Identification of a Hydroxyproline Transport System in the Legume Endosymbiont *Sinorhizobium meliloti*

Allyson M. MacLean, Catharine E. White, Jane E. Fowler, and Turlough M. Finan

Center for Environmental Genomics, Department of Biology, McMaster University, Hamilton, Ontario, Canada

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Hydroxyproline-rich proteins in plants offer a source of carbon and nitrogen to soil-dwelling microorganisms in the form of root exudates and decaying organic matter. This report describes an ABC-type transport system dedicated to the uptake of hydroxyproline in the legume endosymbiont *Sinorhizobium meliloti*. We have designated genes involved in hydroxyproline metabolism as *hyp* genes and show that an *S. meliloti* strain lacking putative transport genes ($\Delta hypMNPQ$) is unable to grow with or transport *trans*-4-hydroxy-L-proline when this compound is available as a sole source of carbon. Expression of *hypM* is upregulated in the presence of *trans*-4-hydroxy-L-proline and *cis*-4-hydroxy-D-proline, as modulated by a repressor (*HypR*) of the GntR/FadR subfamily. Although alfalfa root nodules contain hydroxyproline-rich proteins, we demonstrate that the transport system is not highly expressed in nodules, suggesting that bacteroids are not exposed to high levels of free hydroxyproline in planta. In addition to *hypMNPQ*, we report that *S. meliloti* encodes a second independent mechanism that enables transport of *trans*-4-hydroxy-L-proline. This secondary transport mechanism is induced in proline-grown cells and likely entails a system involved in L-proline uptake. This study represents the first genetic description of a prokaryotic hydroxyproline transport system, and the ability to metabolize hydroxyproline may contribute significantly toward the ecological success of plant-associated bacteria such as the rhizobia.

Members of the order *Rhizobiales* (including *Sinorhizobium*, *Mesorhizobium*, *Rhizobium*, and *Bradyrhizobium* spp.) participate in a symbiotic partnership with plant species within the legume family. The soil-dwelling and free-living bacteria are attracted by plant-derived flavonoids and isoflavonoids, which induce a set of bacterial genes (*nod*) involved in the synthesis of lipochitin oligosaccharides referred to as Nod factors (Cooper 2007). Plant recognition of symbiotically relevant Nod factors triggers root hair deformation, cell division, and the production of an infection thread that is necessary for the invasion of the host plant with the endosymbiont (Geurts et al. 2005). These events culminate in the development of root-borne nodules, which house the nitrogen-fixing bacteria. The plant offers the rhizobia a source of carbon in the form of reduced photosynthate in exchange for a supply of fixed nitrogen.

Hydroxyproline (primarily in the form of *trans*-4-hydroxy-L-proline) commonly occurs in plant tissues in the form of hydroxyproline-containing proteins, including hydroxyproline-

rich glycoproteins (HRGP), that are a component of plant cell walls and contribute to a structural support mechanism (Khashimova 2003). HRGP consist of at least three related groups of glycosylated and hydroxyproline-containing proteins, including extensins, arabinogalactan-proteins, and proline- or hydroxyproline-rich proteins. Hydroxyproline has been detected in the root nodules of leguminous plants (Benhamou et al. 1991; Cassab et al. 1985; Frueauf et al. 2000), and hydroxyproline-rich extensin has been reported as the predominant structural protein of root cell walls and nodules of *Medicago truncatula* (Frueauf et al. 2000). A subclass of extensins (referred to as root nodule extensins) that are specific to legumes and are expressed in both uninfected root cells and nodules have also been reported (Rathbun et al. 2002). These hydroxyproline-rich extensins represent a significant component of the infection thread lumen in pea root hairs, and are secreted during even the earliest stages of rhizobial infection in *Vicia hirsuta* (Rae et al. 1991, 1992; Rathbun et al. 2002).

The prevalence of hydroxyproline in plant tissue and secretion of hydroxyproline-rich proteins into the rhizosphere offer a potential source of nitrogen and carbon to plant-associated microorganisms (Knee et al. 2001). Additionally, hydroxyproline has been detected in soil samples, humic acids, and decaying leaf litter (Griffith et al. 1976; Lahdesmaki and Phspanen 1989; Morita and Sowden 1981; Sowden et al. 1976). Hydroxyproline catabolism has been documented in several species of soil-dwelling bacteria, including *Pseudomonas putida* (Adams 1973; Gryder and Adams 1969; Jayaraman and Radhakrishnan 1965; Manoharan 1980; Thacker 1969). In this species, hydroxyproline metabolic enzymes and a dedicated uptake system are induced in cells grown in the presence of this compound, and these genes are likely regulated as a single transcriptional unit or share a common regulator (Gryder and Adams 1969, 1970). Hydroxyproline uptake kinetics and growth experiments with *P. putida* indicate that the affinity of the transport system for *trans*-4-hydroxy-L-proline is much greater than that for *cis*-4-hydroxy-D-proline (Gryder and Adams 1969, 1970). Data reported in previous studies, including competition experiments between L-proline and *trans*-4-hydroxy-L-proline, imply the possibility of a shared hydroxyproline or proline uptake system in *Pseudomonas* spp. (Gryder and Adams 1969, 1970; Manoharan 1980). However, the genes encoding a hydroxyproline-specific transport system have yet to be identified in any species.

In this study, we describe an ABC-type transport system (encoded by *hypMNPQ*) that is essential for growth with and transport of hydroxyproline in *Sinorhizobium meliloti*. We establish that the *hypMNPQ* cluster is hydroxyproline inducible and is a high-affinity and specific transporter for hydroxyproline. Although alfalfa nodules contain hydroxyproline-rich proteins such as extensin, the *S. meliloti* hydroxyproline up-

Corresponding author: T. M. Finan; Telephone: +1.905.525.9140 ext. 22932; Fax: +1.905.628.6033; E-mail: finan@mcmaster.ca

take system is not highly expressed in either young or senescent root nodules. This suggests that hydroxyproline does not play a major role in bacteroid metabolism.

RESULTS

Identification of a gene cluster essential for growth with hydroxyproline.

S. meliloti RmF909 carries a large-scale deletion within the pSymB megaplasmid (Charles and Finan 1991) that renders the strain unable to metabolize both *trans*-4-hydroxy-L-proline and *cis*-4-hydroxy-D-proline, whereas wild-type *S. meliloti* is able to utilize both of these compounds as sole carbon and nitrogen sources. To identify the genes required for hydroxyproline catabolism, we screened the *S. meliloti* pLAFR1 clone bank for a cosmid that was able to complement the hydroxyproline-

minus growth phenotype of this strain. We sequenced cosmid DNA originating from six independent RmF909-derivative strains of *S. meliloti* in which the inability of the parental strain to metabolize hydroxyproline was complemented by a cosmid clone. Sequencing reactions performed upon each cosmid yielded identical *S. meliloti* DNA sequences (255,129 to 275,255 nucleotides; pSymB), indicating that the cosmids were siblings. The approximately 20-kb region of *S. meliloti* DNA contained within the cosmid (pTH2439) includes genes annotated to encode a single ABC-type transport system positioned upstream of several metabolic genes (Fig. 1). We have designated the genes involved in hydroxyproline catabolism with the prefix *hyp* and the predicted transport genes as *hypMNPQ* (*smb20263-smb20266*). To determine whether this putative uptake system is required for hydroxyproline transport, an *S. meliloti* strain was constructed carrying a 1.7-kb in-

Fig. 1. Hydroxyproline transport in *Sinorhizobium meliloti*. **A**, Structure of two hydroxyproline isomers and L-proline. *S. meliloti* can use *trans*-4-hydroxy-L-proline and *cis*-4-hydroxy-D-proline as a sole source of carbon and nitrogen. **B**, Schematic diagram of hydroxyproline transport genes encoded on the pSymB megaplasmid. *hypMNPQ* (shaded gray) encodes an ABC-type transport system that is required for efficient uptake of *trans*-4-hydroxy-L-proline in *S. meliloti* (this study). The inverted triangle indicates the insertion of an antibiotic resistance cassette within the GntR-type regulator gene (*hypR*). Gene annotation as follows: *smb20257*, adenylate cyclase; *hypR* (*smb20258*), GntR-type transcriptional regulator; *smb20259*, dihydrodipicolinate synthase; *smb20261*, malate dehydrogenase; *smb20262*, semialdehyde dehydrogenase; *hypM* (*smb20263*), periplasmic solute binding protein; *hypN* (*smb20264*) and *hypP* (*smb20265*), permease protein; *hypQ* (*smb20266*), ATP-binding protein; *smb20267*, D-amino acid oxidase; *smb20268* and *smb20270*, proline racemase; *smb20269* and *smb20271*, conserved hypothetical protein. **C**, Schematic diagram of genetic constructs used in this study.

frame deletion (Fig. 1C, RmP1114) which disrupted *hypM* and *hypQ* and removed the *hypN* and *hypP* genes.

Wild-type *S. meliloti* RmP110 grows readily on *trans*-4-hydroxy-L-proline with a growth rate constant (μ) of 0.20 h⁻¹ and mean generation time (g) of 3.4 h when this compound is

available as a sole carbon source (for *cis*-4-hydroxy-D-proline; $\mu = 0.22$ hour⁻¹ and $g = 3.2$ h) (Fig. 2). Although *S. meliloti* RmP1114 grows at a rate that is comparable with the wild-type strain RmP110 when subcultured with succinate (Fig. 2A) or glycerol (data not shown) as carbon sources, the mutant strain exhibits a considerable lag in growth when either *trans*-4-hydroxy-L-proline or *cis*-4-hydroxy-D-proline are available as a sole source of carbon (Fig. 2B and C). This phenotype was complemented when the genes encoding the transport system (*hypMNPQ*) were provided to RmP1114 in trans (pTH2513), indicating that the putative uptake system is required for wild-type growth with hydroxyproline.

The *S. meliloti* deletion strain RmF909 was unable to utilize either *trans*-4-hydroxy-L-proline or *cis*-4-hydroxy-D-proline as a source of carbon; however, growth was restored upon introduction of the cosmid pTH2439 (Fig. 2B and C). The disparate phenotypes exhibited by RmP1114 (slow growth) and RmF909 (no growth) cultured in hydroxyproline may be attributed to the loss of putative catabolic genes adjacent to *hypMNPQ* in RmF909 (and present in RmP1114). We note that strains carrying the cosmid clone pTH2439 exhibited a markedly reduced growth lag compared with the wild-type RmP110 subcultured into either of the hydroxyproline-containing media (Fig. 2B and C); this effect may be attributed to an increased copy number of hydroxyproline catabolic and uptake genes located on the cosmid.

Expression of *hypM* is induced in the presence of hydroxyproline and is regulated by the GntR transcriptional regulator HypR.

We examined the expression of *hypM* (encoding a putative periplasmic solute binding protein) (Fig. 1B) in strain RmFL7003 carrying an integrated *hypM::gfp⁺/lacZ* reporter gene fusion (Cowie et al. 2006). Based on reporter enzyme assays, expression of *hypM* was induced more than fivefold in the presence of *trans*-4-hydroxy-L-proline and *cis*-4-hydroxy-D-proline compared with growth on succinate (Table 1). A gene encoding a putative transcriptional regulator belonging to the GntR family of proteins (*hypR*) is located approximately 5 kb upstream of the hydroxyproline-inducible ABC-type transport system (Fig. 1B). To determine whether HypR was involved in regulating expression of the uptake system, we constructed a *hypR* insertion mutant in which an Ω cassette was introduced 345 bp downstream of the predicted translational start site. Expression of *hypM::gfp⁺/lacZ* was increased at least 20-fold in the *hypR::\Omega* strain RmP1838 (RmFL7003 *hypR::\Omega*) compared with RmFL7003 when grown with succinate or glucose as carbon sources (Table 1). In addition, *hypM::gfp⁺/lacZ* expression in RmP1838 was no longer hydroxyproline-inducible; gene expression occurred at a comparable high level under all growth conditions tested. The *hypR* gene is located upstream of a putative dihydrodipicolinate synthase gene

Fig. 2. Growth of *Sinorhizobium meliloti* strains subcultured into M9-minimal medium with **A**, 10 mM succinate; **B**, 5 mM *trans*-4-hydroxy-L-proline; or **C**, 5 mM *cis*-4-hydroxy-D-proline as sole carbon sources. Inoculum was from washed cells grown in Luria-Bertani broth supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ and cultures were incubated with shaking at 30°C for 48 h; growth of each strain was assayed in triplicate cultures and shown is the average optical density at 600 nm (OD₆₀₀) with error bars representing standard deviation. The growth rate constant (μ) and mean generation time (g) for wild-type *S. meliloti* in *trans*-4-hydroxy-L-proline ($\mu = 0.20$ h⁻¹; $g = 3.4$ h) and *cis*-4-hydroxy-D-proline ($\mu = 0.22$ h⁻¹; $g = 3.2$ h) were calculated during exponential growth (corresponding to $t_0 = 10.2$ and $t = 18.1$ h) using the following formulae: $\mu = [(\log_{10} N - \log_{10} N_0) \times 2.303]/(t - t_0)$ and $g = (\log_{10} N_t - \log_{10} N_0)/\log_{10} 2$, where N equals the number of cells.

Table 1. Expression of *hypM::gfp⁺/lacZ* in *Sinorhizobium meliloti* wild-type and *hypR::\Omega* backgrounds

Growth condition	GFP ⁺ -specific activity (SD) ^a	
	Wild type ^b	<i>hypR::\Omega</i> ^c
Succinate	599 (45)	12,920 (145)
Glucose	292 (113)	18,111 (290)
Glycerol	311 (81)	22,521 (200)
L-proline	914 (101)	16,592 (442)
<i>trans</i> -4-hydroxy-L-proline	4,431 (173)	17,132 (147)
<i>cis</i> -4-hydroxy-D-proline	5,439 (117)	15,322 (95)

^a GFP = green fluorescent protein and SD = standard deviation.

^b Wild type, RmP110 *hypM::gfp⁺/lacZ*.

^c RmP1838, RmP110 *hypM::gfp⁺/lacZ*, *hypR::\Omega*.

(*smb20259*) and it is formally possible that the insertion of a cassette within *hypR* might exert polar effects upon the expression of this gene. However, the intergenic region between these genes is sufficiently large (121 bp) to encode an independent promoter associated with *smb20259*, and it is unlikely that the regulatory phenotype exhibited by RmP1838 is the result of a polar effect upon the expression of a putative dihydrodipicolinate synthase gene. Thus, our results indicate that *hypR* encodes a product that represses expression of *hypM* and that this repression is relieved by hydroxyproline.

Identification of the *hypM* transcriptional start site.

Primer extension reactions were performed using an oligonucleotide complementary to sequence within the coding region of *hypM* (+27 to +51 nucleotides downstream of the annotated translation start site). One major extension product was obtained using RNA extracted from wild-type (RmP110) cells grown with *trans*-4-hydroxy-L-proline (induced), indicating a transcriptional start site located 38 nucleotides upstream of the predicted start codon associated with *hypM* (Fig. 3A). The absence of a comparable extension product in reactions using RNA derived from glycerol-grown cells (uninduced) is consistent with the hydroxyproline-inducible expression of this gene. Examination of the inferred -35 and -10 hexanucleotide regions associated with this transcriptional start site reveals similarity to the consensus promoter sequence previously described in *S. meliloti* (MacLellan et al. 2006) (Fig. 3C). In fact, the experimentally verified promoter associated with *hypM* and identified in this work was previously predicted based on a search of the *S. meliloti* genome for sequences matching the consensus sequence (MacLellan et al. 2006).

Hydroxyproline-inducible uptake requires *hypMNPQ*.

To directly examine hydroxyproline uptake, we used ³H-radiolabeled *trans*-4-hydroxy-L-proline. Wild-type cells grown

in the presence of *trans*-4-hydroxy-L-proline as a sole nitrogen and carbon source demonstrated the greatest rate of uptake at 10.7 nmols/min/mg of protein (Fig. 4A). In contrast, cells grown with succinate (data not shown) or glycerol (uptake rate of 1.2 nmols/min/mg of protein for the wild type) were unable to efficiently transport hydroxyproline, indicating that the system involved is hydroxyproline inducible. The strain *S. meliloti* RmP1114 (Δ *hypMNPQ*) was unable to transport *trans*-4-hydroxy-L-proline when grown in M9-glycerol in the presence of hydroxyproline (<0.5 nmols/min/mg of protein), and uptake was restored to wild-type levels upon introduction of the *hypMNPQ* genes in trans on the plasmid pTH2513 (Fig. 4B). Thus, *hypMNPQ* is required for the uptake of this compound in hydroxyproline-grown cells (Fig. 4B). We examined the kinetics of uptake in hydroxyproline-grown *S. meliloti* RmP110, and have determined the K_m for *trans*-4-hydroxy-L-proline to be $0.56 \pm 0.28 \mu\text{M}$, and a V_{max} of 6.8 ± 2.1 nmols/min/mg protein. These values indicate that *hypMNPQ* has a high affinity for *trans*-4-hydroxy-L-proline and is comparable to other ABC-type amino acid transport systems (Hosie et al. 2002).

Experiments performed using unlabeled competitor versus labeled *trans*-4-hydroxy-L-proline indicate that both *cis*-4-hydroxy-D-proline and L-proline compete with *trans*-4-hydroxy-L-proline for binding or uptake in *S. meliloti* hydroxyproline-grown cells (Table 2). Proline reduced the uptake of labeled *trans*-4-hydroxy-L-proline as effectively as unlabeled *trans*-4-hydroxy-L-proline; in contrast, the *cis* epimer was less effective as a competitor. Similar findings have been reported in *P. putida*, where L-proline also strongly reduces the transport of *trans*-4-hydroxy-L-proline (Gryder and Adams 1970). In contrast, L-ornithine and pyrroline-2-carboxylic acid do not compete for hydroxyproline uptake, because the presence of these solutes did not substantially inhibit *trans*-4-hydroxy-L-proline transport (Table 2).

Fig. 3. Analysis of the *hypM* promoter. **A**, Primer extension reactions were performed using RNA isolated from *Sinorhizobium meliloti* wild-type strain RmP110 grown in M9-minimal medium with glycerol (lane 1) or *trans*-4-hydroxy-L-proline (lane 2) as a source of carbon (*trans*-4-hydroxy-L-proline was also added as a sole source of nitrogen). Sequencing reactions are included on the left, with the corresponding nucleotides indicated above each lane, and the extension product is indicated with an arrow. **B**, The promoter region associated with *hypM*. The inferred -35 and -10 hexanucleotide regions are underlined and the transcriptional start site is indicated with an arrow bent in the direction of transcription. The translational start site of *hypM* is underlined. **C**, The promoter of *hypM* is aligned with six promoters previously identified in *S. meliloti* (MacLellan et al. 2006).

Hydroxyproline uptake in *S. meliloti* cells grown with L-proline.

The effectiveness of L-proline as a competitor against *trans*-4-hydroxy-L-proline in uptake assays raised the possibility that proline and hydroxyproline may share a common transport

system, as has been suggested in *P. aeruginosa* (Manoharan 1980). To investigate whether proline induces expression of a hydroxyproline uptake mechanism, transport assays were performed on cells cultured in growth medium containing L-proline as a sole source of carbon and nitrogen (Fig. 4C). Proline-grown *S. meliloti* wild-type RmP110 cells transported labeled *trans*-4-hydroxy-L-proline at approximately one-half the rate observed in *trans*-4-hydroxy-L-proline-grown cells (3.2 versus 8.2 nmols/min/mg of protein, respectively). This rate of uptake is significantly greater than that exhibited by uninduced control cells grown in M9-glycerol (<1 nmol/min/mg of protein). We also examined uptake of *trans*-4-hydroxy-L-proline in *S. meliloti* RmP1114 (Δ hypMNPQ) grown in the presence of *trans*-4-hydroxy-L-proline or L-proline. Although RmP1114 did not transport labeled *trans*-4-hydroxy-L-proline when grown in M9-glycerol plus *trans*-4-hydroxy-L-proline, proline-grown RmP1114 did transport *trans*-4-hydroxy-L-proline at a rate (2.6 nmols/min/mg of protein) comparable with that observed in proline-grown wild-type cells. These results are consistent with the presence of a second mechanism for hydroxyproline uptake into *S. meliloti* that is induced by L-proline and, therefore, is likely to entail a proline transport system.

The hydroxyproline uptake system is not highly expressed in alfalfa root nodules.

Expression of *hypM* was monitored in alfalfa nodules through the use of a transcriptional fusion to the reporter gene *gusA* in *S. meliloti* RmP1886 (Fig. 1C). As a positive control, alfalfa seedlings were inoculated with strain RmFL5810,

Fig. 4. Uptake of labeled *trans*-4-hydroxy-L-proline (nmols/mg of protein) in *Sinorhizobium meliloti*. **A**, Hydroxyproline uptake was observed in *S. meliloti* wild-type strain RmP110 grown in M9-minimal medium with carbon sources as indicated. **B**, Hydroxyproline uptake was observed in *S. meliloti* strains grown in M9-minimal medium with 0.5% glycerol + 5 mM *trans*-4-hydroxy-L-proline. **C**, Hydroxyproline uptake was observed in *S. meliloti* wild-type RmP110 and RmP1114 (Δ hypMNPQ) strains grown in M9-minimal medium with carbon and nitrogen sources as indicated. Uptake was not assayed in RmP1114 grown in M9-glycerol or M9-*trans*-4-hydroxy-L-proline. Assays were performed using 10 μ M *trans*-4-hydroxy-L-proline [³H(G)]. In all assays, ammonium chloride was present in growth medium as a source of nitrogen unless otherwise indicated. Trans-hyp: *trans*-4-hydroxy-L-proline. Sole C/N: *trans*-4-hydroxy-L-proline or L-proline is present as a sole source of carbon and nitrogen.

Table 2. Effect of competitors on *trans*-4-hydroxy-L-proline uptake

Type of competitor	Inhibition of <i>trans</i> -4-hydroxy-L-proline uptake in presence of competitor (%) ^a		
	10 μ M	50 μ M	100 μ M
<i>trans</i> -4-hydroxy-L-proline	58 (5)	85 (6)	91 (<1)
<i>cis</i> -4-hydroxy-D-proline	18 (3)	57 (3)	74 (1)
L-proline	60 (6)	88 (4)	95 (1)
L-ornithine	5 (2)	7 (1)	14 (<1)
Pyrroline-2-carboxylic acid	0	0	0

^a Results are reported as percentage of inhibition (standard deviation) of labeled *trans*-4-hydroxy-L-proline observed in the presence of unlabeled competitor. Uptake in the absence of assayed competitor averaged 6.4 nmols/min/mg ($\pm$ 1.6) in seven independent experiments and assays were performed using hydroxyproline-grown cells and 10 μ M-labeled substrate.

Table 3. Expression of *hypM::gusA* in young and senescent alfalfa root nodules

Strain	Gene fusion	Days post inoculation	β -Glucuronidase activity (SD) ^a
RmP110	None	42	5 (<1)
		84	7 (<1)
		30	41 (1)
		42	28 (1)
		52	27 (<1)
RmP1886	<i>hypM::gusA</i>	66	30 (<1)
		84	28 (<1)
		42	53 (3)
		84	68 (2)
		42	7 (<1)
RmFL5810	<i>minD::gusA</i>	84	8 (<1)
RmP778 ^b	<i>smb20568::lacZ</i>	42	
		84	

^a Results are representative of two independent experiments using nodules obtained from a minimum of 14 alfalfa plants inoculated per strain; β -glucuronidase activity (SD = standard deviation) is the average of assays performed in triplicate.

^b RmP778 contains a *gusA* fusion in the opposite orientation with respect to *P*₂₀₅₆₈ and serves as a negative control for endogenous β -glucuronidase activity.

which contains a *gusA* fusion with *minD*; this gene is known to be expressed in nodules (Cheng et al. 2007). As negative controls, we used the wild-type strain RmP110 and a strain (RmP778) in which *gusA* is integrated into the *S. meliloti* genome in the antisense orientation to a promoter unrelated to hydroxyproline metabolism. RmP778 was included to reflect the level of β -glucuronidase activity associated with a single copy of the reporter gene in the *S. meliloti* genome.

β -Glucuronidase activity was measured in nodule extracts obtained from alfalfa plants 30 to 84 days post inoculation (dpi) (Table 3). Our results indicate a level of *hypM* expression that is approximately fourfold greater than that observed in nodules inoculated with the negative controls RmP110 (no *gusA* fusion) or RmP778 (*gusA* in antisense orientation to *smb20568*). Nodules harvested from plants inoculated with the positive control (*minD::gusA*) exhibited a higher level of expression (at least sevenfold greater than the controls), consistent with previous data obtained regarding expression of this gene in nodules (Cheng et al. 2007). To investigate whether expression was localized to particular regions of the nodule (Timmers et al. 2000), we stained longitudinal sections of 30- and 66-dpi nodules for β -glucuronidase activity (Fig. 5). β -Glucuronidase activity was observed primarily within the distal and central portions of the nodules, corresponding to the infection or prefixing zone II and nitrogen-fixing zone III.

DISCUSSION

The legume endosymbiont *S. meliloti* is able to utilize hydroxyproline as a carbon and nitrogen source. To identify loci involved in hydroxyproline metabolism and transport, we isolated a cosmid clone that was able to complement an *S. meliloti* deletion strain for growth at the expense of hydroxyproline. Sequencing reactions revealed that the cosmid contained approximately 20 kb of *S. meliloti* DNA (255,129 to 275,255 nucleotides; pSymB), including a single ABC-type transport system encoded by genes *hypM* (periplasmic solute binding protein), *hypN* and *hypP* (transporter permease proteins), as well as *hypQ* (ATP-binding protein).

We have presented data to show that *S. meliloti hypMNPQ* encodes an ABC-type transport system for the high-affinity uptake of *trans*-4-hydroxy-L-proline. Mutant strain RmP1114 carrying a deletion spanning the *hypMNPQ* genes failed to transport *trans*-4-hydroxy-L-proline and grew slowly with this compound as a carbon and nitrogen source (Figs. 2 and 4). The growth and transport phenotypes exhibited by RmP1114 were complemented when *hypMNPQ* was supplied in trans; thus, the in-frame deletion only disrupted expression of the uptake system and not expression of any downstream metabolic genes (Fig. 1B). In contrast, *S. meliloti* RmF909 carries a large deletion that encompasses *hypMNPQ* and adjacent metabolic genes. Our data indicate that these genes encode products relevant to the catabolism of *trans*-4-hydroxy-L-proline (C. E. White and T. M. Finan, unpublished data), and the complete lack of growth exhibited by RmF909 at the expense of this compound is consistent with a role in hydroxyproline metabolism.

Expression of *hypM* and, we infer, the complete *hypMNPQ* gene cluster, was induced by growth in the presence of either *trans*-4-hydroxy-L-proline or *cis*-4-hydroxy-D-proline (Table 1). Our uptake assays indicated that *cis*-4-hydroxy-D-proline is a less effective competitor of labeled *trans*-4-hydroxy-L-proline than either L-proline or *trans*-4-hydroxy-L-proline itself. In *P. putida*, the affinity of the transport system for *trans*-4-hydroxy-L-proline is much greater than that for *cis*-4-hydroxy-D-proline (Gryder and Adams 1969, 1970). Likewise, *P. aeruginosa* PAO is impermeable to *cis*-4-hydroxy-D-proline and cannot grow at the expense of this compound, although the strain

readily transports and metabolizes *trans*-4-hydroxy-L-proline (Manoharan 1980). Although we did not directly examine uptake of *cis*-4-hydroxy-D-proline, our data are consistent with the *S. meliloti* hydroxyproline transport system having a greater affinity for *trans*-4-hydroxy-L-proline than the *cis* epimer. Saprophytic or plant-associated bacteria such as *S. meliloti* and *Pseudomonas* spp. are more likely to encounter *trans*-4-hydroxy-L-proline in a natural habitat because this isomer is the predominate form of hydroxyproline present in plant (and animal) tissue (Adams and Frank 1980). Accordingly, the evolution of an uptake system with a greater affinity for *trans*-4-hydroxy-L-proline than *cis*-4-hydroxy-D-proline may reflect the relative abundance of each stereoisomer in the habitats occupied by these soil-dwelling species.

The finding that growth of *S. meliloti* with L-proline enables immediate and efficient uptake of *trans*-4-hydroxy-L-proline in both the wild-type and the Δ *hypMNPQ* strain RmP1114 (Fig. 4C) suggests that an L-proline uptake system can also transport hydroxyproline. The apparent inability of this unidentified uptake system to rescue growth of RmP1114 in M9-minimal medium with hydroxyproline, as well as the lack of *trans*-4-hydroxy-L-proline uptake observed in succinate- and glycerol-grown cells, emphasizes the requirement for induction via proline metabolism. In *S. meliloti*, proline is known to be transported into the cell by the ABC-type transport system Hut; however, expression of this system is not induced by proline (Boncompagni et al. 2000). The osmoprotectant proline betaine is transported by the ABC-type transporter Prb in *S. meliloti*, yet expression of this system is not induced by proline and there is no evidence to indicate that this system imports proline (Alloing et al. 2006). The *Rhizobium leguminosarum* ABC-type transport systems Bra and Aap are amino acid permeases with broad and overlapping substrate specificities, and there is evidence that these systems transport proline (Hosie et al. 2002; Walshaw and Poole 1996). *S. meliloti* encodes several transport systems with sequence similarity to Bra, and it is possible that one or more of these may mediate hydroxyproline uptake in the *hypMNPQ* mutant strain RmP1114. In enteric bacteria such as *Escherichia coli*, a proline permease gene (*putP*) is divergently transcribed with respect to the proline dehydrogenase (*putA*) (Mogi et al. 1986); however, *putA* in *S. meliloti* is not associated with a permease (Soto et al. 2000). Consequently, the identity of the proline-induced transport system remains unresolved.

Alfalfa and other legume nodules contain high amounts of hydroxyproline-rich proteins; however, it is unclear to what degree nodule-inhabiting bacteroids encounter hydroxyproline in a form that is available for metabolism. At all stages tested (from 30 dpi to senescence), nodules induced by a strain containing an *hypM::gusA* fusion yielded an average fourfold greater reporter enzyme activity than that observed in the negative controls (Table 3). Nodule sections were stained for β -glucuronidase activity and we observed uniform staining throughout nodules from plants inoculated with RmP1886 (*hypM::gusA*). Particularly, the distal and central regions of the nodule (prefixing zone II and nitrogen-fixing zone III) showed evidence of gene expression in both 4- and 9-week-old nodules (Fig. 5). In summary, our results indicate that the expression of *hypM* is a fraction of that exhibited by moderately expressed *minD* (60%) and highly expressed *nifH* (16%) (data not shown). In addition, the hydroxyproline uptake gene cluster is not required for nodulation or nitrogen fixation (data not shown). Accordingly, it is unlikely that the hydroxyproline uptake genes play an essential role in the plant-microbe symbiosis.

Identification of the transcriptional start site associated with *hypM* (Fig. 3A) reveals a hydroxyproline-inducible promoter exhibiting sequence similarity to that of consensus promoters

previously described in *S. meliloti* and *R. etli* (MacLellan et al. 2006; Ramirez-Romero et al. 2006). In fact, the promoter identified upstream of *hypM* was accurately predicted by MacLellan and associates, thus validating the promoter predic-

tion methodology employed by that study. The consensus sequence identified in *R. etli* is recognized by SigA (σ^{70}) (Ramirez-Romero et al. 2006). By extension, it may be expected that the promoter element upstream of *hypM* is simi-

Fig. 5. Histochemical staining for β -glucuronidase activity in alfalfa nodules. **A**, Four-week-old nodule inoculated with RmP1886 (*hypM::gusA*) shows evidence of staining throughout a longitudinal section, including the prefixing zone (zone II; black asterisk) and nitrogen-fixing zone (zone III; white star). **B**, Four-week-old nodule inoculated with RmP778 (*gusA* in antisense orientation) shows little evidence of staining. **C**, Nine-week-old nodule inoculated with RmP1886. Slight staining was occasionally observed in the saprophytic zone of the senescent nodule (zone V; black star). **D**, Nine-week-old nodule inoculated with RmP778 shows no evidence of staining.

larly recognized by the *S. meliloti* housekeeping σ factor RpoD; however, expression of this gene is also repressed by the GntR regulator HypR (discussed below). The observation that *hypM* expression is regulated via repression (as opposed to activation) may in part underlie the sequence similarity between the *hypM* and consensus promoter sequences, because a high level of *hypM* expression under permissive conditions is likely dependent upon RNA polymerase σ^{70} recognition of the promoter element in the absence of any transcriptional activator. The promoter prediction methodology proposed by MacLellan and associates (2006) was established using (primarily) factor-independent promoters, and it is possible that such predictions may also be biased toward a subset of promoters subject to negative regulation.

HypR is a member of the GntR-type family of transcriptional regulators that was initially described by Haydon and Guest (1991). This comprises a group of proteins that participates in the regulation of a diverse range of processes, including sporulation (Rigali et al. 2006), plasmid maintenance and transfer (Kataoka et al. 1994; Lee et al. 2003), the acquisition of phosphorus (Gebhard and Cook 2008), and utilization of sugars (Franco et al. 2006), aromatic compounds (Gerischer 2002; Morawski et al. 2000), organic acids (Georgi et al. 2008), amino acids (Wiethaus et al. 2008), and fatty acids (Fujita et al. 2007). GntR proteins have been classified into one of six subfamilies (FadR, MocR, HutC, YtrA, PlmA, and AraR) based on sequence divergence between members at the C-terminal effector-binding and oligomerization domains (Franco et al. 2006; Lee et al. 2003; Rigali et al. 2002). As a means of determining which subfamily the hydroxyproline regulator belongs to, we constructed a phylogenetic tree using the full-length amino acid sequences of proteins representing members of each subfamily (Fig. 6A). Based upon these analyses, the regulator encoded by *hypR* clusters within the FadR subfamily of GntR proteins. Consistent with the classification of HypR, this subfamily comprises many members involved in the regulation of amino acid metabolic genes in both gram-positive and -negative species (Rigali et al. 2002).

The FadR subfamily of proteins recognizes binding sites established upon a (tnTGn₃ACna) consensus motif (Rigali et al. 2002). We identified a possible regulator binding site (5' TTTTGTATAC₃) that approximates the FadR consensus motif, upstream of the *hypM* promoter. An examination of adjacent metabolic genes identified additional putative binding sites exhibiting marked sequence similarity with the *hypM* motif (Fig. 6B). Further studies are required to determine the role of these sites and the putative metabolic genes in hydroxyproline catabolism.

We recently reported the identification of a TRAP-T uptake system whose expression was induced by hydroxyproline (Mauchline et al. 2006). Disruption of the genes encoding the TRAP-T system does not affect cell growth upon hydroxyproline (data not shown) and we conclude that the TRAP-T system is not essential for hydroxyproline uptake. It is possible that expression of the system is induced by a metabolite generated during hydroxyproline catabolism; however, this was not investigated further.

This report represents the first genetic identification of a hydroxyproline uptake system in prokaryotes; however, it is likely that other soil-dwelling and plant-associated bacteria encode hydroxyproline uptake and catabolic genes. The ability to catabolize hydroxyproline may be particularly relevant to plant-associated bacteria such as the rhizobia due to the abundance of hydroxyproline-rich glycoproteins in plant tissue, including the nodules, seeds, and roots of legumes (Cassab et al. 1985; Frueauf et al. 2000; Knee et al. 2001; Rae et al. 1991, 1992; Rathbun et al. 2002; Wisniewski et al. 2000; Wood et al.

1991). Natural processes such as root nodule senescence may even result in an enrichment of hydroxyproline in the surrounding soil. Thus, the ability to catabolize hydroxyproline may confer a selective advantage to the saprophytic bacteria that colonize the rhizosphere, thereby contributing toward the ecological success of these organisms.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

E. coli and *S. meliloti* strains used in this study are described in Table 4. *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth or agar plates incubated at 37°C. *S. meliloti* strains were grown aerobically in LB broth supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBmc) or in M9-minimal medium (Difco Laboratories, Detroit) at 30°C. M9-minimal medium was supplemented with 1.0 mM MgSO₄, 0.25 mM CaCl₂, D-biotin at 1 µg/ml, and CoCl₂ at 10 ng/ml. Carbon sources were added to M9-minimal medium as follows: 10 mM glucose, 10 mM succinate, 0.5% (vol/vol) glycerol, 5 mM L-proline (hydroxyproline-free) (Sigma-Aldrich, St. Louis), 5 mM *trans*-4-hydroxy-L-proline (Sigma-Aldrich), or 5 mM *cis*-4-hydroxy-D-proline (Sigma-Aldrich). When required, antibiotics were added to growth medium at the following concentrations

Fig. 6. A, Phylogenetic tree of GntR family member proteins. The unrooted tree was based on an alignment of the full-length amino acid sequences obtained for each protein. The multiple alignment was performed using ClustalW (Thompson et al. 1994) with manual adjustment of the aligned sequences. Distances between proteins were determined based upon the Jones-Taylor-Thornton matrix (Jones et al. 1992) using PRODIST (Felsenstein 1989). **B**, Alignment of putative HypR binding sites. The translational start site associated with *hypR* and -35 hexanucleotide region upstream of *hypM* are underlined. Nucleotide positions relative to the annotated start codons are indicated on the right, with the exception of *hypM*, in which the position is indicated with respect to the transcriptional start site. Invariant residues are indicated by an asterisk.

for *S. meliloti*: streptomycin (Sm), 200 µg/ml; spectinomycin (Sp), 200 µg/ml; gentamicin (Gm), 60 µg/ml; tetracycline (Tc), 5 µg/ml; and rifampicin (Rf), 20 µg/ml.

Isolation of a pLAFR1 cosmid capable of complementing an Hyp[−] strain of *S. meliloti*.

Spot matings with RmF909 as a recipient strain were performed using the *S. meliloti* pLAFR1 clone bank (Friedman et al. 1982) and transconjugants were plated on M9-minimal media with *trans*-4-hydroxy-L-proline as a sole carbon source and neomycin (Nm) to counter-select the *E. coli* donor strain. Ten Hyp⁺Nm^r *S. meliloti* colonies were patched onto LB agar containing Tc to confirm the presence of the cosmid. Spot matings with six of these Hyp⁺ colonies were performed with *E. coli* recipient strain DH5α to facilitate cosmid DNA extraction. Cosmid DNA was isolated from the six independent *E. coli* strains and DNA sequencing reactions were performed by Mobixlab (McMaster University, Hamilton, Canada) using primers complementary to the cosmid.

Construction of an in-frame deletion of the hydroxyproline uptake system in *S. meliloti*.

An in-frame deletion within the putative hydroxyproline transport system encoded by *hypMNPQ* was generated as follows. A 2,723-bp fragment spanning *hypM* and *hypQ* was polymerase chain reaction (PCR) amplified using primers 5' AAGCGGCCGCAACAACGAACCGATCG 3' and 5' AAGCGGCCGAGGCGAAAATCTGATCG 3' and cloned into the suicide vector pJQ200 uc1 (Quandt and Hynes 1993) via *NotI* to yield pTH2130. This plasmid was then digested with *SalI* to excise a 1,689-bp fragment encompassing *hypNP*, in addition

to portions of *hypM* and *hypQ*. The linearized plasmid was purified from an agarose gel and incubated with T₄ DNA ligase to generate a plasmid carrying the *S. meliloti* sequence minus the approximately 1.7-kb *SalI* fragment (pTH2131). The suicide plasmid pTH2131 was introduced into *S. meliloti* wild-type derivative strain RmP110 via conjugation using the *E. coli* helper strain MT616 (triparental mating). Transconjugants in which plasmid pTH2131 had integrated into the pSymB megaplasmid via homologous recombination were selected by plating onto LB agar supplemented with Sm plus Gm. A single *S. meliloti* colony resistant to Sm and Gm (Sm^rGm^r) was purified (RmP1113) and inoculated into LBmc broth for incubation overnight in the absence of antibiotic selection. Aliquots of the culture were plated upon LB agar plus 5% sucrose to select for cells in which the suicide plasmid (encoding *sacB*) had excised from the genome; sucrose^r colonies were screened for susceptibility to Gm (Gm^s) to confirm loss of the plasmid. The genotypes of 15 sucrose^r/Gm^s colonies were screened via whole-cell PCR for the presence of a 1.7-kb deletion within *hypMNPQ*; of these, 7 colonies yielded PCR products consistent with such a deletion. One of the seven strains was purified and designated as RmP1114. The primers used to identify the deletion mutants via whole-cell PCR correspond to a sequence external to the region cloned into pJQ200.

To provide a means of complementing the in-frame deletion of the ABC-type transport system in trans, plasmid pTH2513 was constructed as follows. A region encompassing the entire uptake system encoded by *hypMNPQ* was amplified by PCR using primers 5' ATTAAGCTTCGTCGCTTACCAGAACATGC 3' and 5' ATTATGCATGCAATTGCAGCCGTGAGG 3' and cloned via *HindIII* and *NsiI* restriction sites into a modified

Table 4. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>Sinorhizobium meliloti</i>		
Rm1021	Sm ^r derivative of wild-type strain SU47	Meade et al. 1982
Rm5000	SU47 <i>rif</i> ^r ; Rf ^r	Finan et al. 1984
RmF909	Rm1021 ΔΩ5085-5047::TnV; Sm ^r Nm ^r	Charles and Finan 1991
RmFL7003	RmP110 <i>hypM</i> :: <i>gfp</i> ⁺ / <i>lacZ</i> via int. of pFL7003 (268,250 to 268,699 nucleotides); Sm ^r Gm ^r	Cowie et al. 2006
RmP110	Rm1021 with wild-type <i>pstC</i> ; Sm ^r	Yuan et al. 2006
RmP319	Rm1021 <i>nifH</i> :: <i>gusA</i> ; Sm ^r Gm ^r	Cheng et al. 2007
RmP778	RmP110 with <i>gusA</i> in the antisense orientation to <i>smb20568</i> via integration of pTH1961; Sm ^r Gm ^r	This study
RmP1113	Integration of pTH2131 into RmP110 (single cross-over); sucrose ^s Sm ^r Gm ^r	This study
RmP1114	RmP110 Δ <i>hypMNPQ</i> (Δ <i>smb20263-smb20266</i>) via excision of pTH2131 to generate in-frame deletion; sucrose ^r Sm ^r Gm ^s	This study
RmP1406	Rm5000 <i>hypR</i> ::Ω (<i>smb20258</i> ::Ω); Rf ^r Sm ^r Sp ^r	This study
RmP1724	RmP110 <i>hypR</i> ::Ω; Sm ^r Sp ^r	This study
RmP1838	RmFL7003 <i>hypR</i> ::Ω; Sm ^r Sp ^r	This study
RmP1886	RmP110 <i>hypM</i> :: <i>gusA</i> via int. of pTH2494; Sm ^r Gm ^r	This study
Plasmids		
pJP2	Broad-host range plasmid with <i>gusA</i> reporter gene; Tc ^r	Prell et al. 2002
pJQ200 uc1	Suicide vector with <i>sacB</i> to select for excision of integrated plasmid; Gm ^r	Quandt and Hynes 1993
pFL7003	449-bp fragment encompassing the <i>smb20262/hypM</i> intergenic region into pTH1522; Gm ^r	Cowie et al. 2006
pLAFR1	IncP cosmid cloning vector; Tc ^r	Friedman et al. 1982
pUCP30T	Cloning vector, unable to replicate in <i>S. meliloti</i> ; Gm ^r	Schweizer et al. 1996
pTH1522	Reporter vector used in construction of <i>S. meliloti</i> reporter gene fusion library; Gm ^r	Cowie et al. 2006
pTH1582	Modified version of pJP2; introduction of promoterless <i>gusA</i> from pFus1; Tc ^r	Yuan et al. 2005
pTH1705	pTH1522 with expanded MCS; Gm ^r	Cowie et al. 2006
pTH1961	627-bp polymerase chain reaction (PCR)-amplified region encompassing <i>smb20568/smb20569</i> IG region into pTH1705 in the antisense orientation to <i>gusA</i> ; Gm ^r	A. M. MacLean and T. M. Finan, unpublished data
pTH2130	2,723-bp <i>NotI</i> PCR product encompassing <i>hypMNPQ</i> in suicide vector pJQ200; Gm ^r	This study
pTH2131	pTH2130 with 1,689-bp deletion via <i>SalI</i> digest; Gm ^r	This study
pTH2216	953-bp <i>HindIII</i> PCR product encompassing <i>hypR</i> in vector pUCP30T; Gm ^r	This study
pTH2217	ΩSm/Sp ^r from pHP45Ω into pTH2216 via <i>XhoI</i> ; Gm ^r Sm ^r Sp ^r	This study
pTH2439	pLAFR1 derivative of <i>S. meliloti</i> cosmid complementing Hyp [−] phenotype of RmF909; Tc ^r	This study
pTH2494	370-bp PCR product encompassing <i>smb20262-hypM</i> intergenic region into pTH1705 via <i>BglII/KpnI</i> ; Gm ^r	This study

^a Sm^r, Rf^r, Nm^r, Gm^r, Sp^r, sucrose^r, and Tc^r = resistant to streptomycin, rifampicin, neomycin, gentamicin, spectinomycin, sucrose, and tetracycline, respectively; sucrose^s and Gm^s = susceptible to sucrose and gentamicin, respectively.

version of the vector pJP2 (pTH1582). The 3,240-bp amplified fragment extends 241 bp upstream of the predicted translational start site of *hypM* and includes the intergenic region upstream of this gene, thus allowing expression of the operon to be determined by its native promoter. The replicating plasmid pTH2513 was transferred to *S. meliloti* strains by triparental conjugation; transconjugants were selected by plating upon LB agar supplemented with Tc and Sm.

Construction of an *S. meliloti* *hypR::Ω* strain.

An Ω cassette specifying Sm and Sp resistance from pHP45 Ω (Prentki and Krisch 1984) was introduced into an *Xho*I site located 345 bp downstream of the translational start site as follows. A 953-bp fragment encompassing the *Xho*I site was PCR amplified using *S. meliloti* Rm1021 genomic DNA as a template with primers 5' CCAAGCTTGCATCTGTGCTGATTTTCG 3' and 5' CCTAAGATCACAGAGCAATTTTCG 3'; this fragment was cloned into plasmid pUCP30T to create pTH2216. The Ω cassette was PCR amplified using pHP45 Ω as a template and the cassette was cloned into pTH2216 via the *Xho*I restriction site to create pTH2217. This plasmid was transferred through conjugation into *S. meliloti* Rf^r strain Rm5000; Rf^r and Sp^r transconjugants were selected to isolate strains in which the plasmid (which is unable to replicate in *S. meliloti*) had integrated into the genome by homologous recombination. Rf^r/Sp^r colonies were screened for Gm^s to identify double recombinants in which the plasmid had simultaneously excised from the megaplasmid. To confirm the genotype of the strains, Southern hybridization was performed with genomic DNA isolated from five Rf^r/Sp^r and Gm^s strains digested with *Sac*II. Digested DNA was hybridized with a labeled probe corresponding to *hypR*; in each case, an approximately 2-kb increase was noted in the putative *hypR::Ω* strains (approximately 6.9 kb) compared with the wild-type control (approximately 4.8 kb), consistent with the incorporation of the Ω cassette into the gene and subsequent excision of the vector backbone. In contrast, DNA isolated from two control Rf^r/Sp^r and Gm^r strains (consistent with a single recombination event) identified two fragments (approximately 5.6 and 6.5 kb) which hybridized with the labeled probe. One of the five Rm5000 *hypR::Ω* strains was purified and designated as RmP1406. The *hypR::Ω* allele was transferred from RmP1406 into RmP110 via transduction by selecting for Sp^r transductants; this strain (RmP110 *hypR::Ω*) is referred to as RmP1724.

Reporter enzyme assays.

β -Glucuronidase and β -galactosidase enzyme assays were performed as previously described (MacLean et al. 2006). *S. meliloti* RmFL7003 belongs to a reporter gene fusion library described by Cowie and associates (2006). The reporter vector includes a variant of green fluorescent protein (GFP+) (Scholz et al. 2000) and fluorescence was assayed using an excitation wavelength of 485 nm and emission wavelength of 510 nm. Assays for GFP+ were performed using overnight LBmc cultures of *S. meliloti* that were subcultured into M9-minimal medium supplemented with a carbon source as indicated and incubated at 30°C for 4 to 6 h. As a means of quantifying fluorescence, the emission output was divided by its optical density at 600 nm (OD₆₀₀) to calculate the specific activity.

Primer extension.

RNA was isolated from *S. meliloti* wild-type strain RmP110 grown in M9-minimal medium with glycerol and ammonium chloride as sole sources of carbon and nitrogen (uninduced sample) or *trans*-4-hydroxy-L-proline as a source of nitrogen and carbon (induced sample). The cultures were grown for 12 h with aeration at 30°C to an OD₆₀₀ of approximately 0.4. Cells

were harvested and total RNA extracted as previously described (MacLellan et al. 2005). The oligonucleotide 5' CGT CAGAGCGGTGGCGGCAACGAGG 3' was end-labeled with γ -³²P-dATP (Amersham, Tokyo) and T₄ polynucleotide kinase (Fermentas, Vilnius, Lithuania), and then purified using a QIAquick nucleotide removal column (Qiagen, Basel, Switzerland). For each primer extension, 2 × 10⁵ counts per minute of the labeled primer was combined with approximately 20 μ g of RNA, 20 nmoles each of all four dNTPs, and reverse transcriptase buffer (Invitrogen) in a total volume of 16 μ l. Each reaction mixture was heated at 65°C for 15 min and cooled to 50°C over approximately 30 min. Then, 2 μ l of 100 mM dithiothreitol (DTT) and 1 μ l of RNaseOUT (Invitrogen, Carlsbad, CA, U.S.A.) were added to each reaction, which were then incubated at 42°C for 2 min before adding 1 μ l of Superscript III reverse transcriptase (Invitrogen). Primer extensions were incubated for 50 min at 50°C, and an equal volume of 2× Stop buffer (Sequenase version 2.0 kit; USB, Cleveland) was added to each sample to terminate the reaction. DNA sequencing reactions were performed using the Sequenase version 2.0 DNA sequencing kit (USB), with purified plasmid pFL7003 as a template and the same oligonucleotide used to prime the extension reactions. Extension products and sequencing reactions were separated by electrophoresis in an 8% polyacrylamide gel containing 7.7 M urea. Data were analyzed using a Storm820 phosphorimager (Amersham).

Hydroxyproline transport assays.

S. meliloti strains were grown overnight in LBmc and subcultured (1:100) into M9-minimal medium supplemented with glycerol, L-proline, or *trans*-4-hydroxy-L-proline as sources of carbon. Where indicated, proline and hydroxyproline were also added as a sole source of nitrogen through the omission of ammonium chloride, which is normally present in M9 growth medium. Cultures were typically harvested after 14 to 18 h of incubation at 30°C via centrifugation at 4°C (OD₆₀₀ approximately 0.8 to 1.2). Cells were washed twice in cold M9-minimal medium and suspended in M9-minimal medium lacking ammonium chloride (M9 – N) to an OD₆₀₀ of 2.0. These cells were kept on ice prior to use in transport assays. Assays were performed with 25 μ l of cells (OD₆₀₀ 2.0) added to 450 μ l of M9 – N, and equilibrated at 30°C for 5 min in a water bath. Assays were initiated by the addition of 25 μ l of labeled substrate (*trans*-4-hydroxy-L-proline [³H(G)]; 20 Ci/mmol) (American Radiolabeled Chemicals, Inc.) added to a final concentration of 10 μ M; cells were vortexed gently and incubated at 30°C for the duration of the assay. Then, 100- to 200- μ l aliquots of the assay mixture were removed at timed intervals and rapidly filtered through 0.45 μ m nitrocellulose filters (catalog number HAWP02500; Millipore, Bedford, MA, U.S.A.), which were presoaked in M9 – N. Filtered cells were immediately washed with approximately 10 volumes of M9 – N. Counts per minute were determined from the dried filters using a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin-Elmer, Norwalk, CT, U.S.A.). For competition assays, unlabeled competitor was added 30 s prior to the addition of labeled substrate. Assays were performed up to 1 min following addition of substrate and the rate of uptake was always linear within this period of time. Protein concentration was determined from sonicated cell lysates according to the method developed by Bradford (1976). Activity was also assessed using induced cells treated with toluene (100 μ l of toluene added to 2 ml of cells, OD₆₀₀ = 2.0). Uptake was not observed in toluene-treated cells, and this control was used to estimate background counts per minute, which was subtracted from values obtained in assays with untreated cells. Transport assays were performed in triplicate, and values reported represent the mean and standard deviation

of the three assays. All reported data are representative of a minimum of two independent experiments.

To examine transport kinetics, assays were performed using 9 to 13 different concentrations of *trans*-4-hydroxy-L-proline (0.05 to 25 μ M) and transport velocity was determined using three time points per assay. K_m and V_{max} were determined as the average of results obtained from three independent experiments.

Plant assays.

Alfalfa seed (*M. sativa* cv. Iroquois) were surface sterilized with 95% ethanol (5 min) followed by 2.5% sodium hypochlorite (20 min) and rinsed with distilled, autoclaved water for 1 h. Seed were germinated on water agar plates (1.5% agar) in the dark for 2 days, and seven seedlings were then transferred to nitrogen-free vermiculite and sand (1:1 [wt/wt]) and inoculated with approximately 10^7 to 10^8 *S. meliloti* cells suspended in 10 ml of sterile, distilled water. Plants were grown in a Conviron plant growth chamber with an 18-h (21°C) day and 6-h (17°C) night. Plants were watered as required using autoclaved, distilled water and nodules were harvested for expression assays 4 to 12 weeks after inoculation and stored at -80°C.

β -Glucuronidase activity was quantified in root nodules as follows. First, 5 to 10 nodules were placed into an Eppendorf tube that was prechilled on ice. Then, 750 μ l of cold MMS buffer (40 mM MOPS, 20 mM KOH, 2 mM $MgSO_4$, 0.3 M sucrose; pH 7.0) was added and nodules were crushed with mini pestles. Plant tissue was pelleted via centrifugation at $400 \times g$ for 2 min and 500 μ l of supernatant was transferred to a fresh tube on ice. Sodium dodecyl sulfate was added to a final concentration of 0.01% and samples were incubated on ice for 5 min. Lysate was used at 100 μ l per assay, and each reaction included 890 μ l of buffer (50 mM sodium phosphate, 50 mM DTT, 1 mM EDTA; pH 7.0). Samples were equilibrated in a water bath at 37°C for 10 min and the assay was initiated by the addition of 10 μ l of 4-nitrophenyl β -D-glucuronide (35 mg/ml) (Sigma-Aldrich). Reactions were terminated by the addition of 200 μ l of reaction mixture to 700 μ l of 0.46 M Na_2CO_3 . Specific activity was calculated as (absorbance at 405 nm $\times$ 1,000)/(time [minutes] $\times$ milligrams of protein) $^{-1}$. The protein concentration of each lysate was determined by the method established by Bradford (1976).

Histochemical staining to detect β -glucuronidase activity was based on a previous method (Boivin et al. 1990). In all, 5 to 10 nodules were obtained from plants inoculated with each *S. meliloti* strain. The fresh nodules were immediately mounted upon a specimen plate using crazy glue (Instant Crazy Glue) (Elmer's Products Canada, Toronto) and nodules were sliced using a vibrating blade microtome (LEICA VT1000; Leica, Wetzlar, Germany) into 90- μ m longitudinal sections. Nodule sections were transferred into staining buffer (200 mM sodium phosphate, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 1.5 mM $K_4Fe(CN)_6$, pH 7.0) containing 0.08% (wt/vol) 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (Sigma-Aldrich). Sections were stained overnight (typically 12 to 18 h) at 30°C and then washed twice in 200 mM sodium phosphate buffer (pH 7.0) for several hours. Prior to microscopic examination, sections were cleared in a solution of 1% sodium hypochlorite for 5 min. Images were captured using a Nikon TE2000 inverted microscope.

For the purpose of monitoring expression of *hypM* in alfalfa root nodules, *S. meliloti* RmP1886 was constructed as follows. Primers 5' ATCAGATCTCATGTGCGATTTCGACGTTTCCTGC 3' and 5' TAGGTACCATGATGTCGTCCAGCTTGTCG 3' were used to PCR amplify a 370-bp fragment encompassing the intergenic region upstream of *hypM*. This fragment was cloned into pTH1705 (Cowie et al. 2006) via *Bgl*III/*Kpn*I to

create pTH2494. The plasmid was transferred via conjugation into *S. meliloti* RmP110. Recombinants in which the plasmid integrated into the genome were isolated by plating transconjugants upon LB agar containing Sm and Gm.

S. meliloti RmP778 was included in all assays as a negative control to reflect the endogenous level of β -glucuronidase activity associated with a single copy of the reporter gene in the *S. meliloti* genome. To construct RmP778, a promoter (associated with a gene unrelated to hydroxyproline metabolism; *smb20568*) was cloned in the antisense orientation with respect to *gusA* in pTH1705 via *Bgl*III/*Kpn*I. This plasmid was transferred into RmP110, and a purified recombinant was designated as RmP778.

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