Enhanced Nodulation and Nodule Development by *nolR* Mutants of *Sinorhizobium medicae* on Specific *Medicago* Host Genotypes

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The *nolR* gene encodes a negatively acting, transcriptional regulatory protein of core Nod-factor biosynthetic genes in the sinorhizobia. Although previous reports showed that *nolR* modulates Nod-factor production and enhances nodulation speed of *Sinorhizobium meliloti* on alfalfa, there have been no reports for the symbiotic function of this gene in the *S. medicae*–*Medicago truncatula* symbiosis. Here, we constructed an *nolR* mutant of *S. medicae* WSM419 and evaluated mutant and wild-type strains for their nodulation ability, competitiveness, host specificity, and density-dependent nodulation phenotypes. When the mutant was inoculated at low and medium population densities, it showed enhanced nodule formation during the initial stages of nodulation. Results of quantitative competitive nodulation assays indicated that an *nolR* mutant had 2.3-fold greater competitiveness for nodulation on *M. truncatula* ‘A17’ than did the wild-type strain. Moreover, the nodulation phenotype of the *nolR* mutant differed among *Medicago* genotypes and showed significantly enhanced nodule development on *M. tricycla*. Taken together, these results indicated that mutation of *nolR* in *S. medicae* positively influenced nodule initiation, competitive nodulation, and nodule development at later nodulation stages. This may allow *nolR* mutants of *S. medicae* to have a selective advantage under field conditions.

The root and stem nodule bacteria, collectively called the rhizobia, fix dinitrogen gas in symbioses with legume plants. The symbiotic interaction is important for agricultural productivity, especially in less developed countries. However, nodulation ability and competitiveness have practical importance in agricultural production, because the inoculation of efficient rhizobia is often unsuccessful, owing in large part to the presence of more competitive populations of ineffective indigenous rhizobia in soils (Streeter 1994; Toro 1996; Triplett and Sadowsky 1992).

Sinorhizobium meliloti forms symbiotic nitrogen-fixing nodules on *Medicago truncatula* (barrel medic) and *M. sativa* (al-

falfa). During this interaction, rhizobial nodulation genes (*nod*, *nol*, and *noe*) are expressed, and synthesize and secrete lipochito-oligosaccharide molecules (Nod-factors) that induce the formation of nodules on the host plant root (Long 1989; Long et al. 1985). The expression of the nodulation genes and nitrogen fixation are regulated in a positive and negative manner for optimal nodulation and efficient plant metabolism in the presence of fixed N (Kondorosi et al. 1989; Loh and Stacey 2003). Positive transcriptional regulation is mediated by the products of the *nodD* and *syrM* genes (Long 1996). The NodD protein is activated by specific flavonoid molecules secreted by the host root and seed coat, and interact with upstream nod-box regulatory elements to induce common-, host-, and genotype-specific nodulation genes (Loh and Stacey 2003; Sadowsky et al. 1991).

In *S. meliloti*, negative regulation is mediated by a repressor protein encoded by the *nolR* gene. This gene was first identified in *S. meliloti* AK631 as a negative transcriptional regulator of core Nod-factor biosynthetic genes (*nodABC*) (Kondorosi et al. 1989, 1991). The NolR protein binds to the overlapping promoter region of *nodD*, and *nodA* genes at the RNA polymerase binding region (Cren et al. 1995). Mutants of *S. meliloti* AK631 lacking an *nolR* gene overproduced and unsuccessfully decorated Nod factors. This, in turn, resulted in less efficient nodule initiation of alfalfa plants (Kondorosi et al. 1989). Moreover, results from proteomic analyses indicated that the NolR protein likely regulates expression of other genes (Chen et al. 2005).

In *S. fredii* HH103, a close relative of *S. meliloti* which nodulates soybean (Sadowsky et al. 1987; Sugawara et al. 2013), NolR influences symbiosis-related signals, such as secretion of signal-responsive proteins and the production of exopolysaccharides (Vinardell et al. 2004). Thus, it appears that the *S. meliloti* NolR is both a global regulatory protein used to fine tune intracellular metabolism and a key player in symbioses with host leguminous plant (Chen et al. 2000, 2005; Vinardell et al. 2004).

Chen and associates (2005) showed that the expression of the *S. meliloti nolR* gene is regulated in a population-density-dependent manner. The *nolA* gene, a negative transcriptional regulator of *nod* genes by *Bradyrhizobium japonicum*, is also expressed in a population-density-dependent manner and enhances the nodule number on soybean genotypes (Jitackorn and Sadowsky 2008; Loh and Stacey 2003). However, the influence of *nolR* on population-density-dependent nodulation in the sinorhizobia has not been investigated thus far. Although rhizobia live in nitrogen-fixing nodules at high cell densities, (approximately 10¹⁰ cells/nodule) (Bergersen 1982), it is expected that *nolR* is also expressed at high levels. Thus, it is possible that *nolR* influences not only initial nodulation, as has

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been previously reported (Kondorosi et al. 1989, 1991), but also later stages of nodulation that occur after infection, such as the development of nodule primordia and symbiotic N₂ fixation.

A functional *nolR* gene is conserved in most *Sinorhizobium* spp. and strains (it is not functional in Rm1021) (Sugawara et al. 2013), and this bacterium generally uses NodR as a regulatory protein for expression of large numbers of genes. Our recent comparative genomics studies showed that *Sinorhizobium* genomes comprised many accessory genes (Sugawara et al. 2013), some of which likely alter host specificity (Crook et al. 2012). In addition, *S. medicae* and *S. meliloti* are taxonomically related, have differences in gene content, and displayed different nodulation patterns on diverse *Medicago* host plants (Sugawara et al. 2013). Consequently, we hypothesized that *nolR*-regulated genes may have different influences on symbiotic performance and intracellular metabolism in *S. medicae* and *S. meliloti* strains.

In this study, we report on the functional characterization of the *nolR* gene in *S. medicae*. We focused our studies on nodulation competitiveness, host specificity, and population-density-dependent nodulation. We also report on the differences in *nolR* mutant phenotypes among *S. medicae* and *S. meliloti* strains on diverse host genotypes.

RESULTS

Expression of *nodC* in *Sinorhizobium nolR* mutant strains.

The *nolR* mutants of *S. medicae* WSM419 and *S. meliloti* Rm41 were constructed by insertion of a kanamycin/neomycin resistance gene (*aph*) into *nolR* (Supplementary Figs. S1 and S2). To investigate the influence of NodR on expression of Nod-factor biosynthesis, β -galactosidase assays were done using an *nodC-lacZ* fusion plasmid (pRmM57). The results show that β -galactosidase activity (Miller units) by *nolR* mutants of both species were greater than those seen in the wild-type strains, when 5 μ M luteolin was added as an inducer in Y minimal medium (Table 1). The activity level of *nodC* in the *S. medicae* WSM419 *nolR* mutant (815.2 ± 13.1 units) was nearly 4.5-fold greater than that seen in the *S. meliloti nolR* mutant (176.3 ± 4.4 units) (Table 1). Moreover, the ratio of the activity value (*nolR* mutant/wild-type) in *S. medicae* was higher (19.5-fold) than that seen in *S. meliloti* (6.8-fold). This showed that the *nolR* gene in *S. medicae* is a negative regulator of *nod* gene expression and the regulation of *nod* gene expression in *S. medicae* is likely tightly regulated by the *nolR* gene product.

Mutation in *S. medicae nolR* enhances nodulation speed on *M. truncatula* ‘A17’ and *M. sativa* ‘Agate’.

The influence *nolR* on symbiotic performance of mutant and wild-type strains was investigated using *M. truncatula* ‘A17’ and *M. sativa* ‘Agate’. Results shown in Supplementary Table S1 indicated that there was no difference between plant phenotypes between wild-type and *nolR* mutant strains, in either sinorhizobial species, after 4 weeks of incubation.

Table 1. Expression of an *nodC-lacZ* fusion in wild-type and *nolR* mutant strains of *Sinorhizobium*

<i>Sinorhizobium</i> strain	Miller units ^a	
	With luteolin ^b	Without luteolin
<i>S. medicae</i> WSM419 wild type	41.9 $\pm$ 0.6	14.8 $\pm$ 0.7
<i>S. medicae</i> WSM419 Δ <i>nolR</i>	815.2 $\pm$ 13.1	26.7 $\pm$ 2.2
<i>S. meliloti</i> Rm41 wild type	25.8 $\pm$ 1.9	17.6 $\pm$ 0.6
<i>S. meliloti</i> Rm41 Δ <i>nolR</i>	176.3 $\pm$ 4.4	19.7 $\pm$ 0.9

^a Values are means $\pm$ standard deviations of data for three biological replicates.

^b Luteolin was added at 5 μ M final concentration.

However, because previous reports showed that a mutation in *S. meliloti* AK631 of *nolR* leads to a decrease in nodulation speed on *M. sativa*, we investigated whether the *nolR* mutants made in other sinorhizobial strains and species also influenced the timing of nodule formation at initial stages of nodulation. All *Sinorhizobium* strains were inoculated onto *Medicago* seedlings at 10⁷ cells per plant, and roots were examined daily for the appearance of nodules. The percentage of *Medicago* plants nodulated by the wild-type, *nolR* mutant, and the *nolR* complemented strains at each day after inoculation is shown in Figure 1. Less time was required for the *S. medicae* WSM419 *nolR* mutant strain to initiate nodule formation on *M. truncatula* A17 and *M. sativa* Agate relative to that seen with the wild-type strain (Fig. 1A and B). Nodules appeared 4 days after the *S. medicae nolR* mutant was first inoculated onto both plants. In contrast, nodulation by the wild-type strain did not begin until 5 days after inoculation (DAI). The proportion of nodulated plant by the *nolR* mutant was significantly greater (*P* < 0.05) than that seen on plants inoculated with the wild-type strain at 4 and 5 DAI. Moreover, the nodulation speed of the *nolR* mutant reverted to that of the wild type in the complemented strain, Δ *nolR* (pMS20), which harbored the pMS20 plasmid (Fig. 1A and B). Although the *nolR* mutant nodulated 70 to 80% of *Medicago* plants at 5 DAI, only 10 to 30% of the plants were nodulated by the *nolR*-complemented strains on both *Medicago* host plants (Fig. 1A and B). In contrast, mutation of the *nolR* gene in *S. meliloti* Rm41 did not significantly influence nodulation timing on Agate relative to that seen with the wild-type strain or the *nolR*-complemented strain harboring pMS19 (Fig. 1C). Because *S. meliloti* Rm41 formed inef-

Fig. 1. Percentage of *Medicago* plants nodulated by a wild-type *Sinorhizobium* sp. (●), an *nolR* mutant (○), and its *nolR* complemented strain (△). **A**, Percentage of *Medicago truncatula* ‘A17’ plants nodulated by *Sinorhizobium medicae* WSM419 strains; **B**, percentage of *M. sativa* ‘Agate’ nodulated by *S. medicae* WSM419; and **C**, percentage of *M. sativa* Agate nodulated by *S. meliloti* Rm41 strains. Approximately 20 to 25 plants were analyzed for each treatment at each time point.

fective (small and white-colored) nodules on *M. truncatula* A17, we did not try this experiment using this strain–host combination. Taken together, these results indicated that mutation of the *nolR* gene in *S. medicae* WSM419 enhanced nodulation at the initial stages of nodulation. Moreover, it suggested that the influence of *nolR* gene product on the speed of nodulation in *S. medicae* is greater than that seen in *S. meliloti*.

The mutation of *nolR* in *S. medicae* enhances competitiveness of nodulation.

The influence of the *nolR* gene on competitive nodulation ability on *Medicago* plants was investigated by co-inoculation with the wild-type strain and the *nolR* mutant. Nodule occupancy rates by each strain are presented in Table 2. The nodule occupancy rates of the *S. medicae* WSM419 wild-type strain on *M. truncatula* A17 and *M. sativa* Agate were 27.8 and 23.9%, respectively, when *S. medicae* WSM419 and its *nolR* mutant were co-inoculated at a 1:1 ratio. In contrast, the *nolR* mutant showed 72.2% (A17) and 69.6% (Agate) nodule occupancy rates. The occupancy rates by the *nolR* mutant and the wild-type strain were significantly different at $P < 0.01$ (Table 2). These results indicated that the mutation of *nolR* in *S. medicae* leads to enhance competitiveness for nodulation. In contrast, no significant influence of *nolR* gene on *S. meliloti* Rm41 nodule occupancy rates was observed (Table 2).

The competitive ability for nodulation by the *nolR* mutant of *S. medicae* on *M. truncatula* A17 was quantified by using seven inoculant mixtures, with the proportion of wild-type and *nolR* mutant strains ranging from 1:1,000 to 1,000:1. Nodule occupancy rates by the two strains resulting from each inoculant mixture were obtained and plotted in Figure 2A. In this experiment, the *nolR* mutant also showed significantly greater nodule occupancy rates ($P < 0.01$) against the wild-type strain when these strains co-inoculated at a 1:1 ratio. The relation-

ships between the logarithm of the ratio of the numbers of nodules formed by the *nolR* mutant and the wild-type strain ($N_{\Delta nolR}/N_{WT}$) and the logarithm of the ratio of the numbers of cells of the two strains in the inoculum ($I_{\Delta nolR}/I_{WT}$) are shown in Figure 2B. The $C_{\Delta nolR:WT}$ value for the *nolR* mutant/wild-type was estimated at 2.29. Thus, this result indicated that the mutation of *nolR* in *S. medicae* WSM419 enhances nodulation competitiveness approximately 2.3-fold on *M. truncatula* A17.

Mutation of *nolR* in *S. medicae* enhances nodule development on specific *Medicago* genotypes.

The influence of the *S. medicae* *nolR* gene on host-specific nodulation was investigated by inoculation of wild-type and mutant strains onto nine genotypes of *M. truncatula* and one genotype each of *M. sativa*, *M. tricycla*, and *M. littoralis*. Nodule numbers and dry weights on each *Medicago* genotype inoculated with wild-type WSM419 and its *nolR* mutant are shown in Table 3. These results show that the *nolR* mutant formed significantly ($P < 0.05$) fewer nodules on the four *M. truncatula* genotypes (SA22322, SA28064, F83005-5, and Salses71B), relative to those seen with the wild-type strain. However, interestingly, the nodule dry weights per plant were not significantly different between the *nolR* mutant and the wild-type strain, except for F83005-5 (Table 3). In addition, these effects were more clearly observed in *M. tricycla* R108-C3. These results indicated that the *nolR* mutant formed relatively larger-sized nodules on the *Medicago* plants than those seen with the wild-type strain. Therefore, we hypothesized that mutation of the *nolR* gene in *S. medicae* enhances not only initiation of nodulation but also nodule development at later symbiotic stages.

To test this hypothesis, we investigated differentiation of nodule numbers and dry weights on *M. tricycla* R108-C3 at each time point that plants were inoculated with the WSM419 *nolR* mutant and wild-type strain. Although nodule

Table 2. Competitive nodulation phenotypes of wild-type and *nolR* mutants of *Sinorhizobium medicae* WSM419 and *S. meliloti* Rm41 on *Medicago* host plants

<i>Sinorhizobium</i> strain	Host plant	Nodule occupancy by strain (%)			<i>P</i> value ^a	Nodules tested ^b
		Wild type	<i>nolR</i> Mutant	Both		
<i>S. medicae</i> WSM419	<i>Medicago truncatula</i> A17	27.8	72.2	0	<0.01	72
	<i>M. sativa</i> Agate	23.9	69.6	6.5	<0.01	92
<i>S. meliloti</i> Rm41	<i>M. sativa</i> Agate	42.7	53.8	3.4	0.221	117

^a *P* values were obtained by χ^2 statistical analysis.

^b Number of nodules tested.

Fig. 2. Quantification of nodule occupancy by co-inoculation of wild-type *Sinorhizobium medicae* WSM419 and its *nolR* mutant on *Medicago truncatula* ‘A17’. **A**, Relationship between percentage of nodule occupancy and cell ratio in inoculum. The *S. medicae* WSM419 wild type (●) and the *nolR* mutant (○) were co-inoculated on *M. truncatula* A17. The *S. medicae* strains were isolated from each nodule and nodule occupancy was determined by neomycin resistance. Each data point represents the mean of 96 nodules per treatment. **B**, Relationship between the logarithm of the ratio of the number of nodules formed by the *nolR* mutant ($N_{\Delta nolR}/N_{WT}$) and the logarithm of the ratio of the numbers of cells of the two strains in the inoculum ($I_{\Delta nolR}/I_{WT}$).

numbers were not significantly different ($P = 0.05$) between the *nolR* mutant and wild-type strain after 7 and 14 days of inoculation, significantly ($P < 0.05$) fewer nodules were formed on the plants inoculated with the *nolR* mutant after 21 days (Fig. 3A). In contrast, nodule dry weights were not significantly different ($P = 0.05$) between the strains at each time point (Fig. 3B). However, nodules formed by the *nolR* mutant on the primary root were relatively larger than that seen with the wild-type strain (Fig. 3D). Moreover, the size and dry weight of the plant inoculated with the *nolR* mutant were significantly greater ($P < 0.05$) than those seen with the wild-type strain after 42 days (Fig. 3C and E). These results suggest that mutation of the *nolR* gene in *S. medicae* enhances nodule development and plant growth on certain *Medicago* plant genotypes.

The influences of population density on *nolR* gene expression and nodulation.

To investigate whether the expression of the *nolR* gene occurs in a population-density-dependent manner, transcriptional *nolR-gusA* gene fusion plasmids were constructed and β -glucuronidase (GUS) assays were done on cells sampled in the early, middle, and late log phases of growth. GUS activity in both *S. medicae* WSM419(pMS18) and *S. meliloti* Rm41(pMS17) cells gradually increased with increasing population density, and the greatest activity was observed at the late log phase of growth (Table 4). These results suggested that *nolR* gene expression in both strains is regulated by a quorum-sensing factor, similar to what was previously reported for the *nolR* gene in *S. meliloti* AK631 (Chen et al. 2005) and *nolA* in *B. japonicum* USDA 110 (Jitaksorn and Sadowsky 2008; Loh et al. 2002).

Table 3. Nodulation phenotype of wild-type and *nolR* mutant strains of *Sinorhizobium medicae* WSM419 inoculated onto several *Medicago* plant genotypes^a

<i>Medicago</i> spp.	Genotype	Nodule number		Nodule dry mass (mg)	
		Wild type	<i>nolR</i> mutant	Wild type	<i>nolR</i> mutant
<i>M. truncatula</i>	SA22322	22.0	12.0*	5.1	5.0
	SA28064	28.3	12.3*	8.2	5.1
	ESP105-L	12.0	13.8	3.3	2.7
	DZA045-6	24.3	36.3	3.8	4.7
	DZA315-16	16.3	12.0	5.0	4.2
	F83005-5	14.3	8.0*	4.4	2.7*
	Salses71B	13.3	8.0*	2.6	2.4
	DZA012-J	19.8	17.0	9.9	7.0
	A20	9.5	7.5	8.8	7.2
<i>M. sativa</i>	Garst6Rm415	35.0	35.5	5.0	5.8
<i>M. tricycla</i>	R108-C3	10.0	2.8*	4.2	3.5
<i>M. littoralis</i>	F11013-27	0	0	0	0

^a Values are means of results for four replicate experiments. Nodule number and dry mass values are per plant. Asterisks indicated a significant difference between four biological replicates of the wild-type and *nolR* mutant strains by analysis of variance at $P < 0.05$.

Fig. 3. Symbiotic phenotypes of *Medicago tricycla* 'R108-C3' inoculated with wild-type and *nolR* mutant strains of *Sinorhizobium medicae* WSM419. **A**, Number of nodules formed; **B**, nodule dry weight; and **C**, plant dry weight per plant were plotted at each time point. Asterisks indicate significant difference between the wild type and the *nolR* mutant at $P = 0.05$ as tested by analysis of variance. Error bars indicate standard deviation of data for six replicates. NT = not tested. **D**, Nodules formed on *M. tricycla* R108-C3 plant roots inoculated with wild-type and *nolR* mutant strains of *S. medicae* WSM419. White arrows indicate nodules formed on primary root of *M. tricycla* after 42 days of inoculation. Bar = 0.5 cm. **E**, *M. tricycla* R108-C3 plants inoculated with the wild type and *nolR* mutants of *S. medicae* WSM419 and a noninoculated control after 42 days of incubation.

We investigated population-density-dependent nodulation of *M. truncatula* A17 by inoculating *S. medicae* WSM419 and its *nolR* mutant at 10^3 , 10^5 , 10^7 , or 10^9 cells per plant. First nodules appeared 6 DAI when the wild-type strain was inoculated at 10^3 cells and at 5 DAI when plants received inoculation of 10^5 cells (Fig. 4A). Plants formed nodules earliest, after 4 days, when inoculated with 10^7 or 10^9 cells. Moreover, 100% of *M. truncatula* A17 seedlings showed nodulation at 5 DAI, the earliest seen, when inoculated with 10^9 cells. Thus, nodulation by *S. medicae* WSM419 was enhanced with increasing population density. When the *nolR* mutant was inoculated at 10^3 to 10^7 cells per plant, nodulation speed increased with increasing population density, similar to what was seen with the wild-type strain. However, nodulation was slower when the mutant was inoculated at 10^9 cells compared with the 10^7 -cell inoculum (Fig. 4B). Thus, the nodulation speed by the *nolR* mutant was reduced when the strain was inoculated at high population density.

The root tip marking (RTM) technique (Bhuvaneswari et al. 1980) was used to investigate nodule-forming speed and efficiency at different cell densities. Nodule distances of the wild-type strain gradually increased with increasing population density (Fig. 4C). In contrast, although plants inoculated with the *nolR* mutant had nodules that increased in distance from the RTM with increasing cell density from 10^3 to 10^7 cells, distance to the RTM decreased when the *nolR* strain was inoculated at 10^9 cells. These results are consistent with results of previous nodulation timing assays (Fig. 4A and B). Taken to-

gether, these results indicate that mutation of the *S. medicae* *nolR* gene enhanced nodulation speed at lower population density but inhibited nodulation at high population density.

DISCUSSION

Although the presence of *nolR* has been known for years, it is naturally disrupted by a single-base insertion in strain Rm1021, the first sequenced *Sinorhizobium* strain (Cren et al. 1994). Previous reports showed that the lack of an *nolR* gene in *S. meliloti* AK631 leads to less efficient nodule initiation on *M. sativa* L. var. Nagyszénás (Kondorosi et al. 1989). In contrast, results of our studies indicate that a mutation of the *S. medicae* WSM419 *nolR* gene significantly enhances initiation and competition for nodulation on both *M. truncatula* A17 and *M. sativa* Agate, when the *Sinorhizobium* cells were inoculated at 10^7 cells per plant (Fig. 1; Table 2). Although the mechanism by which this occurs is presently unknown, the differences in nodulation phenotypes between *S. medicae* WSM419 and *S. meliloti* AK631 may be due to differences in the plant genotypes tested and bacterial species or inoculant density used. Interestingly, *NolR* in some rhizobial species acts in a plant genus-specific manner. For example, inactivation or overexpression of *nolR* in *S. fredii* HH103 decreased the capacity of the strain to nodulate *Glycine max* but enhanced its ability to nodulate *Vigna unguiculata* (cowpea) (Vinardell et al. 2004). In addition, our results also show that nodule initiation on *M. truncatula* A17 was inhibited when *nolR* mutant cells were inoculated at high population density (Fig. 4). Taken together, the alterations of efficiency of initial nodulation by *nolR* are likely dependent on the rhizobial strain and legume used and bacterial population density in soil.

Results from the studies presented here also show that mutation of *nolR* in *S. medicae* WSM419 enhances nodule development on the roots of specific *Medicago* plant genotypes (Table 3; Fig. 3). Thus, these results suggest that the *nolR* in *S. medicae* WSM419 is involved in host-specific nodulation, and suppresses the expression of unknown symbiosis-related genes at a later stage of nodulation on the genotypes. Although the molecular mechanisms of enhancement of nodule development by the *nolR* gene remain thus far unresolved, it is a clear example of a new function for the *sinorhizobial* *nolR* gene on symbiotic

Table 4. Growth phase-dependent expression of an *nolR* -*gusA* fusion in *Sinorhizobium medicae* WSM419 and *S. meliloti* Rm41

Growth phase	<i>S. medicae</i> WSM419		<i>S. meliloti</i> Rm41	
	OD ₆₀₀ ^a	GUS (units) ^b	OD ₆₀₀ ^a	GUS (units) ^b
Early log	0.195	378 ± 9.8	0.179	426 ± 16
Middle log	0.764	530 ± 3.5	0.789	534 ± 26
Late log	1.685	1,004 ± 27	1.480	786 ± 52

^a OD₆₀₀ = optical density at 600 nm. Strains were grown in tryptone-yeast extract medium.

^b β-Glucuronidase (GUS) activity. Values are means ± standard deviations of data for four biological replicates. β-Glucuronidase activity (unit) was expressed as 1,000 × OD₄₀₅/OD₆₀₀/volume (ml)/reaction time (min).

Fig. 4. Percentage of *Medicago truncatula* 'A17' plants nodulated by wild-type and *nolR* mutant strains of *Sinorhizobium medicae* WSM419 at different population densities. The percentage of *Medicago* plants nodulated by **A**, wild-type *S. medicae* WSM419 and **B**, the *nolR* mutant at each time point is shown. **C**, Influence of population density on distance of uppermost nodule from the root tip (RT) mark by the *S. medicae* WSM419 wild-type (●) and the *nolR* mutant (○) strains. Twenty-four plants from each treatment were analyzed at each time point.

phenotypes at later nodulation stages. Further study of this phenomenon using transcriptomic or proteomic approaches on WSM419 *nolR* mutant bacteroids may shed further light on new factors involved in nodule development in the *Medicago-Sinorhizobium* symbiosis.

Our results also show that expression of *nolR* in *S. medicae* WSM419 is enhanced with increasing population density, similar to that reported for *S. meliloti* Rm41 (Table 4). In addition, a previous study showed that the addition of supernatant of stationary-phase culture of *S. meliloti* AK631 also induced the expression of *nolR* (Chen et al. 2005). Thus, these results indicate that a similar quorum-sensing system, and possibly signal molecules, may be acting the same in both species and that this is not a strain-specific phenomenon. Several N-acetyl homoserine lactone (AHL)-type quorum signals and the responsible genes have been identified in the rhizobia (Pongslip et al. 2005; Sanchez-Contreras et al. 2007), and a non-AHL-type quorum signal, bradyoxetin, is produced by *B. japonicum* USDA110, which enhances *nolA* (a negatively acting genotype-specific nodulation gene of soybean) expression at higher population density (Loh et al. 2002). Identification of these *nolR*-inducing quorum signal molecules from culture supernatants would be useful to better understand symbioses with this host plant.

Nodulation competitiveness has practical importance in agriculture, because the inoculation of efficient rhizobia in often unsuccessful owing to the presence of more competitive but inferior N_2 -fixing populations in the soil (Streeter 1994; Toro 1996; Triplett and Sadowsky 1992). Several rhizobial factors that affect nodulation competitiveness are known, including motility (Liu et al. 1989), rhizopines (Gordon et al. 1996), exopolysaccharides (McDermoti and Graham 1990), 1-aminocyclopropane-1-carboxylate deaminase (Uchiumi et al. 2004), and rhizobitoxine (Yuhashi et al. 2000). In addition, of these factors, we found here that mutation of *nolR* significantly enhanced nodulation efficiency and competitiveness of strains on *M. truncatula* and *M. sativa*. Moreover, the positive effects of the *nolR* mutation were observed when the sinorhizobial cells were inoculated at lower population density (10^3 to 10^7 cells per plant). Taken together, the mutation of *nolR* in *S. medicae* enhances symbiotic performance, and this may help improve the use of this bacterium as a legume inoculant for *Medicago* plants.

Finally, previous proteome analyses suggested that the *nolR* gene of *S. meliloti* AK631 encodes a negatively acting transcriptional regulator of core Nod-factor biosynthetic genes, and also acts as a global regulatory protein in response to environmental factors and to fine tune intracellular metabolism (Chen et al. 2000, 2005). In the results presented here, we showed that the *nolR* gene in *S. medicae* is also a negative regulator of *nod* gene expression and the regulation of *nod* gene expression in *S. medicae* is likely more tightly regulated by *nolR* gene product than that seen in *S. meliloti*. In addition, results from carbon source utilization assays by Biolog GN2 plate (Biolog Inc., Hayward, CA, U.S.A.) suggest that the *S. medicae* NolR also globally regulates intracellular metabolic genes (data not shown). Taken together, these results suggest that NolR may globally control gene expression in *S. medicae* more tightly than in *S. meliloti*, and *nolR*-regulated genes likely may be different in *S. medicae* and *S. meliloti*, because these taxonomically related species have large gene content differences (Sugawara et al. 2013).

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotide primers, and growth media.

Bacterial strains and plasmids used in this study are shown in Supplementary Table S2. The oligonucleotide primers used in this study are shown in Supplementary Table S3. The *Sinorhizo-*

bium strains were grown at 30°C in tryptone-yeast extract (TY) medium (Beringer 1974), and *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium (Sambrook and Russell 2001). Growth media were supplemented with antibiotics, as required, at the following concentrations (per milliliter) for *Sinorhizobium*: 150 µg of neomycin (Nm), 25 µg of tetracycline (Tet), and 20 µg of gentamicin (Gm); and, for *E. coli*, 100 µg of ampicillin, 50 µg of kanamycin, and 25 µg of Tet.

Construction of *nolR* mutants of *S. medicae* WSM419 and *S. meliloti* Rm41.

Mobilizable *nolR* inactivation plasmids were constructed as follows. The 1.2-kb *nolR* gene and its flanking regions from both *S. medicae* WSM419 and *S. meliloti* Rm41 strains were amplified by polymerase chain reaction (PCR) using the oligonucleotide primers WSM419_nolR_PstIF and WSM419_nolR_PstIR (for *S. medicae* WSM419) or Rm41_nolR_PstIF and Rm41_nolR_PstIR (for *S. meliloti* Rm41). The PCR products contained newly created *Pst*I sites. The PCR products were digested by *Pst*I and ligated into pBlueScript SKII (+) to create pMS02 (for the *S. medicae* WSM419 *nolR*) and pMS01 (for the *S. meliloti* Rm41 *nolR*). These plasmids were digested by *Nco*I and the 1.2-kb *aph* cassette, obtained from pUC4K (Vieira and Messing 1982) by PCR using the oligonucleotide primers (*aph*_NcoIF and *aph*_NcoIR), was inserted into *nolR* to create plasmids pMS08 (WSM419 *nolR::aph*) and pMS07 (Rm41 *nolR::aph*). Plasmids pMS08 or pMS07 were digested by *Pst*I and the 2.4-kb fragment containing *nolR::aph* region was inserted into *Pst*I site in the suicide vector pSUP202 (Simon et al. 1983) to create pMS11 (for Rm41) and pMS12 (for WSM419). The plasmids pMS11 or pMS12 were introduced into *S. meliloti* Rm41 or *S. medicae* WSM419, respectively, by triparental mating. Mutated strains were selected on TY agar plates containing Gm at 20 µg/ml and Nm at 150 µg/ml. Gene replacement double-crossover mutants were verified by their antibiotic resistance phenotype (Gm and Nm resistant and Tet sensitive), PCR amplification using primers that spanned the insertion sites, and DNA sequence analysis.

Construction of *nolR*-complemented strains.

To complement *nolR* mutants, full-length *nolR*, including the predicted native *nolR* promoter and Shine-Dalgarno sequences, was amplified by PCR using *Sinorhizobium* genomic DNA as template and oligonucleotide primers nolRN_all_PstI and nolRC_all_BglII. The PCR product was digested by *Pst*I and *Bgl*II and inserted into the *Pst*I-*Bgl*II site of pFUS1 (Reeve et al. 1998) to create pMS19 (for Rm41 *nolR*) and pMS20 (for WSM419 *nolR*). Plasmids pMS19 or pMS20 were introduced into the *S. meliloti* Rm41 *nolR* and *S. medicae* WSM419 *nolR* mutants by triparental mating. The *nolR*-complemented strains were selected on TY agar plate containing Gm, Nm, and Tet.

Evaluation of *nolC* gene expression by β -galactosidase assay.

Sinorhizobium wild-type and *nolR* mutant strains harboring the *nolC-lacZ* transcriptional fusion plasmid pRmM57 were assayed for β -galactosidase activity, as previously described (Mulligan and Long 1985). Cultures were washed twice in Y minimal medium (Beringer 1974) and adjusted to an optical density at 600 nm (OD_{600}) of approximately 0.5. 5 µM luteolin was added, and the cultures were incubated for 4 h at 30°C. Units of β -galactosidase activity were measured using the Miller method (Miller 1972).

GUS assay for evaluation of population-density-dependent *nolR* gene expression.

The *nolR* gene expression levels in *Sinorhizobium* strains harboring the *nolR-gusA* transcriptional fusion plasmids pMS17

(for *S. meliloti* Rm41) and pMS18 (for *S. medicae* WSM419) were assayed for GUS activity, as described by Reeve and associates (1998). Cultures were washed twice in TY medium, adjusted to an OD₆₀₀ of approximately 0.05, and 3 ml of the suspension was incubated at 30°C. Aliquots (100 µl) of cells sampled at each phase of growth were mixed with 790 µl of GUS assay buffer (50 mM sodium phosphate [pH 7], 50 mM dithiothreitol, and 1 mM EDTA) and 100 µl of saline. For a blank, 100 µl of saline was added instead of cells. One drop of toluene was added to each tube, the mixture was vortexed, and the tubes were incubated at 37°C for 30 min to remove toluene. The GUS assays were done as previously described (Reeve et al. 1998).

Symbiotic phenotypes of *Sinorhizobium* wild-type and mutant strains.

Medicago seed were prepared as described by Bucciarelli and associates (2006). Plant assays were done in sterile Leonard jar assemblies containing a 1:1 mixture of Sunshine mix number 5 (SunGro Horticulture Inc., Vancouver, BC Canada) and Turface MVP (Profile Product LLC, Buffalo Grove, IL, U.S.A.), as previously described (Sugawara et al. 2013). *Sinorhizobium* strains were grown in TY medium for 2 days, collected by centrifugation at 8,000 × g for 10 min, and washed twice with sterile 0.85% sodium chloride. The resultant cultures were diluted to 10⁷ cells/ml with sterile 0.85% NaCl and aliquots (1 ml) of serial, decimal dilutions of this inoculum were added onto sterilized *Medicago* seedlings. Plants were watered with nitrogen-free plant nutrient solution (Bucciarelli et al. 2006) and incubated in a plant growth chamber at 25°C with a 16-h light condition and at 21°C for 8 h in the dark. Nodule numbers, dry weights, and dry weight of each plant were determined 1 to 6 weeks after inoculation, as required.

Competitive nodulation assay.

Sterilized *Medicago* seedlings were inoculated with 1 ml of inoculum mixed with wild-type and *nolR* mutant strains at a concentration of 5 × 10⁶ per milliliter of each strain (ratio 1:1), and grown under the same condition as described above. After 28 days of cultivation, nodules were randomly picked from each plant root and surface sterilized by immersion in 1% sodium hypochlorite for 5 min. Nodules were washed three times with sterile water and transferred into a 96-well microtiter plate containing 100 µl of TY liquid medium in each well. The surface-sterilized nodule was homogenized by using a sterile toothpick and the suspension was streaked onto TY plates. Three single colonies from each TY plate were transferred onto a TY plates containing Nm at 150 µg/ml. Strains in nodules was determined by Nm resistance (the wild type was Nm sensitive and the *nolR* mutant was Nm resistant).

Quantitative evaluation of competitive nodulation was determined using seven inoculant mixtures, where the proportions of wild-type and *nolR* mutant strains varied from 1:1,000 to 1,000:1. The mathematical model reported by Amarger and Lobreau (1982) was used to quantify the nodulation competition capability of *S. medicae nolR* mutant strains on *M. truncatula* A17. In this model, a linear relationship exists between the logarithmic ratio of the numbers of nodules formed by each of two strains (N_A/N_B) and the logarithm of the ratio of the number of cells of the two strains in the inoculum (I_A/I_B): $\log(N_A/N_B) = \log C_{AB} + \log(I_A/I_B)$. The intercept of the regression line, the C_{AB} value, was reported to be an index representing the competitiveness of strain A in relation to that of strain B. Moreover, this was considered to be equal to the ratio of the number of nodules formed by strain A to the number of nodules formed by strain B, when the two strains were present in the inoculum in equal quantities. Thus, C_{AB} should be close to 1 for two strains of equal competitiveness.

Evaluation of nodulation speed by *Sinorhizobium* strains.

The speed of nodulation and the location of nodules on the primary root (distance from root tip mark) were determined as follows. Briefly, surface-sterilized *Medicago* seedlings (1- to 2-cm root length) were placed on a paper surface (the paper support from seed growth pouches; Mega International, Minneapolis, MN, U.S.A.) and this was placed on the surface of 1.5% agar plates (8 to 10 seedlings per plate) containing nitrogen-free Fahræus medium (Vincent 1970) in square petri dishes (245 by 245 mm; Corning, Corning, NY, U.S.A.). *Sinorhizobium* inoculants were prepared as described above and 0.1 ml of cell suspensions, adjusted as required by cell densities, were inoculated onto each *Medicago* seedling root. At the time of inoculation, the location of the root tip was marked on the outside of the petri dish. The dishes were sealed by using surgical tape and the roots were covered with aluminum foil. Petri dishes were placed on a slope in a growth chamber at 25°C with a 16-h light condition and at 21°C for 8 h in the dark. Roots were examined daily for the appearance of nodules and the distances of uppermost nodule from the root tip were recorded at 10 DAI. Three replicate petri dishes were prepared for each treatment and the data values were averaged from 20 to 25 plants inoculated with each strain or at each population density.

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