deformation in response to Nod factor production) are unaffected by this mutation, as assessed by the formation of immature nodules. None of the genes misregulated in the *ecfS* mutant encode proteins necessary for Nod factor production or have been shown to be induced by the soybean isoflavonoid, genistein (Lang et al. 2008). In support of a role for EcfS and TmrS in the early infection process, only 2 of the 40 misregulated genes identified in both the *ecfS* mutant and *tmrS* OE via microarray have altered expression in differentiated bacteroids compared with free-living cells (Table 1) (Chang et al. 2007; Pessi et al. 2007). Future characterization of the EcfS or TmrS regulon may involve the quantification of nod or genome-wide gene expression in planta.

Interestingly, over time, the nodules formed by plants inoculated with $\Delta ecfS$ ($\pm$ pLAFR3) and *tmrS* OE undergo one of two fates: nodule senescence or the transition back into roots via the formation of a growing root tip (data not shown). Based on

Fig. 6. Summary of transcripts found to be misregulated a minimum of two-fold change above or below wild-type levels in $\Delta ecfS$ /pLAFR3, *tmrS* overexpressor (OE), and $\Delta ecfS$ /pLAFR3. **A**, Proportional Venn diagram of misregulated transcripts in each strain compared with wild-type USDA110. The number of total transcripts represented by each fraction of the diagram is indicated. The breakdown of up- and downregulated genes in each category is shown, where relevant. **B**, Summary of functional groups represented in the 332 $\Delta ecfS$ /pLAFR3 only, 131 *tmrS* OE only, and 40 $\Delta ecfS$ -*tmrS* OE overlapping misregulated transcripts. Functional groups have been derived from the genome annotation put forth by Kaneko and associates (2002).

this observation, we propose that the nodules formed by these plants have not fully differentiated into nodule organs and, upon the apparent halt in infection, they are able to transition back into roots. The protrusion of root tips from immature nodules was similarly reported of nodules induced by *ecfG* mutants (Gourion et al. 2009). However, the *ecfS* mutant phenotype is more severe than that of *ecfG* because, unlike for *ecfG*-inoculated soybean, plants infected with $\Delta ecfS$ do not establish a symbiosis over time (Gourion et al. 2009). We propose that EcfS and EcfG perform different roles in the cell. In support of this, none of the misregulated transcripts identified in the *ecfS* mutant or *tmrS* OE strain have been shown to be

EcfG regulated and no EcfG-binding motifs (GGAAC-N15-GCGT) were identified in the upstream regions of these genes (Gourion et al. 2009).

Based on the precedence of in vitro stress hypersensitivity of ECF σ mutants, we hypothesized that the symbiotic defect of the *ecfS* mutant may be due to a general lack of fitness of this strain. In vitro analysis of the *ecfS* mutant reveals a modest hypersensitivity to oxidative stress, yet sensitivity to extreme pH, detergents, or hypersalinity; extended stationary phase survival; and exopolysaccharide production are unaltered in this strain (Fig. 5) (data not shown). Transcriptome analysis yielded few misregulated chaperone-encoding genes in the *ecfS* mutant

Table 1. List of the 40 *Bradyrhizobium japonicum* transcripts misregulated $\geq$ twofold above or below wild type USDA110 levels in both $\Delta ecfS$ /pLAFR3 and *tmrS* overexpressor (OE) when grown under standard laboratory conditions

Genome ^b	ORF description ^c	<i>n</i> Fold-change (<i>P</i> value) ^a		Functional category ^d
		$\Delta ecfS$ /pLAFR3	<i>tmrS</i> OE	
<i>bl13387</i>	Unknown protein	0.312 (0.00118)	2.388 (0.00385)	Hypothetical/unknown
<i>bs14527</i>	Unknown protein	0.462 (0.00403)	0.456 (0.0055)	Hypothetical/unknown
<i>bs15453</i>	Hypothetical protein	0.303 (6.01e-5)	0.484 (0.00373)	Hypothetical/unknown
<i>bl17424</i>	Hypothetical protein	0.41 (0.000295)	0.46 (0.000645)	Hypothetical/unknown
<i>blr6798</i>	Transcriptional regulatory protein IclR family	0.491 (0.00279)	0.458 (0.000518)	Regulation
<i>blr1064</i>	ABC transporter permease protein	0.304 (0.00307)	0.268 (0.00215)	Transport
<i>blr1065</i>	ABC transporter permease protein	0.272 (0.0072)	0.258 (0.00365)	Transport
<i>blr1066</i>	ABC transporter ATP-binding protein	0.436 (0.00117)	0.408 (0.00194)	Transport
<i>blr2437</i>	ABC transporter ATP-binding protein	0.215 (0.001)	0.473 (0.00313)	Transport
<i>bl13149</i>	Putative oxalate:formate antiporter	0.33 (0.0097)	0.317 (0.00893)	Transport
<i>blr1818*</i>	RhcQ; component of type III secretion system	0.431 (0.00128)	0.441 (0.000954)	Other; protein and peptide secretion/trafficking
<i>blr1819*</i>	RhcR; component of type III secretion system	0.415 (0.000364)	0.384 (7.82e-5)	Other; protein and peptide secretion/trafficking
<i>bl16039</i>	Probable SgaA serine-glyoxylate aminotransferase	0.438 (0.00326)	0.464 (0.00534)	Other; amino acid biosynthesis
<i>bs10728</i>	Hypothetical protein	2.77 (0.000456)	2.065 (0.000461)	Hypothetical/unknown
<i>bl10800</i>	Hypothetical protein	3.14 (0.00039)	2.337 (0.000875)	Hypothetical/unknown
<i>bl10888</i>	Hypothetical protein	6.18 (7.38e-5)	3.496 (7.54e-5)	Hypothetical/Unknown
<i>bsr1784*</i>	Unknown protein	2.35 (0.00335)	2.127 (0.00493)	Hypothetical/unknown
<i>bl12158</i>	Unknown protein	3.62 (0.00235)	2.458 (0.00456)	Hypothetical/unknown
<i>blr2564</i>	Hypothetical protein	3.98 (0.00028)	2.109 (0.00115)	Hypothetical/unknown
<i>blr4131</i>	Unknown protein	2.20 (4.43e-5)	2.045 (7.4e-5)	Hypothetical/unknown
<i>bl15155</i>	Hypothetical protein	3.11 (4.31e-5)	2.267 (0.000225)	Hypothetical/unknown
<i>blr5730</i>	Hypothetical protein	2.02 (0.00182)	2.036 (0.00216)	Hypothetical/unknown
<i>blr5841</i>	Hypothetical protein	2.09 (0.00323)	2.315 (0.00124)	Hypothetical/unknown
<i>blr7300</i>	Unknown protein	3.94 (0.0079)	4.257 (0.00656)	Hypothetical/unknown
<i>bl17395</i>	Hypothetical protein	2.54 (4.05e-5)	2.254 (6.12e-5)	Hypothetical/unknown
<i>bl10620</i>	Two-component response regulator	3.12 (0.000138)	2.135 (2.21e-5)	Regulation
<i>blr0462</i>	Probable ABC transporter permease protein	2.31 (0.00314)	2.154 (0.00231)	Transport
<i>blr1516^{Fe}</i>	AcrB, RND multidrug efflux transporter	3.02 (0.000119)	3.037 (0.000234)	Transport
<i>blr2314</i>	MFS permease	2.10 (0.00157)	3.213 (0.000191)	Transport
<i>blr0149</i>	Cytochrome O ubiquinol oxidase subunit II	2.02 (0.00176)	2.357 (0.00127)	Other; energy metabolism
<i>blr0151</i>	Cytochrome O ubiquinol oxidase subunit III	2.99 (0.00277)	2.826 (0.00311)	Other; energy metabolism
<i>blr0152</i>	Cytochrome O ubiquinol oxidase subunit IV	2.68 (0.00015)	2.49 (0.0011)	Other; energy metabolism
<i>bl10246</i>	Indoleacetamide hydrolase (EC 3.5.1.-)	2.02 (0.00305)	3.214 (0.000437)	Other; biosynthesis of cofactors, prosthetic groups and carriers
<i>blr1201^{Fe}</i>	3-Dehydroquinate dehydratase	2.40 (0.00268)	2.35 (0.00169)	Other; amino acid biosynthesis
<i>blr1253</i>	Dehydrogenase	3.27 (4.62e-5)	2.044 (9.57e-5)	Other; central intermediary metabolism
<i>bl11418</i>	Methionine synthase	2.01 (0.00243)	3.089 (0.000128)	Other; amino acid biosynthesis
<i>bl11419</i>	5,10-Methylenetetrahydrofolate reductase	2.58 (0.00249)	3.578 (0.000371)	Other; amino acid biosynthesis
<i>blr2511</i>	Molybdopterin biosynthesis protein B	2.53 (0.000445)	2.156 (0.000778)	Other; biosynthesis of cofactors, prosthetic groups and carriers
<i>bl15700^{Fe} #</i>	Hypothetical glutathione S-transferase-like protein	2.83 (3.0e-5)	2.653 (0.000271)	Other; detoxification
<i>bl17966^{Fe}</i>	Putative thiosulfate sulfurtransferase	2.42 (0.000506)	2.27 (0.000483)	Other

^a Expression values are reported as *n*-fold change above or below wild-type USDA110 levels. Genes with a $\geq$ twofold change in expression compared with the wild type are listed in numerical order within general functional categories. Corresponding *P* values are shown in parentheses.

^b Genome open reading frame (ORF) number. Genes misregulated in the *ecfS* mutant were cross referenced with those reported to be regulated by EcfG, RegR, NifA, RpoN_{1/2}, Irr, genistein, symbiosis (i.e., in bacteroids), desiccation, oxygen, or iron availability. There was no overlap in the EcfG and EcfS regulons (Gourion et al. 2009). The 11 RegR-regulated transcripts are underlined (Hauser et al. 2006; Lindemann et al. 2007). The one Irr-regulated transcript is indicated by the presence of a pound sign superscript (#) (Yang et al. 2006). The four transcripts previously found to be regulated in bacteroids or microaerophilic conditions are indicated by the presence of an asterisk (*) or caret (^) sign, respectively (Chang et al. 2007; Hauser et al. 2006; Pessi et al. 2007). The four transcripts regulated in *B. japonicum* strain LO under iron limitation are indicated by the presence of an Fe superscript (Yang et al. 2006). None of the transcripts were shown to have altered expression in response to desiccation (Cytryn et al. 2007), genistein (Lang et al. 2008), or the loss of Nif A or RpoN_{1/2} (Hauser et al. 2006, 2007). The fold-change values of the most up- and downregulated genes compared with the wild type are indicated in bold.

^c ORF nomenclature and descriptions are based on those established by Kaneko and associates (2002).

^d General functional categories are those put forth by Kaneko and associates (2002).

and *tmrS* OE strain, none of which were common to both strains (Table 1). These results suggest that the loss of a functional symbiosis in the *ecfS* mutant and *tmrS* OE is not due to compromised general stress responses or cell envelope integrity.

We were unable to ascertain any substantial conserved nucleotide motifs, including the ECF16-like promoter recognition site (TGTAAC(C/A)N16CGAA) (Staron et al. 2009), within the putative promoters of *ecfS*/pLAFR3 or *tmrS* OE misregulated genes; therefore, we propose that they do not compose one uniform regulon but, rather, a collection of direct and indirect targets. Thus, the EcfS regulon could not be determined through these studies. However, extrapolation of our transcriptome data implicates a general defect in the regulation of transport-related functions in the mechanism of the *ecfS* mutant and *tmrS* OE symbiotic defects. Future characterization of the misregulated genes, particularly those encoding hypothetical or unknown functions, will undoubtedly lead to a more thorough mechanistic understanding.

As a culmination of the data presented here, we propose a model in which EcfS functions in an early stage of nodulation.

Based on its predicted sequence and structural similarity to ECF σ factors, we propose a model in which EcfS promotes the symbiosis by binding to and recruiting RNAP core enzyme to the promoters of target genes as an indirect response to an environmental or plant-specific cue encountered during the early infection process. TmrS negatively affects symbiosis, a role consistent with that of an anti- σ factor. Loss of EcfS or hyperactivity of TmrS leads to the same end: the inability of the bacteria to establish themselves in the symbiotic niche. Thus, we propose that the balance of EcfS or TmrS levels within a hypothesized regulatory network is critical for a proper bacterial response. Future work will focus on the elucidation of potential protein–protein interactions of EcfS with TmrS, and RNA Pol core enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes, and chemicals.

All strains and plasmids used in this study are listed in Table 3. *Escherichia coli* strains were routinely grown in Luria-Ber-

Table 2. Predicted *Bradyrhizobium japonicum* USDA110 extracytoplasmic function (ECF) σ and anti- σ factor genes

Genome number ^a	Gene name	ECF subgroup ^a	Associated anti- σ factor gene	Known or putative cocistronic genes ^b	References or other notable features
<i>blr7797</i>	<i>ecfG</i>	15	<i>nepR</i> (<i>blr7796</i>)	<i>nepRecfG</i>	Gourion et al. 2009
<i>blr3038</i>	...	16	<i>blr3039</i>	<i>blr3038-3039</i>	...
<i>blr4928</i>	<i>ecfS</i>	16	<i>tmrS</i> (<i>blr4929</i>)	<i>ecfStmrS</i>	Downstream of the USDA110 ferrichrome receptor (<i>blr4920</i>) (Small et al. 2009)
<i>blr2203</i>	...	18	...	<i>blt2201-2203</i>	Clustered with flagellar synthesis genes (<i>fliI</i> , - <i>J</i>)
<i>blr3694</i>	...	20	...	<i>blr3694-3701</i>	Clustered with flagellar synthesis genes (<i>flaA</i> , <i>flgE</i> , <i>flaN</i>)
<i>blt6484</i>	<i>ecfA</i>	26	<i>blt6483</i>	<i>blt6488-6483</i>	...
<i>blt2628</i>	<i>prtI</i>	26	<i>blt2627</i>	<i>blt2628-2627</i>	...
<i>blt3014</i>	...	26	<i>blt3013</i>	<i>blt3016-3011</i>	...
<i>blr2557</i>	...	29	...	<i>blr2555-2559</i>	...
<i>blr3042</i>	...	33	...	<i>blr3042-3043</i>	...
<i>blt1028</i>	...	33	...	<i>blt1032-1023</i>	...
<i>blr7812</i>	...	41	...	<i>blr7812-7816</i>	...
<i>blt6612</i>	...	42	...	...	...
<i>blt0085</i>	...	42	...	<i>blt0091-0085</i>	...
<i>blt7170</i>	...	42	...	<i>blt7170-7168</i>	...

^a ECF subgroups determined by Staron and associates (2009).

^b Based on Kaneko and associates (2002b).

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>Bradyrhizobium japonicum</i>		
USDA110	Nitrogen-fixing <i>Glycine max</i> symbiont	Guerinot and Chelm 1986
$\Delta ecfS::aacC1$	USDA110 <i>ecfS</i> ORF deletion/replacement strain (Gm ^r)	This work
<i>tmrS</i> overexpressor (OE)	USDA110 <i>tmrS</i> OE strain (Gm ^r , Tet ^r), chromosomal knock-in of pBR322($\Delta ecfS::aacC1$)	This work
61A152	...	EMB Crop Bioscience, Brookfield, WI, U.S.A.
<i>Escherichia coli</i>		
DH5 α	<i>hsdR17 endA1 thi-1 gyrA96 relA1, recA1 supE44 $\Delta lacU169$ ($\phi 80dlacZ\Delta M15$)</i>	Gibco-BRL, Gaithersburg, MD, U.S.A.
Plasmids		
pBR322	Tet ^r , Amp ^r <i>Escherichia coli</i> cloning vector, <i>B. japonicum</i> suicide vector	Bolivar et al. 1977; Watson 1988
pRK2013	Kan helper plasmid for mobilization	Figurski and Helinski 1979
pGEM-T Easy	Amp ^r cloning vector	Promega, Madison, WI, U.S.A.
pLAFR3	Tet ^r broad host range plasmid for <i>B. japonicum</i> expression	Staskawicz et al. 1987
pBBR1-MCS5	Gm ^r broad host range plasmid	Kovach et al. 1995
pAC Ω GM	Plasmid carrying the Gm ^r cassette (<i>accCI</i>)	Schweizer 1993
pGEM-TE (<i>ecfS</i>)	pGEM-T Easy carrying USDA110 <i>ecfS</i> with 500-bp upstream sequence	This work
pGEM-TE ($\Delta ecfS::aacC1-tmrS$)	pGEM-T Easy carrying 750 bp upstream of <i>ecfS</i> fused to <i>aacC1</i> ORF and <i>tmrS</i>	This work
pLAFR3 (<i>ecfS</i>)	Insert of pGEM-TE (<i>ecfS</i>) subcloned into pLAFR3 at the <i>EcoRI</i> site of the MCS	This work

^a ORF = open reading frame; OE = overexpressor; and Gm^r, Tet^r, and Amp^r indicate resistance to gentamycin, tetracycline, and ampicillin, respectively.

tani media at 37°C with shaking. *B. japonicum* USDA110 cultures were grown in arabinose-gluconate (AG), yeast extract media supplemented with xylose, or PSY media with shaking, or on plates at 30°C. When necessary, antibiotics were supplemented as follows: for *E. coli*, ampicillin (100 µg/ml), tetracycline (20 µg/ml), and gentamycin (15 µg/ml); and for *B. japonicum*, tetracycline (200 µg/ml), gentamycin (200–250 µg/ml), and rifampicin (40 µg/ml). T4 DNA ligase and all restriction enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). The pGEM-T Easy vector system was purchased from Promega Corporation (Madison, WI, U.S.A.). Polymerases were purchased from Stratagene (La Jolla, CA, U.S.A.) (PFU-Ultra High Fidelity Polymerase), Takara (Otsu, Japan) (ExTaq), and Roche (Nutley, NJ, U.S.A.) (Amplitaq) while primers were synthesized and purchased from Integrated DNA Technologies (Coralville, IA, U.S.A.).

Molecular techniques and mutagenesis.

Mutant and plasmid constructs are listed in Table 3 and illustrated in Figure 1. Full primer sequences are listed in Supplementary Table S1.

End-point RT-PCR of *ecfStmrS*.

Wild-type USDA110 cells were grown in AG broth to an optical density at 600 nm of 0.6. Cells were harvested by centrifugation and total RNA was extracted using a Quick-RNA MiniPrep kit (following the manufacturer's instructions; Zymo Research, Irvine, CA, U.S.A.) with the addition of a 30-min on-column DNase treatment (RNase-free DNase set; Qiagen, Valencia, CA, U.S.A.). Approximately 1 µg of total RNA was converted to cDNA using a high-capacity cDNA RT kit equipped with MultiScribe RT, 10× RT buffer, 4 mM dNTPs, and random primers (Applied Biosystems, Carlsbad, CA, U.S.A.). A negative control reaction, in which RT was omitted, was run in parallel. The RT reaction conditions were as follows: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and 10°C for 30 min. A fraction of each cDNA reaction (i.e., ±RT) served as a template in end-point PCR with AmpliTaq DNA Polymerase (Applied Biosystems) and primers (P1 and P2) designed to flank the *ecfS*–*tmrS* ORF junction. Additional control reactions contained no template or 100 ng of purified USDA110 chromosomal DNA. One-fifth of each reaction was separated through a 1% agarose gel and visualized via ethidium bromide staining.

Creation of a *B. japonicum* USDA110 *ecfS* nonpolar deletion mutant and a *tmrS* OE strain.

Overlap extension PCR was used to replace the *ecfS* ORF with the gentamycin resistance gene (*aacC1*) to create a synthetic operon of *aacC1* and *tmrS* driven by the endogenous *ecfS* promoter (Fig. 1A). Three DNA fragments were amplified corresponding to 750 bp of *ecfS* upstream sequence (primers P3/P5; presumably containing the *ecfS* promoter and terminating at the *ecfS* GTG start codon), the ORF conferring gentamycin resistance (primers P6/P7; *aacC1*), and 750 bp of *ecfS* downstream sequence (primers P8/P4; including the last 39 bp of the *ecfS* ORF and the entire *tmrS* ORF). Using homologous sequences engineered into each fragment, the three pieces were spliced together using overlap extension PCR (primers P3/P4), creating $\Delta ecfS::aacC1-tmrS$. This product was purified and cloned into pGEM-T Easy using TA overhangs added by ExTaq polymerase, creating pGEM-TE($\Delta ecfS::aacC1-tmrS$). The insert was verified by PCR and sequencing. Next, $\Delta ecfS::aacC1-tmrS$ was subcloned using flanking *Bam*HI sites engineered into the construct, and cloned into a similarly cut *B. japonicum* suicide plasmid, pBR322. pBR322($\Delta ecfS::aacC1-tmrS$) was conjugated into *B. japonicum* USDA110 via tripar-

ental mating. *B. japonicum* clones containing the pBR322 ($\Delta ecfS::aacC1-tmrS$) plasmid recombined onto the chromosome were selected with tetracycline (200 µg/ml), and *E. coli* donor and helper strains were counterselected using rifampicin (40 µg/ml). These clones were verified as single-crossover meridioid strains by confirmation of the mutant allele by screening for gentamycin resistance and PCR. As shown by subsequent microarray analysis, these meridioid strains over-express *tmrS* 3.27-fold over wild-type USDA110 (data not shown), because they contain an additional copy of the *tmrS* ORF (driven by 750 bp of its endogenous promoter) knocked into the *ecfStmrS* locus. One of the meridioid strains, containing the pBR322 backbone on the chromosome, was passaged six times in antibiotic-free AG broth, diluted, and plated for single colonies. These colonies were screened for tetracycline and gentamycin resistance, indicative of a second crossover event leading to loss of the wild-type *ecfS* allele. Gentamycin-resistant, tetracycline-sensitive clones were confirmed as $\Delta ecfS::aacC1$ mutants by colony PCR and Southern blot (data not shown). Herein, this mutant strain is denoted " $\Delta ecfS$ ". The putative *ecfS* promoter sequence, spacing, and *tmrS* upstream sequence (presumably containing the *tmrS* ribosome binding site) is maintained in the *ecfS* mutant. Microarray analysis reveals a 2.6-fold increase in *tmrS* expression in $\Delta ecfS$ compared with the wild type, confirming the presumption that *tmrS* expression is not impaired in this strain.

Complementation of $\Delta ecfS$.

The $\Delta ecfS$ mutant complementation plasmid was made as follows: *ecfS* (including 500 bp of upstream sequence) was amplified, using primers P9/P10, from the *B. japonicum* USDA110 genome using the high-fidelity polymerase, PFU Ultra (Stratagene) (Fig. 1A). The product was initially cloned into the pGEM-T Easy vector system by TA-overhangs added to the PCR product by ExTaq polymerase following amplification, creating pGEMTE(*ecfS*). The insert was released from the pGEM-T Easy backbone with *Eco*RI and subcloned into a similarly cut low-copy plasmid, pLAFR3, for expression in *B. japonicum*. This created complementation plasmid pLAFR3(*ecfS*). Both pLAFR3(*ecfS*) and the empty pLAFR3 vector were conjugated into $\Delta ecfS$ by triparental mating and selected with tetracycline at 200 µg/ml. *E. coli* was counterselected using rifampicin at 40 µg/ml. Resulting *B. japonicum* clones were confirmed to contain the appropriate pLAFR3 complementation plasmids by PCR.

In vitro analysis of the mutant hypersensitivity to environmental stresses.

Some ECF σ factors are known to regulate genes required for survival in the face of environmental stress; therefore, the σ mutants were tested for hypersensitivity to oxidative stress, detergents, and extreme pH. Late-exponential to early-stationary phase AG liquid cultures of *B. japonicum* USDA110 and $\Delta ecfS$ were diluted and plated onto AG plates to create a lawn of bacteria. To test for sensitivity to oxidative stress, detergents, and pH, 3 µl of 2% H₂O₂, 7 µl of 10% SDS, 5 µl of 10 N NaOH, or 10 µl of 1 N HCl were added to the toothpick holes or sterile filter disks (in the case of SDS) in the center of each plate. Following 5 to 10 days of incubation at 30°C, the growth inhibition zones surrounding the added solutions were quantified. In addition to plate assays, a long-term stationary-phase survival assay was performed on the wild-type and mutant strains. In this assay, cultures were grown to stationary phase and viable cell counts were determined at 4, 7, 14, 21, and 28 days postinoculation. Because no difference was observed between the strains, this data has been excluded from this report. There was also no difference in Calcofluor white or Congo Red bind-

ing, or hypersensitivity to salt of the *ecfS* mutant compared with wild-type and complemented strains (not shown).

Symbiosis assays.

Glycine max soybean seed (Yellow butterbean; Johnny's Selected Seed, Ambion, ME, U.S.A.) were sterilized and inoculated with washed *B. japonicum* cultures or mock inoculated with sterile water, as previously described by Benson and associates (2005). Plants were grown for 5 weeks in modified, sterilized Leonard jars containing nitrogen-free medium (Benson et al. 2005). Shoots were separated from roots and weighed. Nodules were counted and weighed. For TEM, 1-mm³ cubes were cut from the centers of nodules infected with *ΔecfS*, *ΔecfS/pLAFR3*, and *ΔecfS/pecfS*. These samples were fixed, thin sectioned (approximately 100 nm thick), stained, viewed with a JEOL 100CX electron microscope operating at 100 kV, and photographed, as previously described (Benson et al. 2005; Roth and Stacey 1989). Chlorophyll extractions were performed as previously described (Benson et al. 2005) using 0.1 g of fresh leaf tissue per plant. Absorbance readings of isolated chlorophyll at 664 and 647 nm were taken and used to calculate total chlorophyll content per gram of leaf tissue as determined by Grann and Ort (1984). To confirm the genotypes of nodulating strains, *B. japonicum* cells were isolated from surface-sterilized, picked nodules and were verified as wild-type USDA110 or complemented mutant strains via colony PCR and antibiotic resistance.

cDNA preparation and microarray hybridization and analysis.

In-planta-derived cells were not used for the microarray due to technical challenges (i.e., the inability to harvest *ΔecfS* cells from inoculated plants). Rather, we elected to perform the microarray analysis on laboratory-grown cells based on previous work showing wild-type expression of *ecfStmrS* under standard culture conditions (Fig. 1B) as well as observed altered growth kinetics of the *ecfS* mutant in culture (i.e., extended lag phase compared with wild-type or complemented strains) (data not shown). Each *B. japonicum* strain included in the study was grown in triplicate in PSY broth to an optical density of 0.5 to 0.6 at 600 nm. Cells were harvested via centrifugation and flash frozen with liquid nitrogen, and RNA was subsequently extracted via a hot phenol method described previously (Babst et al. 1996). RNA was analyzed for integrity and lack of genomic DNA contamination. Approximately 10 to 20 μg of RNA (derived from independent cultures) was spiked with control transcripts and converted to cDNA, fragmented, and end labeled as previously described (Hauser et al. 2007; Pessi et al. 2007).

Approximately 2.3 μg of each sample of fragmented and labeled cDNA was hybridized to a custom-made Affymetrix GeneChip containing 8,266 genes and 5,261 intergenic regions of the *B. japonicum* USDA110 genome (provided and previously described by H. Hennecke and H.-M. Fisher's group) (Hauser et al. 2007; Pessi et al. 2007) and processed accordingly. Triplicate chips of each strain (USDA110, *ΔecfS/pLAFR3*, *ΔecfS/pecfS*, and *tmrS* OE) were performed.

GeneChip hybridization signals were normalized and transformed using a scaling factor (range of scaling factor values of 1.336 to 2.717) and analyzed using the GeneChip Operating System software provided by Affymetrix following standardized processing steps described by Pessi and associates (2007). Briefly, genes were filtered for those that were present or marginal, categories defined by a hybridization signal above background levels in at least two out of three replicates. Those genes that fell below this threshold of expression were removed from further analyses. Analysis of variance statistical analysis was

performed on the remaining data points with a false discovery rate of 0.01 and filtered for ≥twofold change in expression compared with the wild type. The resulting gene lists were then annotated based on the established Kaneko and associates (2002b) nomenclature and gene calls (Kaneko et al. 2002b). Further promoter analysis was performed using motif searches (MEME Suite) (Bailey et al. 2009) and the virtual footprint algorithm (Softberry BPROM analysis tool) (Munch et al. 2005); however, a conserved motif was not identified within the promoters of the genes listed in Table 1.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The MEME Suite motif-based sequence analysis tool: meme.nbcr.net
 Softberry BPROM online promoter prediction tool:
linux1.softberry.com/berry.phtml?topic=bprom&group=help&subgroup=gfindb