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Characterization of MocR, a GntR-like transcriptional regulator, in *Bradyrhizobium japonicum*: its impact on motility, biofilm formation, and soybean nodulation[§]

May Nyan Taw[1], Hae-In Lee[1], Sang-Ho Lee[2], and Woo-Suk Chang[1,3*]

[1]*Department of Biology, University of Texas at Arlington, Arlington, Texas 76019, USA*
[2]*Fusion Technology R&D Division, Korea Institute of Industrial Technology, Ansan 426-910, Republic of Korea*
[3]*Division of Biotechnology, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan 570-752, Republic of Korea*

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Bradyrhizobium japonicum **is a Gram-negative soil bacterium that can fix nitrogen into ammonia by developing a symbiotic relationship with the soybean plant. MocR proteins make up a subfamily of GntR superfamily, one of the most widely distributed and prolific groups of the helix-turn-helix transcription factors. In this study, we constructed a mutant strain for *mocR* (blr6977) to investigate its role in cellular processes and symbiosis in *B. japonicum*. Although growth rate and morphology of the mutant were indistinguishable from those of the wild type, the mutant showed significant differences in motility and attachment (i.e., biofilm formation) from the wild type. The mutant displayed a decrease in biofilm formation, but was more motile than the wild type. The inactivation of *mocR* did not affect the number of nodules on soybean roots, but caused delayed nodulation. Delayed nodulation intrigued us to study competitiveness of the mutant infecting soybeans. The mutant was less competitive than the wild type, indicating that delayed nodulation might be due to competitiveness. Gene expressions of other MocR subfamily members were also compared between the wild type and mutant strains. None of the *mocR*-like genes examined in this study were differentially expressed between both strains.**

***Keywords*:** GntR transcriptional regulator, MocR, *Bradyrhizobium japonicum*, motility, biofilm, symbiotic nitrogen fixation

*For correspondence. E-mail: wschang@uta.edu; Tel.: +1-817-272-3280; Fax: +1-817-272-2855
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Introduction

Bradyrhizobium japonicum is a Gram-negative soil bacterium and an endosymbiont of the leguminous soybean plant (*Glycine max*). In order to establish the symbiosis, *B. japonicum* undergoes a series of steps including a complex signal exchange with its host and the formation of root nodules where nitrogen-fixing bacteroids reside (van Rhijn and Vanderleyden, 1995; Kaneko *et al.*, 2002). In the *B. japonicum*-soybean symbiosis, a number of transcriptional regulators play a role in controlling the expression of nodulation genes. One of the most represented is a LysR family transcriptional regulator, NodD protein, which is activated by soybean isoflavonoids and regulates the expression of common *nod* gene operon *nodYABCSUIJ* for efficient nodulation on soybean roots (Göttfert *et al.*, 1992). In addition to NodD, NolA, a MerR family transcriptional regulator, is required for *B. japonicum* to successfully nodulate soybean roots in a genotype-specific manner (Sadowsky *et al.*, 1991). More interestingly, both regulators NodD and NolA are also involved in cell density-dependent regulation (e.g., quorum sensing) by *B. japonicum* for symbiotic nitrogen fixation with soybeans (Loh *et al.*, 2001).

One of the most widely distributed helix-turn-helix transcription factors among prokaryotes is the GntR superfamily, named after the repressor of the gluconate operon in *Bacillus subtilis* (Fujita and Fujita, 1987; Lord *et al.*, 2014). GntR-like transcriptional regulators contain a highly conserved DNA-binding N-terminus along with a heterogeneous effector binding and/or oligomerization C-terminus (Rigali *et al.*, 2002). The GntR superfamily is further divided into subfamilies such as AraR, DevA, FadR, HutC, MocR, PlmA, and YrA according to the secondary structure prediction based on their amino acid sequences. GntR-like regulators have been found to control many fundamental cellular processes such as carbon metabolism, motility, development, antibiotic production, and virulence (Haine *et al.*, 2005; Hillerich and Westpheling, 2006; Jaques and McCarter, 2006; Hoskisson and Rigali, 2009; Tanaka *et al.*, 2014). Additionally, they can act as activators or repressors of gene expression in other parts of the genome. Furthermore, it has been suggested that microorganisms inhabiting complex environments such as soil possess a greater number of *gntR* genes, making it an interesting aspect to study its involvement in establishing a symbiosis, survival, and competitiveness in the rhizosphere (Rigali *et al.*, 2002). Wang and colleagues showed that the mutants of two GntR-like regulator genes, *gtrA* and *gtrB*, in *Sinorhizobium meliloti* were deficient in growth and motility as

well as nodulation on alfalfa roots (Wang *et al.*, 2008).

Despite the abundance of GntR-like regulators, there have been relatively few studies that have extensively analyzed the protein and their operators to characterize protein function, especially in the MocR subfamily. MocR in *S. meliloti* has been known to be involved in facilitating competition in rhizosphere to initiate nodulation with *Medicago sativa* by regulating the synthesis of the symbiosis-specific compound rhizopine (Rossbach *et al.*, 1994). Despite the possibility that MocR plays a role in the rhizobia-legume symbiosis, a few MocR-type regulators have been characterized in other rhizobia. In this study, we investigated the role of the *B. japonicum* locus blr6977, presumably encoding a MocR transcriptional regulator, by constructing a *mocR* mutant strain and then comparing its physiological and symbiotic properties with the wild type.

Materials and Methods

Strains, plasmids, and growth conditions

The bacterial strains and plasmids used throughout this study are listed in Table 1. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) media (Sambrook *et al.*, 1989). *B. japonicum* strains were grown in arabinose-gluconate (AG) media (Sadowsky *et al.*, 1987) at 30°C and at 200 rpm when grown in liquid media. For phenotype tests such as growth measurement, colony morphology comparison, and motility test, *B. japonicum* was also grown in minimal media (Chang *et al.*, 2007) or RDY media (So *et al.*, 1987). Antibiotics used for strain or plasmid selection were ampicillin (50 μg/ml for *E. coli*, unless indicated otherwise), streptomycin (50 μg/ml for *E. coli*), kanamycin (50 μg/ml for *E. coli* and 150 μg/ml for *B. japonicum*), tetracycline (15 μg/ml for *E. coli* and 50 μg/ml for *B. japonicum*), and chloramphenicol (30 μg/ml for *B. japonicum*).

Construction of a *mocR* mutant and its complementary strain

A 2.5 kb fragment containing the *mocR* gene, blr6977, was amplified by PCR with primers mocR_fw (5′-CTGATCA GCGTGCAGGCAGA-3′) and mocR_rv (5′-AGTGGGTTT CCCTGAGTTTT-3′). The PCR product was inserted into *Xcm*I-digested pKO-Ω (Windgassen *et al.*, 2000). The gene was then disrupted by the insertion of a 1.5-kb kanamycin-resistant gene from pKD4 at the *Bsr*GI site of the *mocR* gene. The resulting plasmid was introduced into the *B. japonicum* USDA110 strain by tri-parental mating with pRK2073 as a helper plasmid (Leong *et al.*, 1982). Transconjugants generated by only double-crossover homologous recombination were screened based on antibiotic selection. Double-crossover mutants are kanamycin-resistant but streptomycin-sensitive, whereas single-crossover mutants are resistant to both kanamycin and streptomycin. Disruption of the *mocR* gene by the kanamycin-resistant gene was confirmed by colony PCR (data not shown).

To restore the function of *mocR* in the mutant, the complemented strain was constructed. A 1.5-kb fragment including the whole *mocR* gene was amplified using primers mocR_comp_fw (5′-CCGGAATTCAGTCGAGACAATAC GCG-3′) and mocR_comp_rv (5′-GGGTTCGAAGATGC AGCGGAAGAGG-3′). The PCR product was cloned into pGEM T-easy vector and the resulting construct was digested with *Apa*I and *Spe*I. The linearized plasmid DNA was then inserted into *Apa*I/*Spe*I-digested pBBR1MCS-3 (Kovach *et al.*, 1994). The complementary vector was transferred into the *mocR* mutant strain by tri-parental mating described above. The complemented strain was selected based on the resistance to both kanamycin and tetracycline and confirmed by colony PCR (data not shown).

Motility test

To compare the motility, swarming test was performed on semi-agar plates. The bacterial strains were grown to an

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristic	Source or reference
B. japonicum strains		
USDA110	Wild-type	USDA
mocR mutant	*mocR*::Km	This study
mocR complement strain	*mocR*::Km with pBBR1MCS3 vector with *mocR* insert	This study
E. coli strains		
DH5α	*supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
JM109	*F′ (traD36, proAB*$^+$ *lacI*^{q}, *Δ(lacZ)*M15) *endA1 recA1 hsdR17 mcrA supE44*λ^- *gyrA96 relA1 Δ(lac*$^-$*proAB) thi-1*	Yanisch-Perron *et al.* (1985)
Plasmids		
pKO-Ω	Sp^R Sm^R	Windgassen *et al.* (2000)
pKO-MocR	*mocR* clone in pKO-Ω; Sp^R Sm^R	This study
pKO-MocR::Km	Km^R	
pRK2073	RK2, Tra$^+$ Sm^R	Leong *et al.* (1982)
pGEM-T easy	Amp^R, vector with MCS within α-peptide coding region of β-galactosidase	Promega
pBBR1MCS3	Broad host range expression vector, Tc^R	Kovach *et al.* (1994)
pGEMTE-MocR	pGEMTE vector containing 1.5-kb fragment including entire *mocR* gene	This study
pBBRIMCS3-MocR	pBBR1MCS vector containing 1.5-kb fragment including entire *mocR* gene	This study

OD_{600} of 0.8. Aliquots of 5 µl of the bacterial cultures were dropped on the surface of AG agar (0.3% and 1% agar) and minimal media agar (0.3% and 1% agar) and incubated at 30°C for 7 days. The diameters of the growth were recorded for quantification of motility.

Biofilm assay

Biofilm-forming ability was measured by a standard microtiter plate method. The bacterial cells grown until early stationary phase were diluted in the minimal media (Chang *et al.*, 2007) to give an optical density at 600 nm (OD_{600}) = 0.001. The cell suspension (200 µl) was transferred into each well of a 96-well microtiter plate. The plate was incubated at 30°C for 6 days. The cell density was measured by reading the absorbance at 600 nm wavelength on a plate reader (Synergy 2; BioTek). Planktonic cells were removed by washing the wells with sterile double-distilled water (ddH_2O) three times, and the plate was air-dried. A 200-µl volume of 1% crystal violet solution was added into each well to stain biofilm cells. The plate was incubated at room temperature for 45 min in the dark condition. Unbound dye was removed by washing with ddH_2O three times. The crystal violet bound to biofilm cells was dissolved in 200 µl of 100% ethanol, then half the volume (i.e., 100 µl) was transferred to a new 96-well microtiter plate. The crystal violet amount for biofilm cells was measured by reading the absorbance at 595 nm. The relative biofilm forming ability was calculated by dividing the A_{595nm} for the total crystal violet by the A_{600nm} for the cell density.

Nodulation assay

Nodulation assay was performed by the method described in the previous study (Lee *et al.*, 2012). Bacterial cultures for nodulation assay were grown to an OD_{600} of 1 at 30°C at 200 rpm and diluted to an OD_{600} of 0.1 in half-strength Broughton and Dilworth (B&D) media (Broughton and Dilworth, 1971), which did not contain nitrogen. Soybean seeds were sterilized by a sequential procedure of immersion in 30% Clorox for 10 min, washing three times with sterile ddH_2O, immersion in 0.1 M HCl solution for 10 min, and washing three times in sterile ddH_2O. The seeds were then germinated in petri dishes with sterile M3 filter paper soaked in 10 ml of sterile deionized water. The germinated seedlings were transferred into CYG seed germination pouches (Mega International) and each was inoculated with 1 ml of the diluted bacterial culture (OD_{600} = 0.1). The pouches were maintained in a growth chamber at 28°C with a light cycle of 16-h day and 8-h night per day and treated with 10–20 ml of half-strength B&D solution when needed. The root nodules were counted after 28 days-post-inoculation (dpi).

Competition assay

Soybean seedlings were inoculated with a mixture of the wild type and the *mocR* mutant cultures (1:1 ratio) and grown

Table 2. Subfamily domains of 14 GntR-like regulators and qRT-PCR primers used in this study

Gene locus	Subfamily domain	Primer	Sequence (5′ to 3′)	Amplicon size (bp)
blr1043	FadR	Forward	GAAGAGCTGCGTGTCCTCA	81
		Reverse	ATAGATCGCGTTGTGGAAGC	
blr1279	FadR	Forward	GAGGCCAATGACGTCGATAC	90
		Reverse	CTCTGCCAGAAACGAGTTGTG	
bll3158	FadR	Forward	AGGAGATTCGTCGTGTCACTG	107
		Reverse	TTGTTCTTGAAGCTGCTCTCC	
blr3319	FadR	Forward	GCGTCGAAATCTCTTAGCAAC	106
		Reverse	GATATCCACCTGCCAACTCG	
blr3325	MocR	Forward	CAAGAACGATGTCTGGGTGAT	124
		Reverse	CGAGAAGGTGCCGAAATAAAT	
blr3551	FadR	Forward	TTCTCGACGATATCTGGATGC	84
		Reverse	GATGCTCGTTCGGATAAATTG	
bll3877	FadR	Forward	CGCATCATCAAGGCTCATATC	93
		Reverse	TCATGATGACCTCTCCTCGAC	
bll4018	FadR	Forward	CCTTCCACGACGAACTCTTT	110
		Reverse	GCTGTATTGCCGCATCTTCT	
bll4154	MocR	Forward	GATCCGATGGTGTGCTGTC	154
		Reverse	CGTGTGCGTGAATAGGTGTC	
bll4368	FadR	Forward	TGGAACTTGCATATTTGGACTG	102
		Reverse	ACGACCTCCTCATGAAGCAT	
blr4427	FadR	Forward	TCGTCTCGGAACTGTCACTG	138
		Reverse	CGCCTTCTCGAAACTCTTGT	
blr6040	FadR	Forward	ATTACGGACGAGGAAATCACC	99
		Reverse	CTGGTTGGTCTGGAAATAGCC	
blr6254	HutC/FarR	Forward	TCGCGCATGACCGTGAACAA	118
		Reverse	TCTTGAGCACCGCGGACTGAT	
blr7971	FadR	Forward	AAGCTCGATACGCCAAAATC	212
		Reverse	AAGACAAAACTGCCGACCTG	

for 4 weeks under the same condition described above. Bacterial cells were isolated from root nodules by the method of Vincent (1970). Briefly, a nodule was detached from the soybean root, rinsed three times in sterile water, exposed momentarily to 100% ethanol, immersed in 0.1% acidified $HgCl_2$ for 10 min, and rinsed again in sterile water at least six times. Subsequently the washed nodule was transferred into a microcentrifuge tube containing 500 µl of sterile water and crushed aseptically. The number of rhizobial cells was measured by serial dilution of the suspension of crushed nodule and plate counting. Each dilution was spread on two different AG agar plates for selection of the mutant cells: one containing only chloramphenicol and the other containing both chloramphenicol and kanamycin. The CFU calculated from the latter was considered as the number of the mutant cells, while the number of the wild type cells was calculated by subtracting the number of the mutant cells from the CFU obtained from the former. For nodules occupied by both the wild type and the mutant, the competition index was calculated by the formula [competition index = $\log_2 (M / W)$], where M is the number of the mutant cells and W is that of the wild type. Zero, negative, and positive indices indicate equal occupation, more wild type cells, and more mutant cells, respectively.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR) analysis

To compare the expression of several *gntR* genes in the *B. japonicum* wild type and the *mocR* mutant strains, quantitative reverse transcription-PCR (qRT-PCR) was conducted. *B. japonicum* wild type and the *mocR* mutant strains were cultured and harvested in minimal media to an OD of 0.5–0.8. Isolation of total RNA was conducted by the hot phenol extraction method as described previously (Bittner *et al.*, 2003). Then DNase treatment and RNA purification were carried out using the RNase-free DNase set and RNeasy mini-kit, respectively (Qiagen). Specific primers were designed for 14 of the *gntR* genes selected and are listed in Table 2. The qRT-PCR process was performed as previously described (Franck *et al.*, 2008). The histidyl-tRNA synthetase (*hisS)* gene was selected as a reference gene to normalize the expression data (Chang *et al.*, 2007).

Results and Discussion

blr6977 encodes MocR, a subfamily of GntR transcription factors

We searched for all GntR superfamily transcription regulator genes in the *B. japonicum* genome. According to the annotation by Kazuza DNA Research Institute, *B. japonicum* has 35 genes encoding putative GntR regulators (http://genome.microbedb.jp/rhizobase/). These GntR regulators are categorized into 4 subfamilies - FadR, HutC, MocR, and PhnF - by conserved domain sequence search (Supplementary data Table S1). Among them, MocR subfamily is distinguished by its lengthy C-terminus, which has high homology to class I aminotransferase enzymes involved in amino acid metabolism (Hoskisson and Rigali, 2009). Class I aminotransferases are dimeric proteins that work by catalyzing the transfer of an amino group to an acceptor molecule such as an aldehyde or keto acid with the cofactor pyridoxal-5′-phosphate (PLP) (Bramucci *et al.*, 2011). This suggests the possibility of catalytic activity in the C-terminal domain of the protein. MocR-type regulators have been extensively studied in bacteria such as *B. subtilis*, *Rhodobacter capsulatus*, and *Corynebacterium glutamicum* (Belisky, 2004; Wiethaus *et al.*, 2008; Jochmann *et al.*, 2011). Surprisingly, the role of MocR subfamily has not been characterized in rhizobia, although some GntR-like transcriptional regulators have been identified in *S. meliloti* (Wang *et al.*, 2008). Thus, blr6977 (MocR subfamily) from *B. japonicum* has been in our interest to be characterized from a nitrogen-fixing symbiotic perspective.

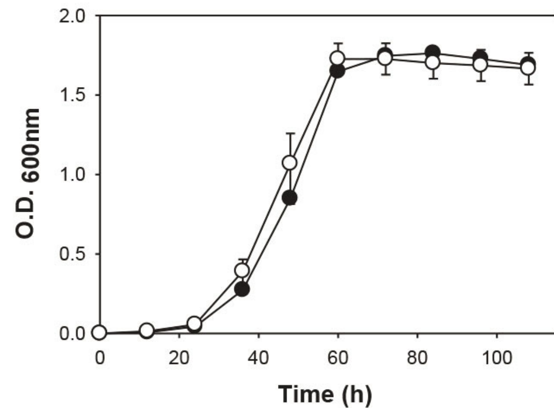

Fig. 1. Growth comparison between the wild type (●) and the *mocR* mutant (○). The bacteria were aerobically grown in AG media at 30°C. Values are means ± standard errors of the means for three biological replicates.

The disruption of *mocR* enhances motility of *B. japonicum*

To investigate the function of *mocR* (blr6977) in *B. japonicum*, we constructed a site-specific mutant and its complementary strain and compared their phenotypic characteristics related with nodulation and nitrogen fixation. First, their growth and morphological phenotypes were examined. No significant difference was observed for generation time between the wild type and the *mocR* mutant (Fig. 1). All three strains including the complemented strain showed ca. 9 h doubling time in AG media. We also checked their growth rates in minimal media and observed no significant difference, showing ca. 20 h doubling time for all three strains. Colony shape between the wild type and mutant strain was indistinguishable in both AG and minimal media (data not shown). In *S. meliloti*, however, the mutation on the two *gntR*-like *gtrA* and *gtrB* genes affected the growth, indicating that the same gntR superfamily, but different subfamily, may have a distinct role in bacterial growth and morphology.

Motility is considered as one of the key factors to determine the ability of rhizobia to successfully nodulate their host plants (Ames and Bergman, 1981; Caetano-Anolles *et al.*, 1988). Thus, motility tests were conducted for the wild type, mutant, and complementary strains on semi agar (0.3%) plates. The *mocR* mutant had a significantly wider mean di-

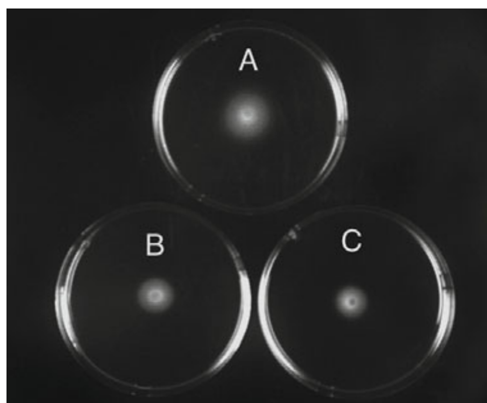

Fig. 2. Motility test on semi-agar plates of minimal media. (A) the *mocR* mutant; (B) the wild type; (C) the complemented strain.

ameter of 23.2 ± 0.2 mm compared to the wild type and the complementary strains which had mean diameters of 16.8 ± 0.2 mm and 12.2 ± 0.2 mm, respectively (Fig. 2). These phenotypic results of increased motility by mutation on the *mocR* gene are contrary to a previous study by Wang and colleagues in which the mutants of two GntR-like regulator genes, *gtrA* and *gtrB*, were deficient in motility (Wang *et al.*, 2008), indicating that MocR in *B. japonicum* might regulate motility-related cellular processes in a different manner from other GntR-like regulators.

The disruption of *mocR* decreases biofilm formation in *B. japonicum*

In addition to motility, it is obvious that the attachment (i.e., biofilm formation) of rhizobial cells to soybean root hairs is essential to establish the soybean-*B. japonicum* symbiosis (Bogino *et al.*, 2013). To check if *mocR* is involved in the biofilm formation of *B. japonicum*, we compared the biofilm forming ability among the wild type, the *mocR* mutant, and the complemented strains by the microtiter plate method. As shown in Fig. 3, relative biofilm amount among three strains do not seem to be different since the different range is within ~ 0.02 between the wild type and the *mocR* mutant. Nevertheless, the *mocR* mutant statistically showed reduced biofilm forming ability compared to the wild type and its complemented strain (Fig. 3). This shows the possibility that the MocR protein may allow *B. japonicum* to successfully attach on soybean root surfaces. However, in planta GntR family proteins are known to play an important role in biofilm formation in some pathogenic bacteria. A *Streptococcus mutans* mutant for *pdxR*, which encodes a member of MocR/GabR family proteins, showed reduced biofilm formation (Liao *et al.*, 2015). As an important pathogenic microorganism in oral microbiology, *S. mutans* can cause the formation of dental cavities. A genetic locus EF1809, encoding a GntR family protein, in *Enterococcus faecalis* is also known to be involved in biofilm formation (Ballering *et al.*, 2009). These results indicate that regardless of pathogenic or symbiotic relationship, GntR superfamily including MocR subfamily is a key factor for bacterial attachment, which is the first step to establish host-microbe interactions.

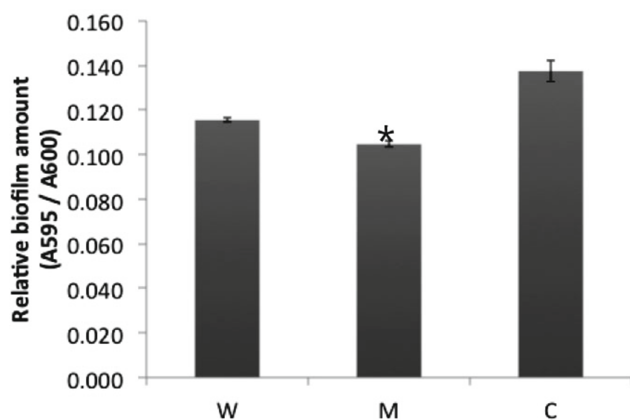

Fig. 3. Biofilm forming ability comparison among the wild type (W), the *mocR* mutant (M), and the complemented (C) strains. The relative biofilm amount was calculated by normalizing the total biofilm amount with the cell density as described in the method section. Data are means ± standard errors from 3 biological replicates with 4 technical replicates. An asterisk above bars indicates statistical difference each other based on Student's *t*-test ($P < 0.05$).

Interestingly, the complementary strain showed the highest biofilm-forming ability (Fig. 3). This could be explained by the expression of the promoter on the broad-host range plasmid vector pBBR1MCS-3 (Kovach *et al.*, 1994) which is presumably stronger than the *mocR* promoter on the *B. japonicum* chromosome.

Increased motility and reduced biofilm formation in the *mocR* mutant are similar to characteristics of *sadB* and *sadC* mutants of *Pseudomonas aeruginosa*, an opportunistic pathogen. Deletion of the *sadB* or *sadC* gene results in defective in biofilm formation, but increased swarming motility (Caiazza *et al.*, 2007; Merritt *et al.*, 2007). Like SadB and SadC, MocR may inversely regulate biofilm formation and motility via exopolysaccharide (EPS) production and control of flagellar machinery. In contrast, several lipopolysaccharide (LPS) mutants of *B. japonicum* showed the opposite phenotype such as reduced motility, but increased biofilm formation (Lee *et al.*, 2010, 2014).

The mutation on *mocR* causes delayed nodulation in *B. japonicum*

Given that nonmotile mutants of rhizobia are typically considered deficient in nodulation on host plants (Ames and Bergman, 1981; Caetano-Anolles *et al.*, 1988), it was expected that the *mocR* mutant would form more nodules on soybean roots as the mutant was more motile than the wild type. To determine the nodulation efficiency of the *mocR* mutant, pouch experiments were conducted. The average number of nodules formed by the wild-type or mutant strain was 9.44 ± 0.70 or 8.16 ± 0.51 nodules per plant, respectively (Fig. 4A). Although the mutant seemed to induce less nodulation, there was no significant difference in the number of nodules between the wild type and mutant strains (Fig. 4A). In addition to counting the number of nodules after a period of 4 weeks, the distance of each nodule from the original root tip (RT) marked at time zero was measured to help determine the timing of nodulation. Nodules that are located close

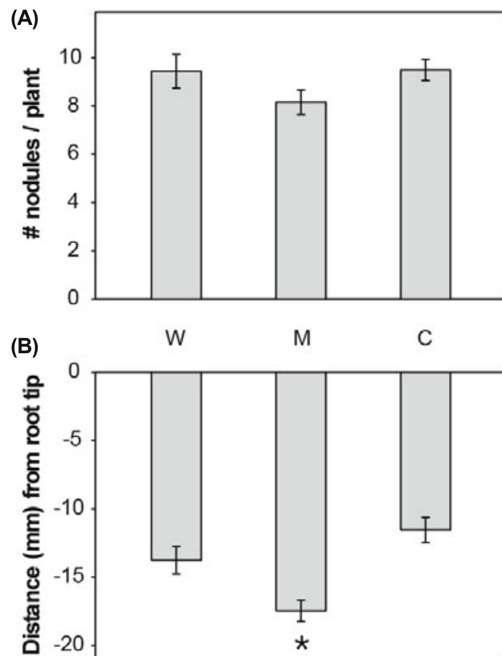

Fig. 4. The effect of *mocR* on nodulation of the wild type (W), the *mocR* mutant (M), and the complemented (C) strains. (A) The average number of nodules per plant. (B) The average distance (mm) of nodules from root tip (RT) marked at the time of inoculation. Values are means ± standard errors of the means. The asterisk above bars indicates statistical difference each other based on Student's *t*-test ($P < 0.05$).

to the RT indicate earlier formation of the nodule, whereas nodules positioned more distantly from the RT points to delayed nodulation. As shown in Fig. 4B, more nodules were formed near the RT in the wild type compared to the mutant. A total of 159 and 165 nodules were examined for the wild-type and mutant strains, respectively, which can eliminate potential bias of sampling size, if any. Therefore, this result indicates that nodule formation is delayed in the mutant strain.

The *mocR* complemented strain, like in the case of the motility and biofilm experiments, was able to restore the wild type phenotype in the nodulation experiment. The average number of nodules formed by the complemented strain was 9.50 ± 0.44 nodules per plant (Fig. 4A) and the average distance from the RT was -11.54 ± 0.92 mm per nodule (Fig. 4B).

The effect of MocR in competitiveness

Delayed nodulation and less biofilm formation in the mutant suggests that MocR transcriptional regulator could be involved in symbiotic competence in the rhizosphere. To investigate competitiveness of the *mocR* mutant infecting soybeans, we performed the competition assay. When the soybean was inoculated with the mixture of the wild type and the mutant at 1:1 ratio, 21.7% of nodules were occupied by the wild type alone, whereas 8.7% were occupied by the mutant, indicating that the wild type outcompeted the mutant. This result is consistent with the previous study by Wang and colleagues. Their finding was that when co-inoculated with the wild type, the mutants for two GntR-type regulators GtrA and GtrB in *S. meliloti* were less competitive in nodulation on alfalfa roots than the wild type (Wang *et al.*, 2008). Less competitiveness in the mutant could be due to the mutation effect on bacterial growth. This is not the case for *B. japonicum* since doubling times between the wild type and mutant strains are not different (Fig. 1). However, less competiveness of the two *S. meliloti gntR*-like *gtrA* and *gtrB* mutants compared to the wild type might be because of growth defect in the mutants.

Interestingly, the other 69.6% of the nodules were occupied by both strains. The average competition index [$\log_2$ (M / W)] for the nodules occupied by both strains was -0.02 ± 0.38. This indicates that neither the wild type nor the *mocR* mutant is predominant over the opponent when concurrently occupying the same soybean nodule.

MocR (Blr6977) does not affect expression of other MocR-like transcriptional regulators

A number of GntR-like regulators can regulate not only other operons but also their own promoter (Hoskisson and Rigali, 2009). To date, MocR-type regulators characterized include GabR, TauR, and PdxR. GabR from *B. subtilis* is a positive transcriptional regulator of *gabTD* operon that encodes key enzymes for γ-aminobutyrate (GABA) utilization (Belitsky, 2004). TauR from *R. capsulatus* is a regulator of two separate operons, *tpa-tauR-xsc* and *tauABC*, which are essential for taurine utilization as a sulfur source and its uptake, respectively (Wiethaus *et al.*, 2008). PdxR regulates the synthesis of pyridoxal 5′-phosphate (PLP) in *C. glutamicum* by positively regulating *pdxST* operon (Jochmann *et al.*, 2011).

In addition, it is possible that GntR-like regulators could regulate other transcription factors. Thus, it was tested if MocR affects expression of GntR-like proteins. The expression of the 14 genes that contain subfamily domains (e.g., FadR, MocR, and HutC/FarR) was compared between the wild type and *mocR* mutant by the qRT-PCR analysis. Of the 14 genes, 11 were homologous to the FadR subfamily, 2 had homologies similar to the MocR subfamily, and 1 was linked to the HutC/FarR subfamily (Table 2). The qRT-PCR analysis revealed that all of the *gntR*-like genes tested did not show a significant fold induction relative to the control *hisS* gene in both the wild type and mutant strains, including the *mocR* gene itself (Supplementary data Fig. S1). Also, there was no significant difference between the both strains for each gene. The only gene that seemed to have a large fold induction in both the *mocR* mutant and wild type strains was the bll3158 gene, a member of the FadR subfamily. Interestingly, expression of the other 2 MocR-type transcriptional regulators (blr3325 and bll4154) was not affected by MocR in *B. japonicum*.

Conclusion

In this study, it was found that mutation on *mocR* caused delayed nodulation and influenced cellular processes such as motility and attachment/biofilm formation. These findings showed that phenotypic changes by the *mocR* mutant of *B. japonicum* were considerably contrary to those by other GntR mutants in previous studies. This suggests that the *B. japo-*

nicum MocR probably has distinct features from two GntR-like regulators in *S. meliloti*, another rhizobium, but possesses a different host range. To the best of our knowledge, this is the first report on characterization of GntR-like regulator MocR from *B. japonicum*.

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