The *Sinorhizobium fredii* HH103 MucR1 Global Regulator Is Connected With the *nod* Regulon and Is Required for Efficient Symbiosis With *Lotus burtii* and *Glycine max* cv. Williams

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Sinorhizobium fredii HH103 is a rhizobial strain showing a broad host range of nodulation. In addition to the induction of bacterial nodulation genes, transition from a free-living to a symbiotic state requires complex genetic expression changes with the participation of global regulators. We have analyzed the role of the zinc-finger transcriptional regulator MucR1 from *S. fredii* HH103 under both free-living conditions and symbiosis with two HH103 host plants, *Glycine max* and *Lotus burtii*. Inactivation of HH103 *mucR1* led to a severe decrease in exopolysaccharide (EPS) biosynthesis but enhanced production of external cyclic glucans (CG). This mutant also showed increased cell aggregation capacity as well as a drastic reduction in nitrogen-fixation capacity with *G. max* and *L. burtii*. However, in these two legumes, the number of nodules induced by the *mucR1* mutant was significantly increased and decreased, respectively, with respect to the wild-type strain, indicating that MucR1 can differently affect nodulation depending on the host plant. RNA-Seq analysis carried out in the absence and the presence of flavonoids showed that MucR1 controls the expression of hundreds of genes (including some related to EPS production and CG transport), some of them being related to the *nod* regulon.

Legumes and a group of soil proteobacteria collectively called rhizobia (Peix et al. 2015) are able to establish a symbiotic interaction in which the microsymbiont infects plant

roots and multiplies inside a new plant organ, the nodule, in which they differentiate into nitrogen-fixing bacteroids (Oldroyd 2013; Suzuki and Kawaguchi 2014). This symbiosis is driven by a complex interchange of molecular signals between both symbionts, and therefore, the participation of diverse regulatory elements is required for a successful interaction (Downie 2010; Janczarek et al. 2015; López-Baena et al. 2016). On the bacterial side, the LysR-type transcriptional regulator NodD is activated by flavonoids exuded by the plant and induces the expression of bacterial genes related to the production of nodulation factors. These molecular signals are perceived by the plant, allowing root infection and triggering the nodule developmental program. In some rhizobia, NodD and plant flavonoids also induce the formation of a symbiotic type III secretion system (T3SS) able to inject effector proteins into plant cells in order to reduce plant defense responses (López-Baena et al. 2016). Diverse bacterial surface polysaccharides also participate in the symbiosis, acting as molecular signals required for one or both infection progression or diminishing defense responses. In addition to NodD (which can be present in different copies having different roles), other transcriptional regulators, such as TtsI (responsible for expression of the symbiotic T3SS), SyrM, and NolR, are necessary for the transition from a free-living to a symbiotically competent state and, therefore, for a successful interaction. In fact, NolR is a global regulatory protein, which, in *S. meliloti*, is required not only for the optimization of nodulation but also for bacterial growth and survival and conjugative transfer of a plasmid (Chen et al. 2005).

The RosR/MucR protein family is composed of transcriptional regulators that contain a Cys₂His₂ type zinc-finger motif (Keller et al. 1995). They are widely distributed in proteobacteria, in which they regulate very diverse processes, such as cell-cycle transition from S-phase to G1 in *Caulobacter* (Fumeaux et al. 2014), alginate production in the opportunistic pathogen *Pseudomonas aeruginosa* (Wang et al. 2015), or expression of the plant oncogene *ipt* in *Agrobacterium* spp. (Chou et al. 1998). In the animal pathogen *Brucella melitensis*, genes encoding a type IV secretion or a quorum-sensing system are downregulated in a *mucR* deletion mutant (Dong et al. 2013). In *Rhizobium leguminosarum* bv. *trifolii* and *Sinorhizobium meliloti*, RosR/MucR were first described as positive regulators of exopolysaccharide (EPS) biosynthesis (Janczarek and Skorupska 2007; Keller et al.

RNA-Seq data have been deposited in the Sequence Read Archive of the National Center for Biotechnology Information (BioProject database) under the BioProjects ID PRJNA313151 and PRJNA324263.

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1995). In addition, in the case of *R. leguminosarum* bv. *trifolii*, inactivation of *rosR* led to ineffective symbiosis with *Trifolium* spp. More recent works showed that the RosR protein of *R. leguminosarum* bv. *trifolii* regulates different processes, such as the synthesis of cell-surface components (including polysaccharides), motility, and bacterial metabolism (Janczarek et al. 2010; Rachwał et al. 2015). Therefore, RosR plays an important role in bacterial adaptation to environmental conditions. During the preparation of this manuscript, Jiao et al. (2016) reported that *Sinorhizobium fredii* CCBAU45436 MucR1 is essential for supporting nitrogen fixation of this strain in *Glycine max* JD17 nodules. In that work, the authors also provided a transcriptomic analysis showing that MucR1 positively regulates the expression of different inorganic ion transporters required for effective nitrogen fixation in CCBAU45436 bacteroids.

In this work, we have studied the regulatory roles of MucR1 in *S. fredii* HH103, a broad host range rhizobial strain able to nodulate American and Asiatic varieties of soybean (López-Baena et al. 2016; Margaret et al. 2011; Vinardell et al. 2015). Inactivation of HH103 *mucR1* decreased EPS and increased external cyclic glucans (CG) production, enhanced biofilm formation at the liquid-air interface (pellicle) and autoaggregation, and led to a severe impairment in symbiosis with *Lotus burtii* and *Glycine max* cv. Williams. Although, in both cases, host plants inoculated with the *mucR1* mutant showed symptoms of nitrogen starvation, the number of nodules formed were significantly decreased with *Lotus burtii* and increased with soybean in comparison with plants inoculated with the wild-type strain. Finally, transcriptomic analysis showed that, in addition to controlling genes involved in EPS and CG production, motility, and chemotaxis, HH103 MucR1 also affects genes included in the *nod* regulon, which could account for the symbiotic phenotype of HH103 *mucR1* with *L. burtii*.

RESULTS AND DISCUSSION

Sinorhizobium fredii HH103 harbors two copies of *mucR*.

Sequence analysis of the genome of *S. fredii* HH103 revealed the presence of two copies of the *mucR* gene (Vinardell et al. 2015; Weidner et al. 2012). One of them, *mucR1* (SFHH103_00694), is located on the chromosome and encodes a 143-amino acid protein that is 99 and 80% identical to the MucR and RosR transcriptional regulators from *S. meliloti* 1021 and *Rhizobium leguminosarum* bv. *trifolii*, respectively. The second copy, *mucR2* (psfHH103d_392), is located on the symbiotic plasmid close to the *nodD1* gene and encodes a protein of 142 residues that is 81% identical to HH103 MucR1. The MucR1 and MucR2 proteins of *S. fredii* HH103 are 100% identical to the corresponding proteins recently described in *S. fredii* CCBAU45436 (Jiao et al. 2016). In strain CCBAU45436, MucR2 is not functionally equivalent to MucR1, due to a frameshift mutation.

In order to analyze the roles of HH103 *mucR1* and *mucR2* in free-living conditions and in symbiosis, the following mutants were constructed as described in Supplementary Methods: SVQ706 (Δ *mucR1*), SVQ719 (*mucR2::lacZAp-Gm^R*), SVQ720 (*\Delta**mucR1 mucR2::lacZAp-Gm^R*), and SVQ721 (*mucR1::lacZAp-Gm^R*). A complemented version of SVQ706, called SVQ706C, was constructed by introduction of a wild-type version of the *mucR1* gene in *cis*.

S. fredii HH103 MucR1 is a positive regulator of EPS production.

The MucR/RosR proteins have been described to be involved in regulation of polysaccharide production in different rhizobia (Janczarek and Skorupska 2011). For this reason, the production of EPS by the different *mucR* mutants of HH103 was analyzed on yeast extract-mannitol agar (YMA) plates. In *S. fredii* HH103, the presence of flavonoids has a negative effect on EPS production (Acosta-Jurado et al. 2016; Vinardell et al. 2004a). For this reason, production of EPS was investigated both in the absence and in the presence of genistein, an effective *nod* gene inducer for HH103 (Vinardell et al. 2004b). Regardless of the presence of genistein, all the mutants affected in *mucR1* showed a rough appearance, suggesting a drastic reduction in EPS production (Fig. 1). The *mucR2* single mutant, instead, was undistinguishable from the wild-type strain, both in the presence or absence of flavonoids. The production of EPS by HH103 Δ *mucR1* in liquid YM medium was also quantified by the anthrone-H₂SO₄ method. These experiments showed that HH103 Δ *mucR1* produced only 17.4 ± 6.2 μ g of sugar per milliliter of supernatant in contrast to the 83.2 ± 16.8 μ g/ml produced by the parental strain HH103. The complemented *mucR1* derivative recovered smooth appearance on YMA plates (Fig. 1) as well as wild-type levels of EPS production (119.2 ± 32.5 μ g/ml). In the presence of genistein, the production of EPS was clearly reduced in the three strains (6.9 ± 0.8 , 4.7 ± 1.0 , and 6.7 ± 5.2 μ g of sugar per milliliter of supernatant for HH103, Δ *mucR1*, and the complemented version of the mutant, respectively).

Production of EPS by HH103 Δ *mucR1* was further analyzed by nuclear magnetic resonance (NMR) spectroscopy. In order to compare NMR spectra from HH103 and HH103 Δ *mucR1*, residual signals from a mannan present in culture media were used as reference (Fig. 2, marked with asterisks). Mutant Δ *mucR1* presented the signals corresponding to wild-type EPS (those corresponding to the anomeric region [zoomed on the left in Figure 2] and those corresponding to acetyl and pyruvate groups), but the intensity of the signals in the mutant was very weak in comparison with those of HH103, confirming that the absence of MucR1 led to a drastic decrease in the production of this polysaccharide. Introduction of a wild-type copy of *mucR1* restored wild-type levels of EPS production. These results confirmed that MucR1 is a positive regulator of EPS production in *S. fredii* HH103, in agreement with previous results in *S. meliloti* 1021, *Rhizobium*

Fig. 1. Colony appearance on yeast extract-mannitol agar (YMA) plates by *Sinorhizobium fredii* HH103 Rif^R (wt) and different *mucR1* or *mucR2* derivatives in the absence (upper row) or the presence (bottom row) of genistein. The *mucR1* deletion is denoted as Δ *mucR1*, whereas mutations constructed by fusions to the *lacZAp-Gm^R* cassette are denoted as *mucR1* or *mucR2*. The complemented version of the Δ *mucR1* mutants is indicated as Δ *mucR1C*.

leguminosarum bv. *trifolii* Rt24.2, and *S. fredii* CCBAU45436 (Janczarek 2011; Jiao et al. 2016). Our results also show that HH103 MucR2 has no role in EPS production, which is in coherence with the fact that this protein is 100% identical to the nonfunctional MucR2 protein of *S. fredii* CCBAU45436 (Jiao et al. 2016).

Inactivation of *S. fredii mucR1* affects biofilm formation ability and autoaggregation.

As inactivation of *mucR1* led to a dramatic decrease of EPS production by HH103, we investigated whether it could also affect the ability of this strain to form biofilms. For this purpose, HH103, its Δ *mucR1* mutant, and the complemented derivative of this mutant were grown in MGM medium (O'Toole and Kolter 1998) in polystyrene microtiter plates. Formation of biofilms was investigated both in the absence and in the presence of genistein (Supplementary Fig. S1). The biomass of the biofilm was measured by staining with crystal violet as described below. The amount of biofilm formed by HH103 Δ *mucR1* was significantly higher than that of the parental strain and was not affected by the presence of genistein. The same results were obtained with the *mucR1::lacZ* Δ p-Gm^R and with the double *mucR1 mucR2* mutant (data not shown). The introduction of a wild-type copy of *mucR1* in the Δ *mucR1* mutant restored normal levels of biofilm formation. The increased ability for biofilm formation exhibited by HH103 Δ *mucR1* was surprising, since, in previous works, we have shown that EPS is an important trait for biofilm formation by *S. fredii* HH103 (Acosta-Jurado et al. 2016; Rodríguez-Navarro et al. 2014). In addition, in the microtiter wells inoculated with the Δ *mucR1* mutant (especially in the cultures grown in the absence of genistein), a floating and translucent layer was observed. Thus, the increased biofilm formation capacity exhibited by HH103 Δ *mucR1* appears to correspond to the formation of an aggregate of cells in the liquid-air interface (also called pellicle) rather than to a biofilm of cells attached to the liquid-covered plastic surface of the well.

The effect of *mucR1* inactivation on biofilm formation was also investigated on glass surfaces. For this purpose, plasmid

pSKDSRED (Kelly et al. 2013), containing a constitutively expressed red fluorescent protein, was introduced by conjugation into HH103, its Δ *mucR1* derivative, and the complemented version of this mutant (Δ *mucR1C*), and the resultant strains were grown in MGM medium in glass Coplin staining jars containing glass slides (discussed below; Supplementary Fig. S2). The biofilms formed on glass slides were scored at 24 and 96 h postinoculation by epifluorescence microscopy. Three different sections of the glass slide were examined, lower, medium, and upper, located at 5, 28, and 49 mm from the bottom of the slide, respectively. Thus, the lower and medium sections were sunken into the culture medium, whereas the upper section was located at the liquid-air interface. Results, presented in Supplementary Fig. S3 and Figure 3, revealed that the Δ *mucR1* mutant showed increased attachment with respect to both HH103 and Δ *mucR1C* and that this attachment was localized mostly at the liquid medium-air interface. Thus at 24 h postinoculation, both HH103 and Δ *mucR1C* showed a dispersed pattern of attachment in the three sections scored, whereas Δ *mucR1* displayed a layer of cells attached to the upper section. At 96 h (Fig. 3), this layer of cells was also formed by HH103 and Δ *mucR1C*, but it was much more evident for Δ *mucR1*. These results confirmed that the Δ *mucR1* derivative of *S. fredii* HH103 exhibits increased capacity to form biofilms onto inert surfaces in the liquid-air interface.

We have also analyzed whether the changes promoted by *mucR1* inactivation could influence bacterial autoaggregation capacity as described below (Supplementary Fig. S4). After 96 h of growth in liquid MGM medium with continuous shaking (180 rpm), the three *mucR1* mutants tested (Δ *mucR1*, *mucR1::lacZ* Δ p-Gm^R, and the double *mucR1 mucR2* mutant) showed elevated autoaggregation capacities (90.0 ± 3.1 , 76.4 ± 4.8 , and $87.6 \pm 1.7\%$ respectively), in clear contrast to the wild type, the *mucR2::lacZ* Δ p-Gm^R, and Δ *mucR1C* strains (1.0 ± 0.7 , 1.1 ± 0.7 , and $2.7 \pm 3.0\%$, respectively). In order to check whether the decreased production of EPS exhibited by the different *mucR1* mutants of HH103 could be the reason for the increased autoaggregation capacity observed in these mutants, strain SVQ530, an *exoA::lacZ* Δ p-Gm^R EPS-defective mutant of

Fig. 2. Nuclear magnetic resonance (¹H-NMR) (500 MHz, 353 K) spectra of exopolysaccharide (EPS) isolated from *Sinorhizobium fredii* HH103 Rif^R (marked as wt), HH103 Δ *mucR1*, and HH103 Δ *mucR1C*. Signals from acetyl (Ac) and pyruvate (Pyr) groups are indicated. Signals corresponding to the anomeric region of EPS are zoomed on the left. Residual signals from a mannan present in culture media were used as reference (marked with asterisks).

HH103, was also included in these studies, showing an intermediate autoaggregation capacity ($29.4 \pm 1.3\%$), which was significantly different (P value < 0.05) from those of the wild type and the $\Delta mucR1$ mutant strains. Thus, our results indicate that $mucR1$ inactivation results in an increased autoaggregation capacity of HH103, which is in agreement with the results, reported by Rachwał et al. (2015), for a *RosR* mutant of *R. leguminosarum* bv. *trifolii*. To elucidate whether this increase in autoaggregation depends only on a decrease in EPS or also in additional bacterial traits requires further investigation.

Inactivation of *S. fredii mucR1* does not affect lipopolysaccharide (LPS), K-antigen polysaccharide (KPS), and intracellular CG production but enhances secretion of external CG.

In rhizobia, several genes are involved in the production of more than one surface polysaccharide. For example, the *S. fredii rkp-3* region is involved in both KPS and LPS synthesis (Margaret et al. 2012). Because of this, we decided to investigate whether $mucR1$, $mucR2$, or both could affect the production of other surface polysaccharides in *S. fredii* HH103. For this purpose, both LPS- and KPS-enriched fractions of bacterial crude extracts from HH103 and its $\Delta mucR1$, $mucR1::lacZ\Delta p$ -Gm^R, $mucR2::lacZ\Delta p$ -Gm^R, and $\Delta mucR1 mucR2::lacZ\Delta p$ -Gm^R derivatives were prepared and were analyzed by polyacrylamide gel electrophoresis (PAGE). As shown in Supplementary Fig. S5, the LPS and KPS electrophoretic profiles of all the mutants tested were undistinguishable from those of the parental strain HH103. These results are in contrast to those reported by Janczarek et al. (2010), who found that *rosR* inactivation affected LPS O-antigen composition in *R. leguminosarum* bv. *trifolii*.

S. fredii HH103 $\Delta mucR1$ bacterial crude extracts were also analyzed by ¹H-NMR, which provides structural information about KPS and internal CG (Supplementary Fig. S6), showing

that inactivation of *mucR1* did not affect the production of these polysaccharides in *S. fredii* HH103.

On the other hand, the ¹H-NMR analysis of extracellular polysaccharides produced by HH103 $\Delta mucR1$ revealed that this strain, in addition to a decreased EPS production, showed higher amounts of extracellular CG than the parental strain HH103 (Fig. 4, signals shaded in gray). Complementation of the mutation led to wild-type levels of external CG. In order to elucidate whether this phenomenon was specifically due to *mucR1* inactivation or was a consequence of reduction of EPS production, supernatants of HH103 *exoA* were also analyzed by ¹H-NMR (Fig. 4). The *exoA* mutant of HH103 also exhibits enhanced production of external CG, suggesting that, in HH103 $\Delta mucR1$, this phenotype might be an indirect effect of the diminution of EPS synthesis.

MucR1 is an important regulator of symbiosis of *S. fredii* HH103 with *Glycine max* cv. Williams and *Lotus burtii*.

In previous investigations, the inactivation of *mucR* orthologs showed different effects on symbiosis depending on the host plants (Janczarek et al. 2009, 2010; Keller et al. 1995). A *mucR* mutant of *S. meliloti* was not affected in symbiosis with *Medicago* spp., whereas inactivation of the *R. leguminosarum* bv. *trifolii mucR* ortholog (*rosR*) led to a significant decrease in nodule number and nitrogen fixation with *Trifolium* plants. In this work, we decided to investigate the symbiotic effect of the mutation of HH103 in *mucR1* on symbiosis with two host legumes, *Glycine max* (the American variety Williams) and *Lotus burtii* (Margaret et al. 2011; Sandal et al. 2012). It is worthwhile to note that *S. fredii* HH103 infects these two host plants by different mechanisms, i.e., infection threads in soybean and crack-entry with *Lotus burtii* (A. Acosta-Jurado, D. N. Rodríguez-Navarro, Y. Kawaharada, J. Fernández Perea, A. Gil Serrano, H. Jin, Q. An, M. A. Rodríguez-Carvajal, S. U. Andersen, N. Sandal,

Fig. 3. Biofilm formation ability of *Sinorhizobium fredii* HH103 Rif^R (wt), its *mucR1* derivative ($\Delta mucR1$), and the complemented version of this mutant ($\Delta mucR1C$) harboring plasmid pSKDSRED on glass slides after 96 h of growth in MGM (O'Toole and Kolter 1998) medium. The amount of biofilm can be estimated as the fluorescence emitted by the attached bacterial population. Photos were taken at different heights of the glass slide (as indicated on the left), using Nikon Eclipse Ti-U epifluorescence microscopy with an excitation and emission wavelength of 554 and 586 nm, respectively.

J. Stougaard, J. M. Vinardell, and J. E. Ruiz-Sainz, unpublished; Margaret et al. 2011). Additionally, the *mucR2* and the double *mucR1 mucR2* mutants were also tested in soybean. Results presented in Tables 1 and 2 show that, in *S. fredii* HH103, inactivation of *mucR1* led to a severe impairment of symbiotic effectiveness with the two legumes tested. In fact, in both cases, plant shoot weights of plants inoculated with HH103 Δ *mucR1* were not different from those of noninoculated plants. On the other hand, inactivation of *mucR2* did not affect the symbiotic

Fig. 4. Nuclear magnetic resonance (^1H -NMR) (500 MHz, 303 K) spectra of fractions containing cyclic glucans (CG) after isolation from *Sinorhizobium fredii* HH103 Rif^R (marked as wt), HH103 Δ *mucR1*, HH103 Δ *mucR1C*, and an *exoA* derivative of HH103. Signals from CG are shaded in gray.

Table 1. Responses of *Glycine max* cv. Williams to inoculation with *Sinorhizobium fredii* HH103 and its *mucR1* and *mucR2* derivatives^a

Inoculant ^b	Nodule number	Nodule fresh weight (mg)	Plant shoot dry weight (g) ^c
HH103 Rif ^R	58.4 ± 10.6	834.6 ± 208.6	2.55 ± 0.74
HH103 Δ <i>mucR1</i>	126.2 ± 49.0*	766.6 ± 268.0	1.20 ± 0.42*
HH103 Δ <i>mucR1C</i>	52.6 ± 12.2	792.6 ± 147.1	2.74 ± 0.78
HH103 <i>mucR2</i> :: <i>lacZ</i> -Gm ^R	60.4 ± 6.7	829.2 ± 168.7	2.33 ± 0.97
HH103 Δ <i>mucR1</i> <i>mucR2</i> :: <i>lacZ</i> -Gm ^R	118.8 ± 21.6*	749.2 ± 39.9	1.54 ± 0.41*

^a Data represent averages of five Leonard jars, each one containing two soybean plants. Determinations were made 42 days after inoculation. All the treatments were individually compared with the values of the parental strain, using the nonparametric test of Mann-Whitney. The presence of an asterisk denotes a significant difference with a *P* value < 0.05.

^b Bacteria isolated from 12 nodules formed by each inoculant showed the expected resistance markers.

^c Plant shoot dry weight of noninoculated plants was 1.24 ± 0.21 g per jar.

performance of *S. fredii* HH103 with soybean ‘Williams’. The double *mucR1 mucR2* mutant exhibited the same phenotype as the Δ *mucR1* mutant. The complemented derivative of HH103 Δ *mucR1* was undistinguishable from the parental strain HH103 in the different parameters tested in their interaction with both soybean and *L. burtii*.

Strikingly, the nodule numbers induced by the Δ *mucR1* mutant were significantly higher with soybean but significantly lower with *L. burtii*, if compared with the nodule numbers induced by the parental strain. Acetylene reduction assays revealed that, in the case of soybean nodules, nitrogenase activity of the Δ *mucR1* derivative was ten-fold reduced with respect to that of the parental strain (309.8 ± 20.2 and 3,424.7 ± 971.2 nmoles ethylene per plant per hour, respectively). In *L. burtii*, the reduction of nitrogenase activity due to *mucR1* inactivation was less pronounced (95.0 ± 27.7 versus 295.6 ± 80.8).

During the preparation of this manuscript, an article describing the symbiotic role of the *S. fredii* CCBAU45436 *mucR1* and *mucR2* genes in their interaction with the Asiatic variety of *Glycine max* JD17 was published (Jiao et al. 2016). Jiao et al. (2016) found that inactivation of *mucR1* led to increased nodulation with this soybean variety but, also, to a dramatic impairment in nitrogen-fixation activity. The *mucR2* derivative of CCBAU45436 was not affected in its interaction with soybean JD17. Thus, our results are in agreement with those reported by Jiao et al. (2016), showing that, in *S. fredii* strains, *mucR1* but not *mucR2* is essential in the interaction with both Asiatic (as JD17) or commercially improved American (as ‘Williams’) varieties of soybean. In addition, our work shows that *mucR1* is also essential for HH103 symbiosis with another legume, *L. burtii*. However, in this legume, the symbiotic defect (a decrease in both nodule number and nitrogen fixation) appears to be different from that of the interaction of *S. fredii* with soybean and similar to that described for the *rosR* mutant of *R. leguminosarum* bv. *trifolii* with *Trifolium* spp. (Janczarek et al. 2010).

S. fredii HH103 MucR1 represses its transcription by binding to its own promoter.

Previous results have demonstrated that *S. meliloti* *mucR* and *R. leguminosarum* bv. *trifolii* *rosR* are negatively autoregulated (Janczarek and Skorupska 2007; Keller et al. 1995). In this work, we studied whether *S. fredii* HH103 MucR1 has an effect on its own transcription. A 408-bp fragment containing the upstream sequence of the *mucR1* gene as well as its start codon was fused to the *egfp* gene (coding for an enhanced version of the green fluorescent protein) present in plasmid pPHU231 (discussed below), which allows *mucR1* expression to be analyzed by measurement of fluorescence. The resultant plasmid, called pMUS1196, was introduced in both *S. fredii* HH103 Rif^R and its Δ *mucR1* derivative. In the *mucR1* mutant background, GFP expression (the ratio of fluorescence to optical density at

Table 2. Responses of *Lotus burtii* to inoculation with *Sinorhizobium fredii* HH103 and its *mucR1* derivative^a

Inoculant ^b	Nodule number	Plant shoot fresh weight (mg) ^c
HH103 Rif ^R	78.5 ± 20.6	1180.8 ± 386.8
HH103 Δ <i>mucR1</i>	42.4 ± 12.9*	235.2 ± 36.2*
HH103 Δ <i>mucR1C</i>	70.0 ± 14.8	973.1 ± 379.5

^a Data represent averages of at least four jars containing four plants each. Determinations were made 56 days after inoculation. All the treatments were individually compared with the values of the parental strain, using the nonparametric test of Mann-Whitney. The presence of an asterisk denotes a significant difference with a *P* value < 0.05.

^b Bacteria isolated from 12 nodules formed by each inoculant showed the expected resistance markers.

^c Plant shoot fresh-weight of noninoculated plants was 82.6 ± 27.1 mg per jar.

600 nm [OD₆₀₀]) driven by the *mucR1* promoter was 3.5-fold higher (363.19 ± 22.01) than that measured in the wild-type strain (100.00 ± 4.39), which suggests that MucR1 acts as a negative autoregulator.

In order to check whether HH103 MucR is able to bind to its own promoter, this protein was fused to GST (glutathione S-transferase), was overexpressed in *Escherichia coli*, was purified, and was used for EMSA (electrophoretic mobility shift assay) studies. As shown in Supplementary Figure S7, *S. fredii* HH103 MucR1 binds to a 399-bp DNA fragment that covers the upstream sequence (from -405 to -7) of *S. fredii mucR1*. When different internal fragments of this sequence were used in EMSA assays, binding of MucR1 was detected to fragments 2 (302 bp, between -405 and -104, with respect to the start codon) and 3 (271 bp, from -277 to -7), which overlap in 174 nucleotides. Fragment 3 contains a well-conserved MucR-binding (from -50 to -33) site, as defined for the *S. meliloti mucR* promoter (Bertram-Drogatz et al. 1997), but this site is not located in the sequence common to fragment 2. In fact, *S. fredii* MucR1 does not bind to fragment 4 (111 bp, from -117 to -7), which contains this putative MucR-binding site. Sequence analysis of the 174-bp sequence common to fragments 2 and 3 showed that it does not contain any well-conserved MucR- or RosR-binding site. Thus, we have detected binding of MucR1 to its own promoter sequence but, surprisingly, this binding appears not to be dependent on a well-conserved MucR binding site or, maybe, in addition to this site, an unidentified sequence is absolutely required for MucR binding. Similar results were found in *S. meliloti*, in which Bertram-Drogatz et al. (1998) found that MucR bound to upstream sequences of *exoH* and *exoY* but failed to find the consensus sequence previously defined for the *mucR* promoter (Bertram-Drogatz et al. 1997).

Altogether, our results show that, as expected, *S. fredii* HH103 MucR1 is a transcriptional regulator that represses its own expression.

S. fredii HH103 MucR1 is a global regulator.

Several studies documented the role of RosR/MucR in regulating different bacterial traits related to interaction with eukaryotic hosts not only in rhizobia but, also, in pathogenic

bacteria such as *Brucella abortus* and *B. melitensis* (Caswell et al. 2013; Janczarek 2011; Janczarek et al. 2010; Mirabella et al. 2013). We have also found that *S. fredii* HH103 MucR1 affects EPS and external GC production, pellicle formation, autoaggregation, and symbiotic performance with the two host plants tested, soybean and *L. burtii*. This prompted us to analyze, by transcriptomics, the set of genes whose expression is affected by MucR1 in *S. fredii* HH103. Since the number of nodules induced is significantly affected in the two host plants tested, we decided to perform the analysis in the presence or absence of genistein, an effective *nod*-inducer flavonoid for HH103 (Vinardell et al. 2004b).

Very recently, transcriptomic studies of the effect of the absence of MucR/RosR in *R. leguminosarum* bv. *trifolii* Rt24.2 and *S. fredii* CCBAU45436 (Jiao et al. 2016; Rachwał et al. 2015) have been published. In the former strain, 1,106 genes were differentially expressed in free-living cells of the wild-type strain and its *rosR* derivative ($\log_2$ of mutant/wild type values > 2). In *S. fredii* CCBAU45436, the numbers of differentially expressed genes (DEG) in the *mucR1* mutant when compared with the wild-type strain ($\log_2$ of mutant/wild type values > 1) were 621 and 597 in free-living cells and in bacteroids isolated from soybean nodules, respectively. The numbers of DEG in HH103 Δ *mucR1* (defined as genes showing a |fold-change| ≥ 3.5 or $\log_2$ of mutant/wild type values > 1.871) were 393 and 904 in the absence and in the presence of genistein, respectively, 265 of them being shared by both conditions (Supplementary Fig. S8; Supplementary Dataset 1). The expression levels of 20 DEG were validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Table 3). Thus, as previously reported for other rhizobia, HH103 MucR1 appears to be a global regulator affecting the expression of hundreds of genes. Detailed information about global gene expression levels in the wild type and the Δ *mucR1* strains in the absence or presence of genistein as well as the lists of DEG in both conditions are given in Supplementary Datasets 2 to 5.

S. fredii HH103 MucR1-dependent genes in the absence of genistein.

In the absence of genistein, 227 and 166 genes were up- and down-regulated, respectively, in HH103 Δ *mucR1*, as compared

Table 3. RNA-Seq data validation using quantitative reverse transcription-polymerase chain reaction (qRT-PCR)^a

Locus tag/promoter sequences	Gene name	Absence of genistein		Presence of genistein	
		RNA-Seq	qPCR	RNA-Seq	qPCR
psfHH103d_118	<i>nodA</i>	4.89	5.13 $\pm$ 0.07	1.18	-1.29 $\pm$ 0.42
psfHH103d_126/NB8		-1.35	-1.23 $\pm$ 0.5	-3.60	-1.88 $\pm$ 0.28
psfHH103d_208/NB17		9.31	4.38 $\pm$ 0.01	-1.51	-1.26 $\pm$ 0.17
psfHH103d_257/NB15		4.54	3.27 $\pm$ 0.17	-2.05	1.11 $\pm$ 0.21
psfHH103d_373/NB1	<i>mucR2</i>	9.25	11.64 $\pm$ 5.58	-1.52	-1.17 $\pm$ 0.01
psfHH103d_391		3.46	8.68 $\pm$ 4.05	2.16	2.54 $\pm$ 0.14
psfHH103d_448/NB13		7.11	4.54 $\pm$ 0.44	-1.31	1.47 $\pm$ 0.16
psfHH103d_604		-11.25	-5.56 $\pm$ 2.16	-14.04	-9.48 $\pm$ 7.59
SFHH103_00308	<i>fliG</i>	8.04	10.26 $\pm$ 5.69	13.75	4.41 $\pm$ 1.25
SFHH103_00335	<i>rem</i>	5.16	18.02 $\pm$ 1.74	10.33	4.29 $\pm$ 0.06
SFHH103_00387	<i>fbpA</i>	-70.28	-111.75 $\pm$ 26.23	-38.44	-18.51 $\pm$ 1.54
SFHH103_01240	<i>exoR</i>	-4.56	-3.28 $\pm$ 1.45	-4.59	-3.92 $\pm$ 0.08
SFHH103_02000	<i>ftsZ1</i>	4.80	4.98 $\pm$ 0.33	1.67	2.60 $\pm$ 0.18
SFHH103_02172	<i>ptsN</i>	9.89	40.62 $\pm$ 9.17	6.18	17.57 $\pm$ 1.52
SFHH103_02569		51.34	45.0 $\pm$ 17.24	25.69	70.65 $\pm$ 7.32
SFHH103_03584		106.74	124.51 $\pm$ 1.29	28.68	503.2 $\pm$ 41.76
SFHH103_05376	<i>exoA</i>	-2.0	-2.53 $\pm$ 1.19	-4.09	-2.46 $\pm$ 0.19
SFHH103_05378	<i>exoK</i>	-2.82	-3.03 $\pm$ 0.46	-4.13	-2.60 $\pm$ 0.1
SFHH103_05384	<i>exoY2</i>	-11.91	-4.35 $\pm$ 0.38	-8.78	-3.90 $\pm$ 0.97
SFHH103_06390		35.51	23.95 $\pm$ 5.25	14.13	11.89 $\pm$ 0.5

^a The expression of 20 individual genes was measured in *Sinorhizobium fredii* HH103 and in a Δ *mucR1* derivative, both in the presence or absence of genistein. For qRT-PCR, fold change values were calculated using the $\Delta\Delta C_t$ method (Pfaffl et al. 2002) and were normalized to the reference 16S rRNA. Data represent expression level fold changes in HH103 Δ *mucR1* with respect to HH103.

with the wild-type strain. Many of the genes affected (174 of 393) code for hypothetical proteins, including the two genes showing the highest fold-changes (SFHH103_03584 and 06391, +106.7 and +98.2, respectively). In agreement with previous results described for a *rosR* mutant of *R. leguminosarum* bv. *trifolii*, the expression of at least 20 transcriptional regulators belonging to different families (such as GntR and LuxR) was altered (either induced or repressed) in the *mucR1* background compared with the wild-type strain, indicating that part of the DEG detected might not be directly controlled by MucR1. Among these regulatory elements, it is worthwhile to notice the presence of a Crp/FixK-like transcriptional regulator (psfHH103d_604, fold-change -11.3), which is involved in regulation of a large number of genes for the anoxic and microoxic, endosymbiotic, and nitrogen-fixing life styles of *Bradyrhizobium japonicum* (Mesa et al. 2008). Other interesting transcriptional regulators found as DEG are members of two-component systems such as the chemotaxis-related genes *cheY1* and *cheY2* or *feuP* (discussed below). Also interesting is the fact that two of the genes showing the highest levels of downregulation in HH103 Δ *mucR1* were *hmuS* (coding for a hemin transport/degrading protein, fold change -13.5) and *fbpA* (iron deficiency-induced periplasmic protein, -70.3), suggesting a role for MucR1 in regulation of Fe uptake.

Genes related to the synthesis of surface polysaccharides. Concerning genes related to the synthesis of surface polysaccharides, unexpectedly, most *exo* genes were not included as DEG, because they were little or not at all affected. This is the case for genes coding for glycosyl transferases putatively involved in the synthesis of the EPS structural subunit, such as *exoA*, *exoL*, *exoM*, or *exoO* (fold-changes of -2, -2.1, -1.2, and 1.6, respectively) or *exoN* and *exoB*, responsible for the production of UDP-Glc and UDP-Gal, respectively (-2.8 and -1.9). Similar results have been recently reported for a *RosR* mutant of *Rhizobium leguminosarum* bv. *trifolii* Rt24 (Rachwał et al. 2015). Instead, the *exoY2* and *exoF1* genes, which are responsible for the addition of the first sugar residue (UDP-Gal) to the lipid carrier located in the internal membrane, and *exoQ*, whose encoded product is involved in EPS polymerization, were more strongly repressed (-11.9, -5, and -4.1 respectively). Most probably, these three genes constitute an operon (Rodríguez-Navarro et al. 2014). Our results are also in agreement with previous observations carried out in *S. meliloti* MucR, which has also a slight effect on *exo* gene expression but increases the transcription of *exoY*, *exoF*, and *exoQ* (Keller et al. 1995). Surprisingly, the HH103 *exoR* gene, which codes for a repressor of *exo* genes, is also repressed (-4.5) in the Δ *mucR1* background.

Other surface component-related genes that are down-regulated in the *mucR1* mutant background are *lpxC*, coding for a UDP-3-O-acetyl-N-acetylglucosamine deacetylase involved in lipid A biosynthesis, *ndvA*, involved in CG transport from the cytoplasm to the periplasm, and SFHH103_01577, which codes for a CG-modification protein containing the MdoB domain characteristic of phosphoglycerol transferases (fold-changes of -3.9, -5, and -6.2, respectively). Although we have not detected differences between the LPS electrophoretic patterns of HH103 and its Δ *mucR1* derivative, the possibility that the absence of MucR1 could provoke changes in the LPS non-detected by PAGE analyses cannot be discarded. Regarding CG, supernatants of *S. fredii* HH103 Δ *mucR1* exhibited higher amounts of extracellular CG than those of the parental strain. To our knowledge, there is no information available about how CG are secreted to the medium, since *ndvA* is an internal membrane protein involved in the transport of CG from the cytoplasm to the periplasm. On the other hand, at least in *S. meliloti*, external CG appears to be predominantly neutral (Geiger et al. 1991). The fact that both *ndvA* and SFHH103_01577 (which codes for a putative CG phosphoglycerol transferase) are

repressed in HH103 Δ *mucR1* could be related to this enhanced secretion of CG to the medium. We have also demonstrated that a HH103 mutant unable to produce EPS exhibits enhanced CG secretion, suggesting that this phenomenon could be a consequence of depletion in EPS production. However, elucidation of the relationship between EPS and external CG production in *S. fredii* HH103 requires further investigation. Remarkably, in HH103 Δ *mucR1*, the response regulator-encoding gene *feuP* is down-regulated (-5.4). In *S. meliloti*, this gene controls the transcription of at least 16 genes (including *ndvA*) in response to low osmolarity conditions (Griffitts et al. 2008). In addition to *ndvA*, orthologs of seven other genes (all coding for hypothetical proteins) induced by *FeuP* in *S. meliloti* are also clearly down-regulated in the HH103 Δ *mucR1* background, i.e., SFHH103_00611 (-5.8, ortholog of SMB20838), SFHH103_00710 (-4.7, SMc00065), SFHH103_00740 (-5.2, SMc02389), SFHH103_01338 (-8.4, SMc00252), SFHH103_01771 (-5.8, SMc04336), SFHH103_02119 (-5.2, SMc01557), and SFHH103_02145 (-11.0, SMc01586).

Genes involved in motility and chemotaxis. Regarding genes showing upregulation in HH103 Δ *mucR1* in the absence of genistein, it is worth mentioning the presence of a large set of genes related to chemotaxis and motility. This is in clear contrast to the results recently reported by Rachwał and coworkers (2015) for *R. leguminosarum* bv. *trifolii*, in which it was found that *RosR* positively regulates these genes and, in consequence, bacterial motility but in agreement with the fact that *S. meliloti* MucR represses bacterial motility (Bahlawane et al. 2008). The group of upregulated chemotaxis-related proteins in HH103 Δ *mucR1* includes the two-component response regulator receiver proteins *CheY1* and *CheY2* (fold-changes of +11.1 and +7.3), the chemotaxis signal transduction protein *CheW1* (+9.5), the methyltransferase *CheR* (+6.3), and the putative antisigma regulatory factor *CheX* (+5.4). Regarding motility-related genes, different *fli*, *flg*, *fla* (including *flaC*), and *mot* genes involved in motor activity, flagellum-dependent cell motility process, and regulation or flagellum filament components were up-regulated in the *mucR1* derivative. These results prompted us to investigate whether swimming or surface motility could be affected by *mucR1* inactivation, as described below. Surprisingly, neither swimming (13.1 ± 0.6 mm in HH103 Δ *mucR1* versus 13.5 ± 0.7 mm in HH103 after 72 h of incubation) nor surface motility (14.0 ± 7.2 mm versus 12.5 ± 4.0 mm after 72 h of incubation) were significantly affected in the HH103 *mucR1* derivative under our experimental conditions, indicating that the biological significance of the upregulation in this strain of such a high number of genes related to motility requires further investigation.

Genes belonging to the nod regulon. Interestingly, many genes located on the symbiotic plasmid and whose expression is dependent on NodD1 and genistein, since they are driven by *nod* boxes (NB) (Vinardell et al. 2015; Pérez-Montañón et al. 2016), are also up-regulated (fold-change >3.5) in HH103 Δ *mucR1* in the absence of flavonoids (Table 4), i.e., psfHH103d_373, _372 (NB1, putatively involved in hopanoid synthesis, fold-changes +9.2 and +8.5), psfHH103d_118, _116 (NB9, hypothetical proteins, +4.9 and +3.5), psfHH103d_448, _447 (NB13, hypothetical proteins, +7.1 and +4.0), psfHH103d_229, _228, _225 (NB14, hypothetical proteins putatively involved in electron transfer to nitrogenase, +8.1 to +4.5), psfHH103d_257 (NB15, histidinolphosphate aminotransferase involved in indole acetic acid production, +4.8), and psfHH103d_208 (NB17, hypothetical ABC-type transporter, +9.3). In addition, two genes whose expression is dependent on *ttb* boxes are also up-regulated in the Δ *mucR1* background in the absence of genistein, namely, a component of the symbiotic T3SS (psfHH103d_335, TB8, NopX, +4.0) and one of the effectors secreted through this apparatus (psfHH103d_349, TB11, NopP, +3.9). Since the role

of most of these proteins remains to be elucidated, it is difficult to understand the biological significance of their regulation by MucR1. However, it has been reported that inactivation of HH103 *nopP* improved symbiotic performance with soybean (López-Baena et al. 2009), which suggests that overexpression of this gene might affect this interaction negatively.

The presence of genistein increases the number of DEG in HH103 ΔmucR1. In the presence of genistein, the number of DEG found in HH103 *ΔmucR1* was 904, which is 2.3-fold higher than that detected in the absence of this flavonoid. The fact that the numbers of up- and down-regulated genes were 615 and 289, respectively, indicates that MucR1 acts mainly as a repressor. A total of 265 genes were differentially expressed in the *ΔmucR1* mutant both in the presence or absence of genistein. Supplementary Dataset 6 contains the list of these genes as well as their expression fold changes in HH103 *ΔmucR1* in both conditions (absence or presence of genistein). For all 265 genes, the sign (positive or negative) of their differential expression was the same in both conditions.

Genes related to surface polysaccharide production, motility, and chemotaxis. In the presence of genistein, genes related to EPS, periplasmic anionic CG production, flagella formation, or chemotaxis were also affected, which suggests that the regulatory effect of MucR1 in these bacterial traits is not dependent on the presence of flavonoids. Although there are additional *exo* genes

(*exoA*, *exoL*, *exoK*, *exoU*, *exoB*, fold changes varying between –3.6 and –4.3) that appear as downregulated in the *mucR1* mutant in the presence of genistein, these genes are also slightly repressed in the absence of genistein (ranging between –2 and –2.8). Regarding genes related to motility, it is worthwhile to note that the *flgA* gene (coding for a putative periplasmic chaperone that assists in the formation of the flagellar P ring) is 111.0 overexpressed in the *mucR1* mutant, as compared with the wild-type strain, in the presence of genistein, in contrast to a 2.8-fold induction in the absence of flavonoids. However, this difference could be an overestimation due to the fact that transcription of this gene was very low in the wild-type strain in the absence of genistein but not detected at all in the presence of the flavonoid. Other motility-related genes that were more highly expressed in the *mucR1* mutant in the presence of flavonoid were *fliL* (flagellar transmembrane protein, +7.3 versus +2.3) and the flagellar motor protein-coding genes *motB* (+17.2 versus +2.9) and *motC* (+15.2 versus +3.1). Remarkably, in the presence of genistein, these three genes are repressed in the wild-type strain (fold changes –2.9, –2.6, and –2.0, respectively), suggesting a complex regulation of motility-related genes in *S. fredii* HH103. We plan to study further the effect of flavonoids and the role of *mucR1* in HH103 motility and chemotaxis abilities in the near future.

Genes belonging to the nod regulon. Most of the NB-depending genes that were found overexpressed in the *ΔmucR1* mutant in the

Table 4. Effect of *mucR1* inactivation on the expression of the *nod* regulon in the absence or presence of genistein

NB ^a	Locus-tag (gene name)	Description	Fold change ^b	
			Without genistein	With genistein
NB1	psfHH103d_373	Hypothetical protein (putative steroid Delta-isomerase)	+9.2	–1.5
	psfHH103d_372	Squalene/phytoene synthase	+8.5	–1.2
	psfHH103d_371_5 (<i>hpnD</i>)	Putative squalene/phytoene synthase	+2.6	+1.3
	psfHH103d_371	Flavoprotein oxidoreductase	+2.2	+1.7
	psfHH103d_370 (<i>shc</i>)	Squalene-hopene cyclase	+1.8	+1.3
NB2	psfHH103d_381 (<i>nodZ</i>)	Nod factor fucosyl transferase	–3.0	–3.3
	psfHH103d_380 (<i>noeL</i>)	GDP-D-mannose dehydratase (involved in the synthesis of GDP-L-fucose for Nod factor decoration)	+1.1	–1.5
	psfHH103d_379 (<i>noIK</i>)	NAD-dependent nucleotide sugar epimerase/dehydrogenase (involved in the synthesis of GDP-L-fucose for Nod factor decoration)	+1.6	+1.1
NB3	psfHH103d_384	Hypothetical protein	+3.0	+1.3
NB8	psfHH103d_126 (<i>nodA</i>)	Nod factor acyl-transferase	–1.3	–3.6
	psfHH103d_127 (<i>nodB</i>)	Nod factor deacetylase	–1.1	–2.5
	psfHH103d_128 (<i>nodC</i>)	Nod factor backbone synthetase	–1.2	–1.4
	psfHH103d_129 (<i>nodI</i>)	Nod factor export ATP-binding protein	+1.2	+1.0
	psfHH103d_130 (<i>nodJ</i>)	Nod factor ABC-transporter permease	+1.0	+1.0
	psfHH103d_131 (<i>noLO</i>)	Nod factor carbamoyl transferase (truncated)	+1.6	+1.3
	psfHH103d_132 (<i>noeI</i>)	Nod factor fucose methylase	+1.6	+1.5
	psfHH103d_118	Conserved hypothetical protein	+4.9	+1.2
NB9	psfHH103d_116	Hypothetical protein (putative dehydrogenase)	+3.5	+1.4
	psfHH103d_161 (<i>psiB</i>)	Conserved hypothetical protein (polysaccharide inhibition?)	+3.1	–1.7
NB10	psfHH103d_448	Hypothetical protein containing a REC domain	+7.1	–1.3
NB13	psfHH103d_408	Conserved hypothetical protein	+4.0	+1.6
	psfHH103d_447	Putative iron-sulfur cluster assembly accessory protein	+8.1	–1.4
NB14	psfHH103d_229	Hypothetical protein	+6.1	–1.4
	psfHH103d_227 (<i>fixA</i>)	Electron transfer flavoprotein beta subunit	+2.2	–1.6
	psfHH103d_226 (<i>fixB</i>)	Electron transfer flavoprotein alpha subunit	+2.4	–1.9
	psfHH103d_225 (<i>fixC</i>)	Oxidoreductase	+4.5	–1.2
	psfHH103d_224 (<i>fixX</i>)	Ferredoxin-like protein	+3.1	–1.2
	psfHH103d_257 (<i>y4wE</i>)	Indole Acetic Acid (IAA) biosynthesis	+4.8	–2.1
	psfHH103d_208	Hypothetical protein (ABC-type transport)	+9.3	–1.5
NB15	psfHH103d_323 (<i>tttI</i>)	Transcriptional activator of the T3SS	+2.1	–1.9
	psfHH103d_324 (<i>rhcC2</i>)	Component of the T3SS apparatus	+1.5	+1.3
NB17	psfHH103d_325	Conserved putative lipoprotein	+1.5	+2.7
	psfHH103d_326	Transposase	–1.3	–1.2
	psfHH103d_367 (<i>syrM</i>)	Transcriptional regulator of symbiotic genes	+2.2	–1.4
	psfHH103d_368	Hypothetical protein containing an HTH domain	–1.3	–2.3
NB18	psfHH103d_369	Hypothetical protein (putative ACC deaminase, partial)	+1.0	–1.9

^a NB = *nod* box, according to Vinardell et al. (2015).

^b Fold induction of *mucR1* mutant versus wild type.

absence of flavonoids are not included in the list of DEG in the presence of genistein, indicating that when NodD1 is activated with flavonoids, the repressor effect of MucR1 on these genes is less pronounced (Table 4). Curiously, in the presence of genistein, the *nodA* gene is repressed in the Δ *mucR1* mutant (fold change -3.6), maybe because the expression of *nodD1* is also slightly reduced (-1.5). In order to further analyze this result, plasmid pMP240, which harbors a copy of the promoterless *lacZ* gene fused to the *Rhizobium leguminosarum nodA* promoter (de Maagd et al. 1988), was introduced in HH103 and its Δ *mucR1* derivative. In the presence of genistein, β -galactosidase activity (Miller units) of HH103 Δ *mucR1* (pMP240) was significantly lower than that of HH103 (pMP240) ($7,298 \pm 3,306$ versus $23,345 \pm 3,977$), confirming that the absence of MucR1 has a negative impact on *nodA* expression in *S. fredii* HH103. This positive effect of MucR1 on *nodD* and, therefore, *nodABC* expression has been previously described in *S. meliloti* (Mueller and González 2011). Remarkably, *nodZ*, which is also involved in Nod factor synthesis, is down-regulated (fold change -3.3) in the Δ *mucR1* background in the presence of genistein (Table 4). Taken together, our results suggest that MucR1 is another important regulator in symbiosis, modulating positively or negatively the expression of different genes belonging to the *nod* regulon. This fact might be related with the phenotype, a clear reduction in symbiotic effectiveness, exhibited by HH103 Δ *mucR1* with the two host legumes analyzed in this work.

In order to better understand the connection between MucR1 and the HH103 *nod* regulon, we looked for MucR- or RosR-binding boxes in the upstream sequences of all the HH103 *nod* box-driven genes. This search, which was carried out by using the *fuzznunc* utility from the EMBOSS software package (Rice et al. 2000), gave no results.

Genes related to inorganic ions transport. As mentioned before, Jiao and coworkers (2016) have very recently described the symbiotic phenotype of a *mucR1* derivative of *S. fredii* CCBAU45436 with soybean JD17. As we have found for HH103 Δ *mucR1* with soybean 'Williams', CCBAU45436 *mucR1* induces the formation of a higher number of nodules than the wild-type strain and these nodules exhibit a severe impairment in nitrogen-fixation capacity. In that work, the authors found that the expression of several ion transporters is down-regulated in bacteroids of a *mucR1* mutant. This might account for the reduction of nitrogen-fixation capacity of this mutant in soybean JD17 nodules. In our study, none of the transporter-encoding genes studied by Jiao et al. (2016) was induced by flavonoids in the wild-type strain and only a few of them were affected in the *mucR1* mutant. In the absence of genistein, *ssuAC* and *tauB* (involved in aliphatic and aminoethane sulphonate transport) were overexpressed (fold changes of +7.3, +4, and +3.5) in the *mucR1* derivative. In the presence of genistein, *modA* and *modC* (related to molybdenum transport) were, strikingly, repressed (-4.8) and induced (+4.7), respectively, in HH103 Δ *mucR1*, whereas *phoC* (phosphate transport) was induced (+4.3). Thus, our results indicate that the downregulation of different ion transporters caused by the lack of MucR1 observed in strain CCBAU45436 in the bacteroid state does not take place in free-living cells, even in the presence of inducing flavonoids.

Conclusions and perspectives.

The rhizobia-legume symbiosis involves a complex molecular dialogue that has to be finely regulated. On the bacterial part, recent studies reveal that the number of genes symbiotically related is higher than expected (Huyghe et al. 2015; Jiao et al. 2016; Lang et al. 2008; Pérez-Montañó et al. 2016) and that their regulation involves the participation of a number of transcriptional regulators. In addition to NodD, TtsI, and SyrM, global regulators such as NodR and MucR also play a role in

this process. *S. fredii* strains, in contrast to *S. meliloti* or *R. leguminosarum*, harbor two copies of the zinc-finger transcriptional regulator MucR, although only MucR1 appears to be functional in strains CCBAU45436 and HH103, due to a frameshift mutation in the 3' end of *mucR2* (Jiao et al. 2016). In this work, we have demonstrated that *S. fredii* HH103 MucR1 is a global regulator that affects the transcription of hundreds of genes, including those related to EPS production and CG export, and, in addition, is clearly connected to the *nod* regulon. In consequence, inactivation of *mucR1* not only affects free-living cells but, also, has negative consequences on symbiotic interaction. Remarkably, the HH103 MucR1 regulator affects differently symbiosis depending on the host plant. Thus, when this protein is absent, the interaction with *L. burtii* is negatively affected in both nodule number and nitrogen fixation, whereas, with 'Williams', soybean nodule number is increased, although nitrogen fixation is dramatically decreased, indicating that MucR1 is essential for *S. fredii* interactions with both American and Asiatic soybeans (Jiao et al. 2016). Our results suggest that MucR1 could either affect different steps of symbiosis in different hosts, affect the same step, such as nodule formation, or both in a different way in different plant species. In addition, the transcriptome analysis carried out in different rhizobia indicates that the same protein (MucR and RosR) can regulate similar sets of genes but in opposite ways in different species, as is the case for motility genes (repressed in *Sinorhizobium* spp., induced in *R. leguminosarum* bv. *trifolii*). Clearly, further research is required to elucidate the different processes controlled by MucR/RosR in rhizobia and how these regulators are integrated into the highly complex rhizobial symbiotic signaling.

MATERIALS AND METHODS

Basic molecular and microbiological techniques.

Sinorhizobium fredii strains were grown at 28°C on tryptone yeast (TY) medium (Beringer 1974), YM medium (Vincent 1970), MGM medium (O'Toole and Kolter 1998), or Bromfield medium (Sourjik and Schmitt 1996). *Escherichia coli* was cultured on Luria-Bertani (LB) medium (Sambrook and Russell 2001) at 37°C. When required, the media were supplemented with the appropriate antibiotics as described by Vinardell et al. (2004a). Genistein was dissolved in ethanol at a concentration of 1 mg/ml and was used at 1 μ g/ml. Plasmids were transferred from *E. coli* to rhizobia by triparental mating, using plasmid pRK2013 as a helper, as described by Simon (1984). β -galactosidase assays were performed as described by Vinardell et al. (2004a).

Recombinant DNA techniques were performed according to the general protocols of Sambrook and Russell (2001). For hybridization, DNA was blotted to Amersham Hybond-N nylon membranes and the DigDNA method of Roche was employed according to manufacturer's instructions. PCR amplifications were performed as previously described (Vinardell et al. 2004b). All primer pairs used in this work are listed in Supplementary Table S1.

Motility assays.

Swimming was examined on plates prepared with Bromfield medium containing 0.3% Bacto agar and inoculated with 3- μ l aliquots of rhizobial cultures grown in TY ($OD_{600} = 1$). Surface motility was analyzed on semisolid minimal medium plates with glycerol as carbon source, containing 0.4% agarose and 22 μ M ferric chloride after sterilization. Plates were supplemented with genistein for surface motility and were inoculated with 2 μ l of washed 10-fold concentrated cultures grown in TY broth to the late exponential phase. The migration zone was determined as the colony diameter (mm) after 24, 48, and 72 h (for swimming and surface motility on semisolid minimal medium) of incubation.

Autoaggregation, biofilm assays, and EPS production.

Bacterial autoaggregation assays were performed as previously described by Sorroche et al. (2012), with some modifications. The bacteria were grown in 4 ml of MGM medium supplemented with appropriate antibiotics, were incubated for 24 h at 28°C, were diluted 1:100 in MGM, and were incubated for 96 h under the same conditions. The bacterial suspensions (5 ml) were then transferred into a glass tube and were allowed to settle for 24 h at 4°C. A 0.2-ml aliquot of the upper portion of the suspension was transferred onto a microtiter plate, and the final OD₆₀₀ (OD_{final}) was measured. A control tube was vortexed for 30 s, and the initial OD₆₀₀ (OD_{initial}) was determined. The autoaggregation percentage was calculated as follows: $100 - [1 - (OD_{final}/OD_{initial})]$. The values of each treatment were compared with those of *S. fredii* HH103, using the Mann-Whitney nonparametrical test.

Assays for biofilm formation on plastic surfaces were carried out as described by Margaret et al. (2013). Data presented are the mean of at least three independent experiments performed in duplicate; in each experiment, at least 12 wells for each treatment were measured. In order to visualize biofilm formation on glass surfaces, *S. fredii* strains harboring plasmid pSKDSRED (Kelly et al. 2013) were cultured in MGM medium (28°C, static conditions) in sterile glass Coplin staining jars containing glass slides, as described by Rodríguez-Navarro et al. (2014). At 1 and 4 days after inoculation, glass slides were collected and air dried, were rinsed with NaCl 0.9%, were air dried, and were observed and photographed, using Nikon Eclipse Ti-U epifluorescence microscopy with an excitation and emission wavelength of 554 and 586 nm, respectively. Three different sections of the glass slide were examined (from the bottom to the upper part of the slide): 5, 28, and 49 mm, the latter corresponding to the liquid-air interface.

For analysis of EPS production in solid media, 20-μl droplets of YMB-grown early log (OD₆₀₀ = 0.4) cultures were placed onto YMA plates (supplemented or not with genistein), were incubated at 28°C for 96 h, and were photographed. For EPS quantification, bacterial cultures were grown on YM for 96 h at 28°C (OD₆₀₀ = 1.2 to 1.3) under shaking conditions. Cells were removed by centrifugation (20,000 × g, 15 min) and total carbohydrate amounts of the EPS-containing supernatants were determined, using the anthrone-H₂SO₄ method, which measures the total reducing sugar content in a given sample, as previously described (Abarca-Grau et al. 2012). Four independent experiments, in duplicate, were carried out. The amounts of EPS produced by each strain were compared with that of the parental strain by the Mann-Whitney nonparametric test.

Isolation of EPS and extracellular CG and analysis by NMR.

Culture supernatant (1 liter) was concentrated to 20% of its original volume on a rotary evaporator and was then mixed with ethanol (3 vol). The resulting precipitate contained the EPS, which was removed, by centrifugation, resuspended, and purified by dialysis. Supernatant was concentrated to 5% of their original volume, and 10 ml of it was mixed with ethanol (10 vol) to precipitate the low-molecular weight fraction that contained CG (Gil Serrano et al. 1993). The resulting precipitate was redissolved in water and was dialyzed with a 10-kDa JumboSep device (Pall Corporation). The diffusate was concentrated on a rotary evaporator and salts were removed by size-exclusion chromatography on BioGel P-2 (85 by 2 cm) with water as eluent. Fractions containing carbohydrates were detected using a refractive-index detector and the orcinol-sulfuric acid method on thin-layer chromatography plates and were then analyzed by NMR. Samples (1 to 5 mg) were deuterium-exchanged several times by freeze-drying from D₂O and were then examined in

solution (1 to 5 mg to 750 ml of 99.90% D₂O). Spectra were recorded on a Bruker AV500 spectrometer operating at 500.13 MHz (¹H). Chemical shifts are given in ppm, using the HDO signal (4.22 ppm at 353 K and 4.71 at 303 K) as a reference (Gottlieb et al. 1997).

Analysis of LPS and KPS.

LPS extraction from bacterial cultures grown on solid TY medium, separation on sodium dodecyl sulfate (SDS)-PAGE gels and silver staining or immuno-staining with the monoclonal antibody NB6-228.22 were as described by Buendía-Clavería et al. (2003). KPS were extracted from bacterial cultures grown on solid TY medium and were analyzed by PAGE as described by Hidalgo et al. (2010). The detection of *S. fredii* KPS and CG by ¹H-NMR was performed as previously described (Crespo-Rivas et al. 2009; Hidalgo et al. 2010).

Nodulation assays.

Nodulation assays on *Glycine max* (L.) Merr. cv. Williams and *Lotus burttii* were carried out, as previously described by Crespo-Rivas et al. (2007) and Sandal et al. (2012), in a plant-growth chamber, with a 16-h photoperiod at 25°C in the light and 18°C in the dark. For soybean, each plant was inoculated with approximately 10⁸ bacteria and, then, was grown for at least 6 weeks. Plant tops were dried at 70°C for 48 h and were weighed. In the case of *Lotus* plants, four germinated seeds were transferred to each Leonard jar assembly and were inoculated with 10⁸ bacteria. Inoculated *Lotus* plants were grown for 6 to 9 weeks. Bacterial isolation from surface-sterilized nodules was carried out as previously described by Buendía-Clavería et al. (2003). Nitrogenase activity of nodules was assessed by ARA (acetylene reduction assay), as described by Buendía-Clavería and associates (1989). For the different parameters analyzed, the values of each treatment were compared with those of *S. fredii* HH103, using the Mann-Whitney nonparametrical test.

eGFP fluorescence assays.

For measuring transcription of *S. fredii* HH103 *mucR1*, its promoter region plus the first codon was PCR-amplified and digested with *Xba*I and *Hind*III and was fused, in frame, to the gene coding for eGFP, contained in plasmid pPHU231 (McIntosh et al. 2008), resulting in plasmid pMUS1196. For eGFP fluorescence assays, the method described by McIntosh et al. (2008) was used, with modifications. Transconjugants of *S. fredii* HH103 Rir^R and its Δ *mucR1* derivative containing plasmid pMUS1196 were first cultivated in YM broth at 28°C, with agitation, until medium exponential phase (OD₆₀₀ = 0.5 to 0.6), were diluted to OD₆₀₀ = 0.04, and 100-μl aliquots were inoculated into microtiter plate wells. The cell density (OD₆₀₀) and eGFP fluorescence (excitation at 485 nm and emission at 528 nm with a 35% scanning rate) were measured using a Tecan Infinite M200 reader (Tecan Trading AG). Data presented (eGFP fluorescence/OD₆₀₀) are the medians of two independent experiments performed in duplicate; in each duplicate, at least six wells for each treatment were measured. The eGFP fluorescence emitted for the corresponding strains carrying the empty vector pPHU231 was subtracted in all cases. The values obtained for HH103 pMUS1196 and HH103 Δ *mucR1* pMUS1196 were compared, using the Mann-Whitney nonparametrical test.

EMSA assays.

Expression and purification of the fusion proteins. The HH103 *mucR1* gene was PCR-amplified, using the attB1*mucR1* and attB2*mucR1* primers, which contain the sequences necessary for Gateway cloning. The amplified DNA fragment was subcloned into plasmid pDONR221, generating the pMUS1203 plasmid. Then, the *mucR1* gene was subcloned into the pGEX-KG

Gateway destination vector, to give pGEX-KG*mucR1*. Vector pGEX-KG added an in frame GST tag to the N-terminus of *mucR1*. Plasmid pGEX-KG*mucR1* was transferred to *E. coli* BL21, following the methodology described by Chung et al. (1989). Expression of the fusion protein was performed, following the general protocols described by Sambrook and Russell (2001). Briefly, *E. coli* BL21 carrying plasmid pGEX-KG*mucR1* was inoculated in 5 ml of LB and was grown overnight at 37°C. Cultures were transferred to 100 ml of LB supplemented with ampicillin and were incubated with shaking at 37°C until late exponential phase (OD₆₀₀ = 0.8) and, then, were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside for at least 4 h and were centrifuged at approximately 2,000 × g for 20 min at 4°C. The supernatants were discarded and the pellets were stored at -80°C until use. Proteins were extracted and purified using a glutathione sepharose 4B resin, following the protocol described by Smith and Johnson (1988), and the purified proteins were quantified using a Nanodrop apparatus (Thermo Fisher Scientific).

Proteins were separated by SDS-PAGE, using the discontinuous buffer system of Laemmli (1970). Electrophoresis was performed on SDS 12% (m/v) polyacrylamide gels, and proteins were visualized using PageBlue (Thermo Fisher Scientific), following the manufacturer's instructions. For immunostaining, proteins were separated and electroblotted to Immobilon-P nitrocellulose membranes (Bio-Rad), using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were blocked with Tris-buffered saline containing 2% (m/v) bovine serum albumin (BSA) and were incubated with antibodies raised against GST (Cell Signaling) diluted 1:1,000 in the same solution. Antirabbit immunoglobulin antibodies (AP-conjugate) were used as secondary antibodies. Reaction results were visualized using nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate.

EMSA analysis. Cy5-labeled DNA fragments 1 to 5 were amplified by PCR, using different primers in such a way that all the fragments were labeled with Cy5. Each labeled DNA fragment in a concentration of 50 ng was mixed with purified MucR1-GST at different concentrations (0, 0.5, and 5 μg) in a reaction buffer containing 50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.1 mM Mg₂SO₄, 4.5% glycerol, and BSA at 0.5 mg/ml, to give a final volume of 25 μl. After 30 min of incubation at 20°C, the mixture was loaded into a 1.5% nondenaturing agarose gel prepared in Tris-glycine, pH 8.3, and prerun for 40 to 60 min in the same buffer at 4°C. The electrophoresis was carried out at 90 V for 2 h at 4°C in Tris-glycine, pH 8.3. Gel images were acquired, using a Typhoon 3000 variable mode imager (Amersham Bioscience).

qRT-PCR.

Results obtained in the RNA-Seq analysis were validated by qRT-PCR of 20 selected genes, which represented DEG and non-DEG in the two strains in the absence and presence of genistein. Total RNA was isolated using the High Pure RNA isolation kit (Roche) and RNase free DNA set (Qiagen), according to the manufacturer's instructions. This (DNA free) RNA was reverse transcribed to cDNA by using PrimeScript RT reagent kit with gDNA Eraser (Takara). qPCR was performed using a LightCycler 480 (Roche, Switzerland) with the following conditions: 95°C, 10 min; 95°C, 30 s; 50°C, 30 s; 72°C, 20 s; 40 cycles, followed by the melting curve profile from 60 to 95°C, to verify the specificity of the reaction. The *S. fredii* HH103 16S rRNA was used as an internal control to normalize gene expression. The fold changes of two biological samples with three technical replicates in each condition were obtained using the ΔΔC_t method (Pfaffl et al. 2002).

RNA-Seq.

The RNA-Seq data discussed in this publication have been deposited in the Sequence Read Archive of the National Center

for Biotechnology Information (BioProject database) under the BioProjects ID PRJNA313151 and PRJNA324263. All samples showed more than 90% of proper pair reads. Three different RNA-Seq metrics for quality control, GC content, duplicate distribution, and the distribution of respective genetic coordinates were performed. A normalization of the quantification data was also carried out before all subsequent analysis to avoid statistical deviations due to differences in library and genetic sizes (Hansen et al. 2012).

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