Sinorhizobium meliloti 1021 Loss-of-Function Deletion Mutation in *chvI* and Its Phenotypic Characteristics

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Bacterial two-component regulatory systems (TCS) are common components of complex regulatory networks and cascades. In *Sinorhizobium meliloti*, the TCS ExoS/ChvI controls exopolysaccharide succinoglycan production and flagellum biosynthesis. Although this system plays a crucial role in establishing the symbiosis between *S. meliloti* and its host plant, it is not well characterized. Attempts to generate complete loss-of-function mutations in either *exoS* or *chvI* in *S. meliloti* have been unsuccessful; thus, it was previously suggested that *exoS* or *chvI* are essential genes for bacterial cell growth. We constructed a *chvI* mutant by completely deleting the open reading frame encoding this gene. The mutant strain failed to grow on complex medium, exhibited lower tolerance to acidic condition, produced significantly less poly-3-hydroxybutyrate than the wild type, was hypermotile, and exhibited an altered lipopolysaccharide profile. In addition, this mutant was defective in symbiosis with *Medicago truncatula* and *M. sativa* (alfalfa), although it induced root hair deformation as efficiently as the wild type. Together, our results demonstrate that ChvI is intimately involved in regulatory networks involving the cell envelope and metabolism; however, its precise role within the regulatory network remains to be determined.

Bacteria have developed signaling systems for eliciting a variety of adaptive responses to their environments. Two-component regulatory systems (TCS) are one of the most important mechanisms for these adaptive responses. They consist of two protein components: a sensor that monitors some environmental parameters and a response regulator that mediates a change in gene expression in response to sensor signals (Li et al. 2002). They are found in the majority of bacteria, and they control housekeeping functions as well as regulate genes important for pathogenesis, stress, and adherence (Azcarate-Peril et al. 2005; Nishino et al. 2005). Recently, more than 4,000 putative TCS have been identified in 145 sequenced bacterial genomes (Ulrich et al. 2005). In *Sinorhizobium meliloti*, at least 31 putative histidine kinase sensors (based on RhizoBase) and 30 putative response regulators have been found during analyses of the *S. meliloti* 1021 genome. The functions of many of these putative systems remain unknown.

The gram-negative α -proteobacterium *S. meliloti*, found in soil, enters into a nitrogen-fixing symbiosis within the roots of leguminous plants such as *Medicago truncatula* and *M. sativa* (alfalfa). A successful symbiosis involves a complex series of

interactions and communication events between the host and bacteria (Long 1989, 2001; Brewin 1991; van Rhijn and Vanderleyden 1995; Jones et al. 2007). Initially, plants release flavonoids that trigger the rhizobial bacteria to produce lipochitin oligosaccharide compounds called Nod factors (Perret et al. 2000). The Nod factors then induce root hair curling and root nodule development. In addition to the Nod factor, *S. meliloti* produces exopolysaccharides (EPS) called succinoglycan (EPSI) and galactoglucan (EPSII) (Leigh et al. 1985; Finan et al. 1986) that is required for the formation of infection threads inside root hairs (Cheng and Walker 1998a). *S. meliloti* cells invade the developing root nodule via a cell division process within the infection thread. Once released from the infection threads, the bacterial cells differentiate into bacteroids and convert atmospheric nitrogen to ammonia (Schultze and Kondorosi 1998; Oke and Long 1999; Pellock et al. 2000).

In *S. meliloti*, the *exoS* and *chvI* genes constitute a TCS that plays a role in controlling EPSI production (Cheng and Walker 1998b). *S. meliloti* Rm1021 mutants that fail to synthesize EPSI form empty nodules that are devoid of bacteria or bacteroids (Leigh et al. 1985; Pellock et al. 2000) and are unable to establish a symbiosis because they are defective in initiating the production of infection threads and in invading the plant (Mendrygal and Gonzalez 2000; Jones et al. 2008). *S. meliloti* *exoS* has been studied extensively. Previous studies suggest that *exoS* and *chvI* are essential genes for growth under standard laboratory conditions, because attempts to generate complete loss-of-function mutations in either *exoS* or *chvI* in *S. meliloti* have been unsuccessful (Osteras et al. 1995; Cheng and Walker 1998b; Wells et al. 2007). Although most insertion mutations in *exoS* and *chvI* were believed to be lethal (Osteras et al. 1995), a Tn5 insertion 57 bp from the N terminus within the open reading frame (ORF) (named *exoS96::Tn5* mutation) results in the production of a constitutively active ExoS sensor kinase which upregulates EPSI levels. This results in the overproduction of EPSI (Doherty et al. 1988), presumably via the cognate response regulator ChvI (Cheng and Walker 1998b), and modulates expression of other *exo* genes (Doherty et al. 1988; Wells and Long 2002; Cheng and Yao 2004). In addition to the regulation of EPSI, the ExoS/ChvI system in *S. meliloti* is also involved in regulating flagellum biosynthesis (Yao et al. 2004). A recent study shows that ExoS/ChvI may interact with ExoR and control the expression of multiple transcriptional regulators (Wells et al. 2007). However, *exoS* mutants that have been studied to date are, in fact, constitutively active rather than null mutants (Cheng and Walker 1998b). No loss-of-function mutations in either *exoS* or *chvI* have been reported in *S. meliloti* thus far.

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Although the outcome is different, common mechanisms exist in pathogenic and symbiotic bacteria to support successful colonization of the host cells (Tsolis 2002). Studies of the ExoS/ChvI homologues in other α -proteobacteria have revealed that they regulate the expression profile of proteins associated with the bacterial cell envelope, and are required for persistence and virulence within host cells (Wells et al. 2007). For example, the ChvG/ChvI system is required for *vir* gene expression, virulence, and growth under acid conditions in *Agrobacterium tumefaciens* (Charles and Nester 1993; Li et al. 2002; Yuan et al. 2008). The homologue BvrS/BvrR is required for intracellular survival and controls the cell invasion and virulence of *Brucella abortus* in animal cells (Sola-Landa et al. 1998). Previous evidence also indicates that BvrS/BvrR controls the expression of several outer membrane proteins and is involved in modulating the properties of bacterial lipopolysaccharide (LPS) (Guzman-Verri et al. 2002; Manterola et al. 2005; Lamontagne et al. 2007).

Herein, we deleted the ORF of the *S. meliloti* Rm1021 *chvI* gene. Further, it is shown that the *chvI* mutant fails to grow in complex media or acidic media. The mutant exhibits symbiotic deficiency but is able to induce root hair deformation. In addition, loss of ChvI function disrupts intracellular polymer accumulation and affects cell motility. These findings provide additional insights into the role of ExoS/ChvI during the early stages of symbiosis.

RESULTS

Construction and phenotype of *chvI* mutant strain SB114.

A complete loss-of-function mutation of *chvI* in *S. meliloti* Rm1021 was generated by crossover polymerase chain reaction (PCR) (Sukdeo and Charles 2003) using the suicide vector pK19mobsacB (Schafer et al. 1994). The presence of the dele-

tion was confirmed by PCR and DNA sequencing. We were unable to generate the knockout *chvI* mutant on tryptone yeast (TY) medium but we generated such a mutant by using M9 + succinate (M9 + suc.) medium. This mutant grew well on YMB (Tombolini et al. 1995) but was unable to grow in media containing tryptone. For complementation analysis, plasmid pCW681 was constructed harboring the complete *chvI* ORF cloned in the broad-host-range vector pBBR1MCS-5 (Kovach et al. 1995). All phenotypic defects such as growth in different media, motility, polymer accumulation, and symbiotic properties in the *chvI* mutant, could be complemented by pCW681.

The *chvI* mutant is inhibited by an acidic environment.

TCS *chvG-chvI* is critical for virulence and is induced under acid conditions in *Agrobacterium* spp. (Yuan et al. 2008), and the *S. meliloti* *exoS* and *chvI* genes are highly similar to the *A. tumefaciens* *chvG* and *chvI* genes, respectively. Therefore, we determined whether the *S. meliloti* *chvI* deletion mutant was acid sensitive by monitoring the growth behavior of both the *chvI* mutant and the wild-type strain Rm1021 (Fig. 1). The *chvI* mutant grew normally compared with the wild-type strain Rm1021 at pH 7.0 but more poorly than Rm1021 at pH 5.5. Also, in comparison with the growth rates of wild-type strain Rm1021 at acidic and neutral conditions, the growth rate was slightly lower at pH 5.5 than at pH 7.0; however, it still exhibited steady-state exponential growth. Thus, the acidic (pH 5.5) growth conditions conferred mild acid stress on the cells.

Loss of ChvI function disrupts LPS production and EPS and poly-3-hydroxybutyrate accumulation.

To test whether ExoS/ChvI is involved in modulating the properties of bacterial LPS, as is the case for BvrS/BvrR in *B. abortus*, we extracted and analyzed the LPS from the wild type and the *chvI* mutant in *S. meliloti*. Resolution of LPS molecules by polyacrylamide gel electrophoresis followed by silver staining revealed striking differences between LPS from the wild-type Rm1021 and that of the *chvI* mutant. The smooth LPS band (higher molecular weight) was not detected in the mutant; however this phenotype was complemented by the plasmid-carried *chvI* gene (Fig. 2). To determine the growth behavior of strains and their ability to synthesize and accumu-

Fig. 1. Growth curve of *Sinorhizobium meliloti* strains in M9 + succinate (M9 + suc.) minimal medium. Log-phase cultures of the wild-type Rm1021 and the *chvI* mutant from M9+suc. were inoculated into fresh M9 + suc. medium (top, pH 7.0; bottom, pH 5.5) with a starting optical density at 600 nm (OD₆₀₀) of 0.04. The cells were grown at 30°C with shaking. The OD₆₀₀ values are the means of three independent experiments.

Fig. 2. Lipopolysaccharide profiles in the wild-type strain Rm1021 and the *chvI* mutant after resolution by sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver staining. Lane 1, the wild-type strain Rm1021; lane 2, the *chvI* mutant strain; lane 3, the complemented strain SB1201. S- and R-LPS = smooth and rough lipopolysaccharide, respectively.

late EPS and poly-3-hydroxybutyrate (PHB), the *chvI* mutant and wild-type strain were cultivated in liquid YMB medium. The wild-type strain and the *chvI* mutant showed similar growth kinetics (Table 1); however, EPS was not detected in the *chvI* mutant. This observation is consistent with an essential role for ChvI in the regulation of EPS synthesis. Surprisingly, we found that the *chvI* mutation resulted in significantly less PHB production than the wild-type strain (Table 1).

Carbon source utilization patterns were also determined on M9 agar with either succinate, fructose, sucrose, glucose, acetoacetate, or pyruvate as the sole carbon source. All strains had similar growth behaviors on succinate, fructose, sucrose, and glucose. However, the *chvI* mutant grew slightly more slowly, forming smaller colonies after 4 days of growth than those of the wild type on acetoacetate or pyruvate (data not shown).

The *chvI* mutation affects apparent cell motility.

Previous studies showed that the *exoS96* mutant is severely impaired in expression of flagellar biosynthesis genes and cell motility in comparison with the wild type (Yao et al. 2004). To examine whether the *chvI* mutation has a phenotype similar to the *exoS96* mutation, we assayed the strains for motility and the presence or absence of flagella. The wild-type strain Rm1021, its EPS-deficient *exoY* mutant Rm7210 (Leigh et al. 1985), and its nonmotile, flagellaless *flp* mutant RmG910 (Finan et al. 1995) were similarly tested on swim plates as controls. Strains capable of motility showed an increased diameter in colony size after 4 days compared with the strain incapable of swimming. All strains tested were capably motile in the swim plates, except for the nonmotile control, *flp* mutant RmG910 (Fig. 3A). Both the wild-type strain Rm1021 and the *exoY* mutant strain Rm7210 formed small, tightly packed colonies, and the *chvI* mutant formed large, diffuse colonies (Fig. 3A), suggesting that large colonies of the *chvI* mutant do not result from EPS production. To determine whether the apparent increase of motility of *chvI* was related to the presence of flagella, wild-type and *chvI* mutant cells were stained for flagella and examined in detail by using transmission electron microscopy (TEM). Both wild-type Rm1021 and *chvI* mutant cells grown in the swim plates containing minimal media (M9 + suc.) were flagellated (Fig. 3B and C). These findings raised the possibility that ChvI may control cell motility genes, directly or through other regulators.

Loss of ChvI function causes symbiotic defects but doesn't affect ability to induce root hair deformation.

To examine the symbiotic phenotype of the *chvI* mutant, plant inoculation experiments were performed in growth pouches and growth chambers on the host legumes *M. truncatula* and alfalfa, using the EPS-deficient *exoY* mutant strain Rm7210 as a control. Five weeks after inoculation, nodulation was observed in both legumes. The seedlings inoculated with the wild-type strain Rm1021 became healthy plants with pink, elongated nodules, whereas the plants inoculated with the *chvI* mutant were unhealthy, with few small, round, and white nodules (Fig. 4). Even though nodules induced by the EPS-deficient *exoY* mutant and the *chvI* mutant looked similar (small, round, white nonfixing nodules), the *chvI* mutant induced

fewer nodules (approximately 15.2%) than the *exoY* mutant, which induced a similar number of nodules as the wild type did on *M. truncatula* and alfalfa in growth pouches (data not shown). The nodulation properties, including the average shoot dry weight (SDW), average number of nodules, and acetylene reduction activity (ARA) elicited by each strain at 5 weeks postinoculation in growth chamber, are shown in Table 2. Both *M. truncatula* and alfalfa plants inoculated with the *chvI* mutant displayed significantly lower SDW than the wild-type strain, and these plants also exhibited severely reduced levels of ARA and number of nodules (Table 2). In addition, similar to the *exoY* mutant, the *chvI* mutant-derived nodules (small, round, white nonfixing nodules) typically yielded 100- to 1,000-fold fewer bacterial colonies than nodules containing Rm1021 when they were crushed and plated on M9 + suc. agar plates (data not shown). However, the symbiotic defect of the *chvI* mutant was fully eliminated by the ectopic expression of *chvI* from pCW681 (Fig. 4 ; Table 2).

Microscopic examination was used to observe whether the *chvI* mutant induced root hair deformation. The root hair responses caused by the *chvI* mutant infection were similar to the wild-type infection at 1, 2, and 3 days after inoculation (dai). *M. truncatula* roots inoculated with wild-type or *chvI* mutant *S. meliloti* were not yet morphologically distinguishable (Fig. 5).

Fig. 3. Motility and flagellar status in *Sinorhizobium meliloti* strains. **A**, Motility was observed by inoculating each strain into 0.22% agar M9 + succinate (M9 + suc.) plates (swim plates). Relevant strain genotypes are indicated. Bacteria are shown after 4 days of growth at 30°C. **B** and **C**, Transmission electron microscope to examine the presence of flagella in **B**, Rm1021 (wild type) and **C**, the *chvI* mutant. Bacteria grown in the swim plates were negatively stained with uranyl acetate and the representative micrographs of the strains are shown at approximately $\times 10,000$ magnification. Scale bars, 1 μ m.

Table 1. Polymer accumulation by the wild-type and mutant^a

Strain	Genotype	EPS/cell dry wt	PHB (% cell dry wt)	Doubling time (h)
Rm1021	Wild type	1.66 $\pm$ 0.03	47.1 $\pm$ 4.46	8.2 $\pm$ 0.1
SB114	<i>chvI</i> ⁻	0	16.4 $\pm$ 1.34	7.7 $\pm$ 0.2
SB1201	<i>chvI</i> ⁻ (pCW681)	1.38 $\pm$ 0.06	39.2 $\pm$ 2.01	7.1 $\pm$ 0.2

^a Doubling time was calculated from the average for three replications, based on the standard procedure. Values are means $\pm$ standard deviations. EPS = exopolysaccharide and PHB = poly-3-hydroxybutyrate.

DISCUSSION

Previously, attempts to generate complete loss-of-function mutations in either *exoS* or *chvI* in *S. meliloti* were unsuccessful, leading to the suggestion that both are essential genes in free-living bacteria (Osteras et al. 1995; Cheng and Walker 1998b; Wells et al. 2007). Here, it was demonstrated that it is possible to generate a loss-of-function *chvI* mutant in *S. meliloti* Rm1021. The mutant strain fails to grow on complex medium, exhibits lower tolerance to acidic conditions, produces significantly less PHB, and is more motile than the wild-type strain Rm1021. The profile of LPS was also altered. Further, this mutant is defective in symbiosis but induces root hair deformation as efficiently as the wild type. Together, these results indicate that ChvI is not only involved in modulating the expression of EPSI and flagella, as previously reported, but also affects regulatory networks involving the bacterial cell envelope and metabolism. Thus, the ExoS/ChvI TCS seems to have a broad role in *S. meliloti*.

Our findings demonstrate that *chvI* is an essential gene for growth in rich media but not an essential gene for growth in minimal media. Considerable evidence suggests that plant partners supply C₄-dicarboxylic acids, such as succinate, malate, or fumarate, to nitrogen-fixing bacteroids (McDermott et al. 1989; Lodwig and Poole 2003). Because attempts to delete either *exoS* or *chvI* were unsuccessful in *S. meliloti* under standard laboratory conditions (rich media), and *S. meliloti* favors succinate and related dicarboxylic acids as carbon and energy

sources, we decided to use the minimal medium M9 + suc. as carbon source to select for the desired mutant.

In the animal pathogen *B. abortus*, BvrS/BvrR, the ExoS/ChvI homologues, not only are critical for cell invasion and virulence (Sola-Landa et al. 1998; Lopez-Goni et al. 2002) but also affect LPS structure and outer membrane proteins (Guzman-Verri et al. 2002; Lamontagne et al. 2007). Although the role that LPS plays in the symbiosis between *S. meliloti* and alfalfa has been studied for several years, its function remains controversial and poorly understood (Clover et al. 1989; Reuhs et al. 1998). Recent studies on mutants with altered LPS demonstrated that alterations in LPS structure can affect the ability of *S. meliloti* to form an effective symbiosis (Campbell et al. 2003). The LPS profile differences between the wild-type strain Rm1021 and the *chvI* mutant suggest that ExoS/ChvI is essential for LPS synthesis or transport in *S. meliloti*.

Our data show that the *chvI* mutant is inhibited by acid. Previous microarray analysis reveals that the ChvG/ChvI TCS in *A. tumefaciens* is induced under acidic conditions (Yuan et al. 2008). The fact that gene expression is acid inducible suggests that the gene plays a role in overcoming growth inhibition under acidic conditions. Therefore, it is not surprising that a strain lacking the gene would be acid sensitive. *S. meliloti* requires ExoS/ChvI TCS to establish a nitrogen-fixing symbiosis with legume hosts. It remains to be determined whether pH plays a role in the symbiotic development process and whether ExoS/ChvI is involved in regulation of the expression of any acid-inducible genes in *S. meliloti* (Li et al. 2002).

Fig. 4. *Sinorhizobium meliloti chvI* is required for efficient nodulation on alfalfa. Alfalfa plants were inoculated with **A**, no bacteria; **B**, wild-type strain Rm1021; **C**, the *chvI* mutant strain SB114; **D**, the succinoglycan-deficient *exoY* mutant strain Rm7210; and **E**, SB114 with complementing plasmid pCW681. Representative nodules were imaged 35 days after inoculation with **F**, Rm1021; **G**, SB114; **H**, Rm7210; and **I**, SB114 harboring pCW681. Symbiotic nitrogen fixation phenotypes (Fix⁺ or Fix⁻) are indicated at the bottom of each image, based on nodule color and morphology and on shoot coloration on the host plant.

Table 2. Symbiotic properties of wild-type and mutant strain on *Medicago truncatula* and *M. sativa* plants^a

Strain	Genotype	Shoot dry weight (mg/plant)		No. of nodules/plant		Acetylene reduction (nmol/h/plant)	
		<i>M. truncatula</i>	<i>M. sativa</i>	<i>M. truncatula</i>	<i>M. sativa</i>	<i>M. truncatula</i>	<i>M. sativa</i>
Rm1021	Wild type	10.20 ± 0.44	15.64 ± 0.58	16.96 ± 0.32	12.94 ± 1.65	13.01 ± 1.53	33.74 ± 0.35
SB114	<i>chvI</i> ⁻	3.75 ± 0.25	1.85 ± 0.25	0.15 ± 0.25	0.26 ± 0.10	0.37 ± 0.12	0.57 ± 0.20
SB1201	<i>chvI</i> ⁻ (pCW681)	9.49 ± 1.02	10.87 ± 0.49	16.32 ± 0.93	12.08 ± 0.52	10.56 ± 1.33	30.35 ± 0.59
Rm7210	<i>exoY210::Tn5</i>	2.38 ± 0.21	2.13 ± 0.13	15.33 ± 1.97	13.33 ± 1.69	0.72 ± 0.42	0.36 ± 0.06

^a Plants were grown and inoculated under growth chamber conditions as described previously (Wang et al. 2007). Dry weights, number of nodules, and acetylene reduction were measured 35 days after inoculation. Twenty-seven plants were used for each strain. Values present the means ± standard deviations.

Flagellar motility is a highly regulated bacterial behavior that is responsive to nutritional availability, population density, and a variety of environmental stimuli (Scharf and Schmitt 2002; Soutourina and Bertin 2003). In *S. meliloti*, EPS production and motility are contrarily regulated (Reed et al. 1991; Yao et al. 2004; Barnett and Fisher 2006; Wells et al. 2007; Hoang et al. 2008). The *exoS96::Tn5* mutant strain, overproducing EPSI by constitutively activated ExoS protein, causes a marked reduction in the biosynthesis of flagella (Barnett et al. 2004) and consequent loss of ability of the cells to swarm and swim. EPSI overproduction is probably not the cause of the suppression of flagellum biosynthesis in the *exoS96* mutant because an *exoY/exoS96* double mutation is nonmotile (Yao et al. 2004). Recent work by Hoang and associates (2008) shows that the ExoS-dependent TCS suppresses motility gene expression through VisN and Rem in parallel to quorum sensing (Hoang et al. 2008). We have found that *chvI* mutant cells are more motile than the wild-type Rm1021 cells on swim plates. Also, both wild-type strain Rm1021 and the EPS-deficient *exoY* mutant Rm7210 formed similar types of colonies on the swim plates. This result demonstrates that the larger colony

morphology of the *chvI* mutant on the swim plates is not due to the EPS phenotype. Furthermore, we confirm that cells of both wild-type Rm1021 and the *chvI* mutant grown on the swim plates containing minimal media (M9 + suc.) have flagella. Based on these data, we propose the following hypothesis: for ExoS/ChvI in *S. meliloti*, ExoS activates ChvI, ChvI controls motility genes directly or through other regulators, and the *exoS96* mutation results in active ChvI and nonmotile cells lacking flagella, as previously reported (Yao et al. 2004); therefore, it is expected that a deletion of *chvI* exhibits greater motility than the wild type. It is still possible, however, that ExoS/ChvI may play some role in regulating chemotaxis in addition to regulating flagellum biosynthesis and EPSI biosynthesis. It should be made clear here that the Rm1021 strain used in our work has an insertion mutation in the *expR* gene. This prevents the formation of EPSII (Pellock et al. 2002).

PHB is a major carbon storage compound in *S. meliloti*. Mutant analysis has demonstrated that PHB metabolism plays a role in *Rhizobium*–legume symbiosis (Willis and Walker 1998; Lodwig and Poole 2003; Trainer and Charles 2006; Wang et al. 2007). It has been hypothesized that PHB accumulated by the

Fig. 5. Root hair deformation. *Medicago truncatula* roots at 1, 2, and 3 days after inoculation (dai) with *Sinorhizobium meliloti* **A**, wild-type Rm1021; **B**, *chvI* mutant; or **C**, uninoculated control. Magnification ×480. All photos were taken of root hairs in the growing root hair zone (Wood and Newcomb 1988).

Table 3. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics ^a	Source or reference
<i>Sinorhizobium meliloti</i>		
Rm1021	Derived from wild-type SU47, Sm ^r	Meade et al. 1982
Rm7210 RmG910	<i>exoY210::Tn5</i> , Sm ^r , Nm ^r 1021:: <i>fliPQ</i> Km	Leigh et al. 1985; Finan et al. 1995
SB114	Rm1021 <i>chvI</i> -precise-deletion	This study
SB1201	SB114 (pCW681)	This study
<i>Escherichia coli</i>		
DH5α	F [−] <i>endA1 hsdR17</i> (rk [−] , mk ⁺) <i>supE44 thi-1 recA1 gyrA96 relA1Δ</i> (<i>argF-lacZYA</i>)U169	Hanahan 1983
DH5α (pRK600)	Φ80 <i>dlacZ</i> ΔM15, λ [−]	Finan et al. 1986
Plasmids		
pGEM-T Easy	Am ^r , for cloning polymerase chain reaction products	Promega
pK19mobsacB	Suicide vector, Nm/Km ^r	Schafer et al. 1994
pJK1	<i>chvI</i> deletion in <i>EcoR</i> I site of pK19mobsac, Km ^r	This study
pBBR1MCS-5	Cloning vector, Gm ^r	Kovach et al. 1995
pCW681	pBBR1MCS-5 containing the <i>chvI</i> gene, Gm ^r	This study

^a Sm^r, Nm^r, Cm^r, Km^r, and Gm^r = resistant to streptomycin, neomycin, chloramphenicol, kanamycin, and gentamicin, respectively.

bacteria prior to infection serves to fuel infection or bacteroid differentiation (Lodwig et al. 2005). A correlation between PHB metabolism and EPS synthesis has been demonstrated (Aneja et al. 2004). However, the mechanisms of regulation of PHB metabolism have not been well characterized. Here, we show that PHB production is dramatically reduced in the *chvI* mutant (Table 1). This raises the possibility that ExoS/ChvI may play a key role in regulating storage polymer biosynthesis and carbon metabolism in *S. meliloti*.

The lack of EPSI biosynthesis by the *chvI* mutant strain could explain the deficient symbiotic phenotype observed but, interestingly, our results clearly show that the quantity of nodules obtained was very different from the *exoY* mutant, deficient in EPSI biosynthesis: the *exoY* mutant induced significantly more nodules than the *chvI* mutant on *M. truncatula* and alfalfa after 5 weeks of inoculation. Also, we showed that there are no morphological differences yet apparent at 1, 2, and 3 dai between *M. truncatula* roots inoculated with the wild type or the *chvI* mutant (Fig. 5). This observation is very similar to the succinoglycan-deficient *S. meliloti* *exoY* mutant, which produces Nod factor in a functional form as efficiently the wild type (Leigh et al. 1985; Klein et al. 1988; Glazebrook and Walker 1989; Jones et al. 2008) and induces root hair curling as efficiently as the wild type (Cheng and Walker 1998a). This result suggests that the *chvI* mutant might have Nod factor gene expression as well as morphological responses similar to the wild-type strain at the early inoculation stage. Put together, the data presented in this work provide evidence that ExoS/ChvI controls a significantly broader set of phenotypes than previously reported. We are pursuing further studies using the loss-of-function *chvI* mutant to identify new functions controlled by the ExoS/ChvI system in *S. meliloti*.

MATERIALS AND METHODS

Bacterial strains and general growth conditions.

The strains and plasmids used in this work are shown in Table 3. All *S. meliloti* strains in this work are derivatives of Rm1021 (wild type) and were grown at 30°C in TY (Beringer 1974), YMB (Tombolini et al. 1995) or M9 minimal medium with carbon sources at 15 mM as previously described (Charles and Finan 1991). Unless otherwise indicated, acid conditions refer to pH 5.5, whereas neutral conditions refer to pH 7.0. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (Miller 1972). Antibiotics were used at the following concentrations: ampicillin (Am), 100 µg/ml; kanamycin (Km), 20 µg/ml; chloramphenicol (Cm), 25 µg/ml; neomycin (Nm), 200 µg/ml; tetracycline (Tc), 10 µg/ml; gentamicin (Gm), 20 µg/ml (for *S. meliloti*) or 10 µg/ml (for *E. coli*); and streptomycin (Sm), 200 µg/ml. Media were solidified by the addition of 1.5% (wt/vol) agar. Liquid cultures were shaken at 220 rpm.

Construction of the *chvI* deletion mutant.

The loss-of-function *chvI* mutant was constructed by cross-over PCR (Sukdeo and Charles 2003) using the suicide vector pK19mobsacB (Schafer et al. 1994), which cannot replicate in *S. meliloti*. In brief, PCR on Rm1021 genomic DNA template was used to generate fragments on either side of the *chvI* sequence targeted for deletion. For amplification of the N-terminal end of *chvI*, primers A (5' TGAATTCGTCGGAACAT ACC 3') and B (5' GCCGTCGACGAGCGAGGCACTTAT TCTACTCCGTTTCGA 3') were used, resulting in an amplification product of 329 bp. For amplification of the C-terminal end of *chvI*, primers C (5' TGCCTCTCGCTCGTCGACGGC GGGAGCGAGCCGGCGGATTG 3') and D (5' GATTTCGAC GTTCGCTACTGC 3') were used, resulting in an amplification

product of 385 bp. Primers B and C contained 21-bp complementary sequences at their 5' ends, as underlined. The PCR products resulting from these two amplifications were gel purified and combined as templates for a PCR reaction using primer pairs A and D. A fragment of the desired 693-bp size was obtained and ligated into the pGEM-T Easy Vector (Promega Corp., Madison, WI, U.S.A.), and the sequence was confirmed to be correct; the fragment was then subcloned into pK19mobsacB to produce plasmid pJK1. Gene replacement in Rm1021 was carried out by first introducing pJK1 by triparental mating, selecting for Sm- and Nm-resistant single-cross-over cointegrants. Subsequent selection for double-crossover events was carried out on M9 + suc. agar containing 5% sucrose, followed by screening for Nm sensitivity. The precise deletion of *chvI* was confirmed by PCR amplification using flanking primers and DNA sequencing. The corresponding strain was designated SB114.

In order to complement the *S. meliloti* *chvI* mutant, a 1,036-bp fragment containing *chvI* under the control of its own promoter was amplified by PCR using primers *chvI*-F09 (5' TTTAAGCTTGGATCAGGTGCTTTTGCCT 3') and *chvI*-R09 (5' TTTGGATCCAAACTGTGTGCGGTTTCC 3') (introduced restriction sites underlined) and Rm1021 genomic DNA as a template. The fragment was digested with *Hind*III and *Bam*HI and ligated into a *Hind*III-*Bam*HI-digested pBBR1MCS-5 plasmid (Kovach et al. 1995), and the resulting plasmid was named pCW681. The plasmid pCW681 was transformed into *E. coli* DH5α competent cells. The clone was confirmed by PCR and DNA sequencing prior to introduction into *S. meliloti* *chvI* mutant by triparental mating using helper plasmid pRK600 (Finan et al. 1986).

Motility tests and TEM.

Motility was assessed by using swim agar plates as described (Wells et al. 2007) but using M9 + suc. medium instead of rich medium. Briefly, colonies were taken from the surface of regular 1.5% agar plates with 10-µl pipette tips, and inserted into 0.22% agar plates. Plates were subsequently dried for approximately 2 h and then transferred to a 30°C incubator, where they were incubated upside down. Motility was scored after 4 days.

For electron microscopy analysis, bacterial cells were picked from the swim agar plates, fixed by adding 2% formaldehyde, washed with phosphate buffered saline, and applied to carbon-coated copper grids. Samples were washed twice with distilled water, negatively stained for 30 s in 0.5% uranyl acetate, and then rinsed three times (10 s each) by submersion in aliquots of Milli-Q water. Imaging was performed with a Zeiss 10CA Transmission Electron Microscope (Carl Zeiss Inc., Oberkochen, Germany) at 60 kV of accelerating voltage at room temperature.

LPS isolation and analysis.

The procedure for preparation and visualization of *S. meliloti* LPS was similar to that employed by Reuhs and associates (1993) with the following modifications: cells were grown to late log phase in M9 + suc. medium, after which 1.5 ml of each sample was pelleted by centrifugation for 5 min at 13,000 × *g*. The pellets were washed twice with water before being resuspended in 50 µl of 65°C water. To the cell suspension, 50 µl of 65°C phenol was added, followed by vortexing, incubation at 65°C for 15 min, and cooling on ice for 10 min. The samples were then centrifuged at 13,000 × *g* for 10 min, the water phase was set aside, and the phenol phase was heated to 65°C. Then, 50 µl of 65°C water was added to the hot phenol phase and the above procedure was repeated. The water phases (containing LPS) were combined and mixed with sam-

ple buffer (62 mM Tris HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% β -mercaptoethanol, 10% glycerol, and 0.03% bromophenol blue) in a 5:1 ratio.

A volume of 5 μ l of the final preparation was then applied to a standard SDS acrylamide minigel (Laemmli 1970) containing 15% acrylamide and visualized by silver staining (Hitchcock and Brown 1983).

Quantification of EPS and PHB.

The EPS content was determined as previously described (Marroqui et al. 2001) from cultures grown in YMB for 4 days at 30°C. The concentration of PHB was determined from cells grown in YMB medium using the spectrophotometric technique (Law and Slepcey 1961) as modified by Peoples and Sinskey (1989).

Root hair deformation phenotype assay.

Root hair responses by *S. meliloti* were analyzed on microscope slides as described by Gage and associates (1996) with the following modifications. *M. truncatula* Jemalong (line A17) seed were sterilized and germinated as described previously (Wang et al. 2007). Two seedlings, 1 to 2 cm long, were placed on top of the microscope slides which had been covered with 2 ml of BNM medium (Ehrhardt et al. 1992) in 1.1% agarose. The seedlings were covered with a single layer of washed dialysis membrane (12,000 to 14,000 molecular weight cutoff) after being inoculated with 0.1 ml of a 1:100 dilution in sterile water of *S. meliloti* cells grown to saturation in M9 + suc. medium and washed once in sterilized water. The slides were then placed in a 250-ml beaker containing 30 ml of liquid BNM medium and covered loosely with Saran Wrap, and the beaker was placed in a growth chamber (26°C, light and dark periods of 16 and 8 h, respectively). Root hairs were examined and photographs were taken as described by Wood and Newcomb (1988) using a Zeiss Axioskop Microscope.

Symbiotic assays.

Symbiotic phenotype assays were performed with *M. truncatula* Jemalong (line A17) and alfalfa (*M. sativa* cv. Iroquois) plants in growth pouches and growth chambers as described previously (Wang et al. 2007). Shoot dry weights, number of nodules, and nitrogen fixation activities (acetylene reduction) were determined 5 weeks after inoculation. Stained nodules were observed and imaged through a $\times 0.8$ objective lens on a Leica Microscope.

Statistical analysis.

All the data presented for PHB, EPS, nodule numbers, acetylene reduction assays, and plant dry weight are presented as means with standard errors. The significance of the results was assessed using Student's *t* test.

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LITERATURE CITED

Aneja, P., Dai, M., Lacorre, D. A., Pilon, B., and Charles, T. C. 2004. Heterologous complementation of the exopolysaccharide synthesis and carbon utilization phenotypes of *Sinorhizobium meliloti* Rm1021 polyhydroxyalkanoate synthesis mutants. FEMS (Fed. Mol. Microbiol. Soc.) Microbiol. Lett. 239:277-283.

Azcarate-Peril, M. A., McAuliffe, O., Altermann, E., Lick, S., Russell, W. M., and Klaenhammer, T. R. 2005. Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 71:5794-5804.

Barnett, M. J., and Fisher, R. F. 2006. Global gene expression in the rhizobial-legume symbiosis. Symbiosis 42:1-24.

Barnett, M. J., Toman, C. J., Fisher, R. F., and Long, S. R. 2004. A dual-genome Symbiosis Chip for coordinate study of signal exchange and development in a prokaryote-host interaction. Proc. Natl. Acad. Sci. U.S.A. 101:16636-16641.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84:188-198.

Brewin, N. J. 1991. Development of the legume root nodule. Annu. Rev. Cell Biol. 7:191-226.

Campbell, G. R., Sharypova, L. A., Scheidle, H., Jones, K. M., Niehaus, K., Becker, A., and Walker, G. C. 2003. Striking complexity of lipopolysaccharide defects in a collection of *Sinorhizobium meliloti* mutants. J. Bacteriol. 185:3853-3862.

Charles, T. C., and Finan, T. M. 1991. Analysis of a 1600-kilobase *Rhizobium meliloti* megaplasmid using defined deletions generated *in vivo*. Genetics 127:5-20.

Charles, T. C., and Nester, E. W. 1993. A chromosomally encoded two-component sensory transduction system is required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. 175:6614-6625.

Cheng, H. P., and Walker, G. C. 1998a. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. 180:5183-5191.

Cheng, H. P., and Walker, G. C. 1998b. Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. J. Bacteriol. 180:20-26.

Cheng, H. P., and Yao, S. Y. 2004. The key *Sinorhizobium meliloti* succinoglycan biosynthesis gene *exoY* is expressed from two promoters. FEMS (Fed. Mol. Microbiol. Soc.) Microbiol. Lett. 231:131-136.

Clover, R. H., Kieber, J., and Signer, E. R. 1989. Lipopolysaccharide mutants of *Rhizobium meliloti* are not defective in symbiosis. J. Bacteriol. 171:3961-3967.

Doherty, D., Leigh, J. A., Glazebrook, J., and Walker, G. C. 1988. *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic calcofluor-binding exopolysaccharide. J. Bacteriol. 170:4249-4256.

Ehrhardt, D. W., Atkinson, E. M., and Long, S. R. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science 256:998-1000.

Finan, T. M., Kunkel, B., DeVos, G. F., and Signer, E. R. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. 167:66-72.

Finan, T. M., Gough, C., and Trucher, G. 1995. Similarity between the *Rhizobium meliloti* fliP gene and pathogenicity-associated genes from animal and plant pathogens. Gene 152:65-77.

Gage, D. J., Bobo, T., and Long, S. R. 1996. Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). J. Bacteriol. 178:7159-7166.

Glazebrook, J., and Walker, G. C. 1989. A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell 56:661-672.

Guzman-Verri, C., Manterola, L., Sola-Landa, A., Parra, A., Cloeckaert, A., Garin, J., Gorvel, J. P., Moriyon, I., Moreno, E., and Lopez-Goni, I. 2002. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the *Rhizobiaceae*. Proc. Natl. Acad. Sci. U.S.A. 99:12375-12380.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.

Hitchcock, P. J., and Brown, T. M. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154:269-277.

Hoang, H. H., Gurich, N., and Gonzalez, J. E. 2008. Regulation of motility by the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti*. J. Bacteriol. 190:861-871.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., and Walker, G. C. 2007. How rhizobial symbionts invade plants: The *Sinorhizobium-Medicago* model. Nat. Rev. Microbiol. 5:619-633.

Jones, K. M., Sharopova, N., Lohar, D. P., Zhang, J. Q., VandenBosch, K. A., and Walker, G. C. 2008. Differential response of the plant *Medicago truncatula* to its symbiont *Sinorhizobium meliloti* or an exopolysaccharide-deficient mutant. Proc. Natl. Acad. Sci. U.S.A. 105:704-709.

Klein, S., Hirsch, A. M., Smith, C. A., and Signer, E. R. 1988. Interaction of *nod* and *exo Rhizobium meliloti* in alfalfa nodulation. Mol. Plant-Microbe Interact. 1:94-100.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd, and Peterson, K. M. 1995. Four new derivatives of

- the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175-176.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lamontagne, J., Butler, H., Chaves-Olarte, E., Hunter, J., Schirm, M., Paquet, C., Tian, M., Kearney, P., Hamaidi, L., Chelsky, D., Moriyon, I., Moreno, E., and Paramithiotis, E. 2007. Extensive cell envelope modulation is associated with virulence in *Brucella abortus*. *J. Proteome Res.* 6:1519-1529.
- Law, J. H., and Slepecky, R. A. 1961. Assay of poly- β -hydroxybutyric acid. *J. Bacteriol.* 82:33-36.
- Leigh, J. A., Signer, E. R., and Walker, G. C. 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proc. Natl. Acad. Sci. U.S.A.* 82:6231-6235.
- Li, L., Jia, Y., Hou, Q., Charles, T. C., Nester, E. W., and Pan, S. Q. 2002. A global pH sensor: *Agrobacterium* sensor protein ChvG regulates acid-inducible genes on its two chromosomes and Ti plasmid. *Proc. Natl. Acad. Sci. U.S.A.* 99:12369-12374.
- Lodwig, E. M., and Poole, P. S. 2003. Metabolism of rhizobium bacteroids. *Crit. Rev. Plant Sci.* 22:37-78.
- Lodwig, E. M., Leonard, M., Marroqui, S., Wheeler, T. R., Findlay, K., Downie, J. A., and Poole, P. S. 2005. Role of polyhydroxybutyrate and glycogen as carbon storage compounds in pea and bean bacteroids. *Mol. Plant-Microbe Interact.* 18:67-74.
- Long, S. R. 1989. *Rhizobium*-legume nodulation: Life together in the underground. *Cell* 56:203-214.
- Long, S. R. 2001. Genes and signals in the *Rhizobium*-legume symbiosis. *Plant Physiol.* 125:69-72.
- Lopez-Goni, I., Guzman-Verri, C., Manterola, L., Sola-Landa, A., Moriyon, I., and Moreno, E. 2002. Regulation of *Brucella* virulence by the two-component system BvrR/BvrS. *Vet. Microbiol.* 90:329-339.
- Manterola, L., Moriyon, I., Moreno, E., Sola-Landa, A., Weiss, D. S., Koch, M. H., Howe, J., Brandenburg, K., and Lopez-Goni, I. 2005. The lipopolysaccharide of *Brucella abortus* BvrS/BvrR mutants contains lipid A modifications and has higher affinity for bactericidal cationic peptides. *J. Bacteriol.* 187:5631-5639.
- Marroqui, S., Zorreguieta, A., Santamaria, C., Temprano, F., Soberon, M., Megias, M., and Downie, J. A. 2001. Enhanced symbiotic performance by *Rhizobium tropici* glycogen synthase mutants. *J. Bacteriol.* 183:854-864.
- McDermott, T. R., Griffith, S. M., Vance, C. P., and Graham, P. H. 1989. Carbon metabolism in *Bradyrhizobium japonicum* bacteroids. *FEMS (Fed. Mol. Microbiol. Soc.) Microbiol. Rev.* 63:327-340.
- Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon mutagenesis. *J. Bacteriol.* 149:114-122.
- Mendrygal, K. E., and Gonzalez, J. E. 2000. Environmental regulation of exopolysaccharide production in *Sinorhizobium meliloti*. *J. Bacteriol.* 182:599-606.
- Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A..
- Nishino, K., Honda, T., and Yamaguchi, A. 2005. Genome-wide analyses of *Escherichia coli* gene expression responsive to the BaeSR two-component regulatory system. *J. Bacteriol.* 187:1763-1772.
- Oke, V., and Long, S. R. 1999. Bacteroid formation in the *Rhizobium*-legume symbiosis. *Curr. Opin. Microbiol.* 2:641-646.
- Osteras, M., Stanley, J., and Finan, T. M. 1995. Identification of *Rhizobium*-specific intergenic mosaic elements within an essential two-component regulatory system of *Rhizobium* species. *J. Bacteriol.* 177:5485-5494.
- Pellock, B. J., Cheng, H. P., and Walker, G. C. 2000. Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. *J. Bacteriol.* 182:4310-4318.
- Pellock, B. J., Teplitski, M., Boinay, R. P., Bauer, W. D., and Walker, G. C. 2002. A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *J. Bacteriol.* 184:5067-5076.
- Peoples, O. P., and Sinskey, A. J. 1989. Poly- β -hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding β -ketothiolase and acetoacetyl-CoA reductase. *J. Biol. Chem.* 264:15293-15297.
- Perret, X., Staehelin, C., and Broughton, W. 2000. Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* 64:180-201.
- Reed, J. W., Glazebrook, J., and Walker, G. C. 1991. The *exoR* gene of *Rhizobium meliloti* affects RNA levels of other *exo* genes but lacks homology to known transcriptional regulators. *J. Bacteriol.* 173:3789-3794.
- Reuhs, B. L., Carlson, R. W., and Kim, J. S. 1993. *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-D-manno-2-octulosonic acid-containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. *J. Bacteriol.* 175:3570-3580.
- Reuhs, B. L., Geller, D. P., Kim, J. S., Fox, J. E., Kolli, V. S. K., and Pueppke, S. G. 1998. *Sinorhizobium fredii* and *Sinorhizobium meliloti* produce structurally conserved lipopolysaccharides and strain-specific K antigens. *Appl. Environ. Microbiol.* 64:4930-4938.
- Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145:69-73.
- Scharf, B., and Schmitt, R. 2002. Sensory transduction to the flagellar motor of *Sinorhizobium meliloti*. *J. Mol. Microbiol. Biotechnol.* 4:183-186.
- Schultz, M., and Kondorosi, A. 1998. Regulation of symbiotic root nodule development. *Annu. Rev. Genet.* 32:33-57.
- Sola-Landa, A., Pizarro-Cerda, J., Grillo, M. J., Moreno, E., Moriyon, I., Blasco, J. M., Gorvel, J. P., and Lopez-Goni, I. 1998. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. *Mol. Microbiol.* 29:125-138.
- Soutourina, O. A., and Bertin, P. N. 2003. Regulation cascade of flagellar expression in Gram-negative bacteria. *FEMS (Fed. Mol. Microbiol. Soc.) Microbiol. Rev.* 27:505-523.
- Sukdeo, N., and Charles, T. C. 2003. Application of crossover-PCR-mediated deletion-insertion mutagenesis to analysis of the *bdhA-xdhA2-xdhB2* mixed-function operon of *Sinorhizobium meliloti*. *Arch. Microbiol.* 179:301-304.
- Tombolini, R., Povolito, S., Buson, A., Squartini, A., and Nuti, M. P. 1995. Poly- β -hydroxybutyrate (PHB) biosynthetic genes in *Rhizobium meliloti* 41. *Microbiology* 141:2553-2559.
- Trainer, M. A., and Charles, T. C. 2006. The role of PHB metabolism in the symbiosis of rhizobia with legumes. *Appl. Microbiol. Biotechnol.* 71:377-386.
- Tsolis, R. M. 2002. Comparative genome analysis of the alpha-proteobacteria: Relationships between plant and animal pathogens and host specificity. *Proc. Natl. Acad. Sci. U.S.A.* 99:12503-12505.
- Ulrich, L. E., Koonin, E. V., and Zhulin, I. B. 2005. One-component systems dominate signal transduction in prokaryotes. *Trends Microbiol.* 13:52-56.
- van Rhijn, P., and Vanderleyden, J. 1995. The *Rhizobium*-plant symbiosis. *Microbiol. Rev.* 59:124-142.
- Wang, C., Saldanha, M., Sheng, X., Shellsell, K. J., Walsh, K. T., Sobral, B. W. S., and Charles, T. C. 2007. Roles of poly-3-hydroxybutyrate (PHB) and glycogen in symbiosis of *Sinorhizobium meliloti* with *Medicago sp.* *Microbiology* 153:388-398.
- Wells, D. H., and Long, S. R. 2002. The *Sinorhizobium meliloti* stringent response affects multiple aspects of symbiosis. *Mol. Microbiol.* 43:1115-1127.
- Wells, D. H., Chen, E. J., Fisher, R. F., and Long, S. R. 2007. ExoR is genetically coupled to the ExoS-ChvI two-component system and located in the periplasm of *Sinorhizobium meliloti*. *Mol. Microbiol.* 64:647-664.
- Willis, L. B., and Walker, G. C. 1998. The *phbC* (poly- β -hydroxybutyrate synthase) gene of *Rhizobium (Sinorhizobium) meliloti* and characterization of *phbC* mutants. *Can. J. Microbiol.* 44:554-564.
- Wood, S. M., and Newcomb, W. 1988. Nodule morphogenesis: The early infection of alfalfa (*Medicago sativa*) root hairs by *Rhizobium meliloti*. *Can. J. Bot.* 67:3108-3122.
- Yao, S. Y., Luo, L., Har, K. J., Becker, A., Ruberg, S., Yu, G. Q., Zhu, J. B., and Cheng, H. P. 2004. *Sinorhizobium meliloti* ExoR and ExoS proteins regulate both succinoglycan and flagellum production. *J. Bacteriol.* 186:6042-6049.
- Yuan, Z. C., Liu, P., Saenkham, P., Kerr, K., and Nester, E. W. 2008. Transcriptome profiling and functional analysis of *Agrobacterium tumefaciens* reveals a general conserved response to acidic conditions (pH 5.5) and a complex acid-mediated signaling involved in *Agrobacterium*-plant interactions. *J. Bacteriol.* 190:494-507.

AUTHOR-RECOMMENDED INTERNET RESOURCE

RhizoBase website: genome.kazusa.or.jp/rhizobase