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e-Xtra*

Sinorhizobium fredii HH103 Invades *Lotus burttii* by Crack Entry in a Nod Factor–and Surface Polysaccharide–Dependent Manner

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***Sinorhizobium fredii* HH103-Rif^r, a broad host range rhizobial strain, induces nitrogen-fixing nodules in *Lotus burttii* but ineffective nodules in *L. japonicus*. Confocal microscopy studies showed that *Mesorhizobium loti* MAFF303099 and *S. fredii* HH103-Rif^r invade *L. burttii* roots through infection threads or epidermal cracks, respectively. Infection threads in root hairs were not observed in *L. burttii* plants inoculated with *S. fredii* HH103-Rif^r. A *S. fredii* HH103-Rif^r *nodA* mutant failed to nodulate *L. burttii*, demonstrating that Nod factors are strictly necessary for this crack-entry mode, and a *noeL* mutant was also severely impaired in *L. burttii* nodulation, indicating that the presence of fucosyl residues in the Nod factor is symbiotically relevant. However, significant symbiotic impacts due to the absence of methylation or to acetylation of the fucosyl residue were not detected. In contrast *S. fredii* HH103-Rif^r mutants showing lipopolysaccharide alterations had reduced symbiotic capacity, while mutants affected in production of either exopolysaccharides, capsular polysaccharides, or both were not impaired in nodulation. Mutants unable to produce cyclic glucans and purine or pyrimidine auxotrophic mutants formed ineffective nodules with *L. burttii*. Flagellin-dependent bacterial mobility was not required for crack infection, since HH103-Rif^r *fla* mutants nodulated *L. burttii*. None of the *S. fredii* HH103-Rif^r surface-polysaccharide mutants gained effective nodulation with *L. japonicus*.**

Rhizobia are α- and β-proteobacteria that are able to establish nitrogen-fixing symbioses with legumes (Peix et al. 2015). The enormous ecological and economic importance of legume crops explains why so many extensive studies on the rhizobial-legume symbiosis have been carried out during the last four decades.

Sinorhizobium fredii is a rhizobial species that has an extremely broad host range. Pueppke and Broughton (1999) showed that *S. fredii* NGR234 nodulates more than 100 genera of legumes and that *S. fredii* USDA257 nodulates a subset of the legumes nodulated by *S. fredii* NGR234. *S. fredii* HH103-Rif^r host range has not been studied in such a detail, but it seems to be closer to that of USDA257 (Buendía-Clavería et al. 1989; Margaret et al. 2011). However, the HH103-Rif^r host range of nodulation shows some significant differences in respect to that of NGR234 and USDA257. For instance, *S. fredii* HH103-Rif^r is able to induce nitrogen-fixing nodules with commercial American soybean cultivars, while *S. fredii* NGR234 and *S. fredii* USDA257 only form ineffective nodules (Buendía-Clavería et al. 1994; Dowdle and Bohlool 1985; Pueppke and Broughton 1999).

Regardless of the rhizobial species that infect *Glycine max* or *Lotus* roots, all the nodules formed are spherical and devoid of an apical meristem. Spherical nodules are also called "determinate nodules" and appear in many other legumes such as *Phaseolus vulgaris* or *Vigna unguiculata*. *L. japonicus* is the model legume used for studying signal exchange and morphogenesis of determinate nodules. *S. fredii* strains are able to nodulate *Glycine* and *Lotus* spp. (Pueppke and Broughton 1999), which offers the possibility for carrying out comparative nodulation studies on these two important legumes. We have previously reported that *Sinorhizobium fredii* HH103-Rif^r is able to induce the formation of nitrogen-fixing nodules on *L. burttii* roots but only ineffective nodules on *L. japonicus* ecotypes Gifu and Miyakojima MG-20 (Sandal et al. 2012). These species are closely related members of the *Lotus corniculatus* clade (Degtjareva et al. 2008; Sz.-Borsos et al. 1972). Three bacterial compatibility groups interacting with members of the genus *Lotus* have been identified. Group 1 is composed of *Mesorhizobium* strains that can efficiently nodulate *L. japonicus*, *L. filicaulis*, *L. burttii*, *L. glaber*, and *L. corniculatus*. Group 2 is composed of *Bradyrhizobium* strains that nodulate *L. pedunculatus* (Gossmann et al. 2012). Group 3 is formed by strains that nodulate both *L. pedunculatus* and *L. corniculatus* (Ward et al. 1989).

Rhizobial Nod factors induce plant responses and facilitate bacterial infection, leading to the development of nitrogen-fixing root nodules on host legumes. Nodule initiation is highly

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dependent on Nod factor structure (Downie 2010). Most of the studies focused on the nodulation process of *L. japonicus* have been carried out using *Mesorhizobium loti* as the microsymbiont partner (Bek et al. 2010; Sullivan and Ronson 1998; Sullivan et al. 1995). Many *Mesorhizobium loti* R7A *nod* mutants have been tested for their symbiotic capacity with different *Lotus* species (Rodpothong et al. 2009). Only mutants affected in *nodA*, *nodC*, and the double mutant *nodD1 nodD2* failed to induce nodule formation. Mutants in the transcriptional regulator *nodD1* induced delayed effective nodules on *L. japonicus* and *L. corniculatus*. Mutants in *nodB*, *nodD2*, *nodM*, *nodO*, *nodS*, and the double mutant *nodO nodS* (it produces Nod factors devoid of the carbamoyl and the *N*-methyl groups on the nonreducing terminal residue) formed nitrogen-fixing nodules on *L. japonicus* and *L. corniculatus* with the same nodulation kinetics as wild-type R7A. In contrast, R7A *nodZ* and *nolL* mutants produce Nod factors without the (acetyl)fucose on the reducing terminal residue and formed delayed effective nodules on *L. japonicus* and uninfected nodule primordia on *L. filicaulis* and *L. corniculatus*. In *L. burttii*, however, the symbiotic properties of *M. loti* R7A and its mutant derivatives *nodZ* and *nolL* were indistinguishable (Rodpothong et al. 2009). Another report has shown that the production of (acetyl)fucosylated Nod factors by a *Rhizobium leguminosarum* isolate from *L. corniculatus* nodules is not required for the formation of infected nodules on *L. burttii* roots (Gossmann et al. 2012).

Other bacterial components that can play a role as symbiotic signals are the diverse bacterial surface polysaccharides present in the different rhizobial species: exopolysaccharides (EPS), capsular K-antigen polysaccharides (KPS), cyclic glucans (CG), and lipopolysaccharides (LPS). The relative symbiotic importance of each particular surface polysaccharide varies among the specific partners (rhizobia and legume) composing the symbiotic partnership (Downie 2010). *M. loti* EPS appears to play a signaling role at the stage of both infection thread (IT) initiation and bacterial release (Kelly et al. 2013). A more recent report shows that the *L. japonicus Epr3* gene encodes a receptor-like kinase that perceives *M. loti* EPS. *Epr3* expression is inducible and induction is dependent on Nod factors. Thus legume-rhizobia symbiotic compatibility and bacterial access to *L. japonicus* roots is regulated by the recognition of Nod factors and EPS signals (Kawaharada et al. 2015).

Information about *M. loti* capsular polysaccharides (KPS) is very limited and does not provide strong evidence for a possible symbiotic role of this surface polysaccharide. *M. loti* NZP2235 produces a capsular polysaccharide (KPS) of unknown structure. This KPS contains sulfate modifications carried out by KpsS, a sulphotransferase. Although NZP2235 *kpsS* mutants exhibited undetectable levels of KPS sulphation, the number of nodules formed on *L. japonicus* roots was similar to that induced by the wild-type strain (Townsend and Keating 2008; Townsend et al. 2006).

M. loti MAFF303099 *cgs* (*ndvB*) mutants do not produce cyclic β-1,2-glucans (Kawaharada et al. 2007, 2008) and are unable to induce the formation of ITs on *Leuresthes tenuis* (nowdays called *L. glaber* Mill.), indicating that *M. loti* requires CG for the infection of *Lotus* roots (D'Antuono et al. 2005). Other reports have shown, however, that the anionic substituents of the *M. loti* CG are not important for the symbiotic bacterial capacity with *L. japonicus* ecotype Gifu (Kawaharada et al. 2010).

The symbiotic capacity of two different *M. loti* mutants affected in LPS production has been studied. The *M. loti lpsβ1* mutant produces LPS totally devoid of O-antigen, while the *lpsβ2* mutant produces LPS with a reduced amount of O-antigen. Although both LPS mutants elicited normal nodules on *L. tenuis* roots, they showed severe reduction in competitiveness when compared with the parental wild-type strain (D'Antuono et al. 2005).

The genome sequence of *S. fredii* HH103 has been determined (Vinardell et al. 2015) and deposited in the European Molecular Biology Laboratory Nucleotide Sequence database. The chemical structure of symbiotic signals produced by *S. fredii* HH103-Rif^r, such as Nod factors and diverse surface polysaccharides, have been previously reported (Gil-Serrano et al. 1997; Hidalgo et al. 2010; Margaret-Oliver et al. 2012; Margaret et al. 2012, 2013; Parada et al. 2006; Rodríguez-Navarro et al. 2014).

To our knowledge, this work describes, for the first time, the symbiotic phenotypes of a collection of *S. fredii* HH103-Rif^r mutants with the model legume *L. japonicus* and *L. burttii*. These bacterial mutants are affected in the chemical structure of Nod factors and also in the production of four different bacterial surface polysaccharides, i.e., CG, EPS, KPS, and LPS. Other HH103-Rif^r mutants, such as purine and pyrimidine auxotrophic (*pur* and *pyr*) mutants or those devoid of flagella-dependent motility (*fla* mutant) were also investigated. Only *S. fredii* HH103-Rif^r mutants unable to produce Nod factors or CG and *pur* and *pyr* mutants failed to nodulate *L. burttii*. It is also concluded that mutations affecting HH103-Rif^r LPS may reduce bacterial nodulation capacity with *L. burttii*. Confocal microscopy studies showed that *Mesorhizobium loti* MAFF303099 invades *L. burttii* roots by ITs formed in root hairs, while *S. fredii* HH103-Rif^r invades *L. burttii* roots through crack entry infections.

RESULTS

Nodulation host-range of *S. fredii* HH103-Rif^r with different *Lotus* species.

S. fredii HH103-Rif^r forms nitrogen-fixing nodules with *L. burttii* but only nodules devoid of bacteria with *L. japonicus* ecotype Gifu (Sandal et al. 2012). Nodulation tests were carried out to further investigate the symbiotic capacity of *S. fredii* HH103-Rif^r with other *Lotus* species belonging to different clades of the *Lotus* genus (Degtjareva et al. 2008). *L. japonicus* Gifu and *L. burttii* (*Lotus corniculatus* clade) plants were used as negative and positive symbiotic controls, respectively. *S. fredii* HH103-Rif^r failed to form nitrogen-fixing nodules with members of all clades tested: Pedrosia clade (*L. creticus* and *L. arenarius*), Lotea clade (*L. cytisoides*), Pedunculatus clade (*L. uliginosus*), and Corniculatus clade (*L. japonicus* ecotypes Gifu and Miyakojima MG-20, *L. filicaulis, L. corniculatus, L. glaber*, and *L. krylovii*). Root outgrowths, varying from elongated root zones to hemispheral structures, were observed in all or some individual plants of the *Lotus* species tested. White ineffective nodules were occasionally observed in *L. filicaulis* and *L. japonicus*. Hence, in our experimental conditions only *L. burttii* plants formed an effective symbiosis with *S. fredii* HH103-Rif^r (Supplementary Table S1). However, *L. burttii* plants inoculated with *S. fredii* HH103-Rif^r were smaller (Supplementary Fig. S1) and the number of nodules formed was slightly lower that that formed by *Mesorhizobium loti* NZP2235 (19.5 ± 6.3 and 25.1 ± 7.8, respectively). In addition, *S. fredii* HH103-Rif^r was clearly outcompeted by *M. loti* NZP2235 to nodulate *L. burttii* plants, since the former only occupied 2 and 4% of the nodules induced in two independent competition experiments (Supplementary Table S2).

Symbiotic capacity of *S. fredii* HH103-Rif^r surface polysaccharides mutants with *L. japonicus* Gifu and *L. burttii*.

Nodulation tests with *L. japonicus* and *L. burttii* were carried out to investigate the symbiotic capacity of diverse *S. fredii* HH103-Rif^r mutants affected in surface polysaccharides (EPS, KPS, LPS, and CG) that are known to play a role in rhizobia-legume interactions. Figure 1 summarizes the chemical structures already determined and the genetic regions mutated in the different *S. fredii* HH103-Rif^r mutants studied in this work. At

least two independent symbiotic tests were carried out for each *S. fredii* HH103-Rif^r mutant investigated in this work.

EPS mutants.

S. fredii SVQ530, an *exoA* mutant derivative of *S. fredii* HH103-Rif^r unable to produce EPS (Parada et al. 2006; Rodríguez-Navarro et al. 2014), formed pink nitrogen-fixing nodules on *L. burttii* roots (Table 1; Fig. 1; Supplementary Fig. S2, panel B). Development of *L. burttii* plants (assessed as shoot height or plant-top fresh weight) inoculated with SVQ530 (15.0 ± 0.9 cm) were similar to those shown by plants inoculated with the parental strain *S. fredii* HH103-Rif^r (14.6 ±

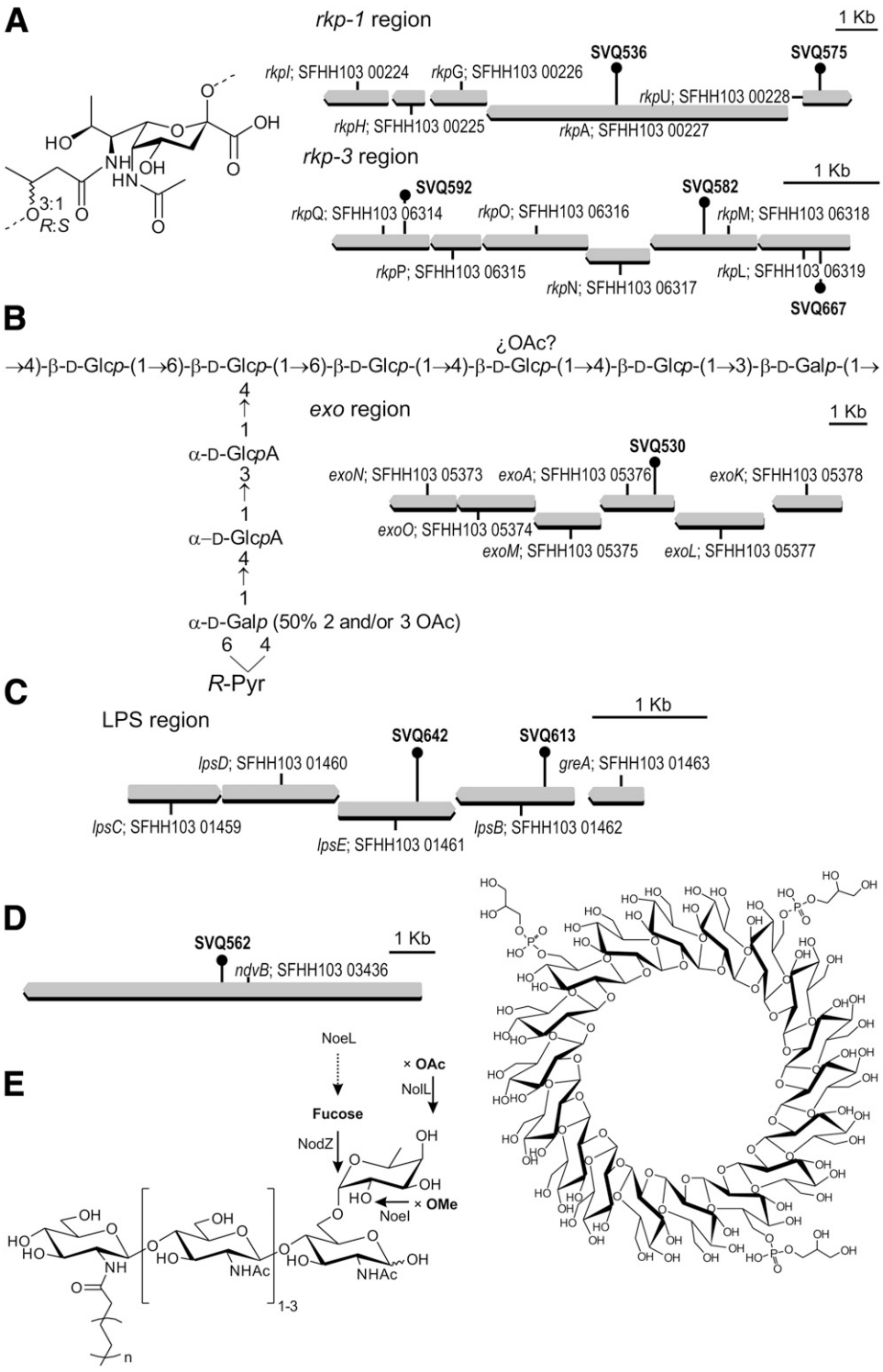


Fig. 1. Chemical structures of *Sinorhizobium fredii* HH103-Rif^r surface polysaccharides and Nod factors and mapping of the mutated genes studied in this work. **A,** Chemical structure of the K-antigen polysaccharide repeating unit and genes of the *rkp-1* and *rkp-3* regions. **B,** Chemical structure of the exopolysaccharide repeating unit and a fragment of the *exo* region containing *exoA*. **C,** Genetic organization of the *lpsCDElpsBgreA* region. **D,** Chemical structure of a cyclic glucan composed of 18 glucosyl residues, the positions of the phosphoglyceryl substituents have not been determined and a fragment of the chromosome containing the *ndvB* (also called *cgs*) gene. **E,** General structure of *S. fredii* HH103-Rif^r Nod factor; arrows indicated the position at which the transferases NodZ y NoeI add the fucosyl residue and the methyl substituent, respectively. The position at which the *Mesorhizobium loti* NolL transferase adds the acetyl substituent is also indicated.

0.7 cm) at 59 days after inoculation (dai). Shoots of uninoculated *L. burttii* plants reached an average height of 2.1 ± 0.7 cm. The kinetics of nodulation of *L. burttii* plants inoculated with SVQ530 and HH103-Rif^r were also similar at 35 dai (Fig. 2, panel A). *L. japonicus* Gifu plants inoculated with SVQ530 failed to form nitrogen-fixing nodules, although macroscopic root outgrowths (MRO) were visible in some plants (Supplementary Fig. S4E and F).

Mutants affected in EPS and LPS.

S. fredii SVQ703 is mutated in a gene homologous to the *S. meliloti rkpK* gene, which codes for a UDP-glucose 6-dehydrogenase that catalyzes the conversion of UDP-glucose into UDP-glucuronic acid (Kereszt et al. 1998). Mutant SVQ703 fails in EPS production and shows an altered LPS profile in polyacrylamide gel electrophoresis (PAGE) experiments (Table 1; Supplementary Fig. S3). *L. burttii* responses to inoculation with SVQ703 were equal to those obtained in plants inoculated with the parental strain *S. fredii* HH103-Rif^r (Table 1). *L. burttii* plants inoculated with SVQ703 or HH103-Rif^r were neither significantly different in the number of nodules formed (23.5 ± 1.2 and 22.2 ± 3.2 nodules per plant, respectively) nor in the height of plant tops (14.2 ± 1.0 and 17.0 ± 2.0 cm, respectively) at 59 dai. *L. japonicus* Gifu plants inoculated with SVQ703 only formed MRO structures and plants were not different from uninoculated controls (Table 1).

KPS mutants.

S. fredii SVQ536 contains a mutation in the *rkpA* gene of the *rkp-1* region (Fig. 1A) and fails to produce the capsular homopolysaccharide (KPS) composed of a pseudaminic acid derivative (Margaret-Oliver et al. 2012). The kinetics of nodulation (Fig. 2B) and the total number of nodules formed in plants inoculated with SVQ536 or HH103-Rif^r were similar. *L. burttii* plants inoculated with SVQ536 or HH103-Rif^r developed green plant tops and the height of all plant shoots was similar (11.5 ± 1.3 and 14.5 ± 1.0 cm, respectively), so that the symbiotic phenotype assigned was Nod$^+$ Fix$^+$ (Table 1). Mutant SVQ536 did not form nitrogen-fixing nodules with *L. japonicus* Gifu, although MRO structures were observed.

Mutants affected in KPS and LPS.

S. fredii SVQ575 carries a mutation in the *rkpU* gene of the *S. fredii* HH103-Rif^r *rkp-1* region (Hidalgo et al. 2010). This mutant does not produce KPS, and its LPS electrophoretic profile is slightly altered in comparison with that of *S. fredii* HH103-Rif^r. These SVQ575 LPS profile alterations are not detectable in 3-day-old cultures (early stationary phase) but in 5-day late stationary phase cultures (Hidalgo et al. 2010). Mutant SVQ575 formed nitrogen-fixing nodules with *L. burttii* and the height of plants inoculated with SVQ575 (13.3 ± 1.0 cm) was similar to that of plants inoculated with HH103-Rif^r (14.5 ± 1.0 cm).

S. fredii SVQ582 (*rkpM*) is mutated in the *rkp-3* region (Fig. 1A), fails to produce KPS and its LPS electrophoretic pattern is altered in comparison with that of HH103-Rif^r (Margaret et al. 2012). *L. burttii* plants inoculated with SVQ582 formed nitrogen-fixing nodules. The kinetics of nodulation of SVQ582 in *L. burttii* plants was delayed in comparison with that of HH103-Rif^r, although the total number of nodules formed by both strains was similar at 36 dai (Fig. 2C). However, at 59 dai, nodulation (15.7 ± 3.3 nodules per plant) and shoot height (9.3 ± 3.1 cm) of *L. burttii* plants inoculated with SVQ582 was reduced in comparison with plants inoculated with HH103-Rif^r (22.2 ± 3.2 nodules per plant; 14.5 ± 1.0 cm shoot-height). This decrease of the symbiotic capacity of SVQ582 with *L. burttii* was further confirmed in another nodulation test (64 dai), in which the shoot fresh weight of plants inoculated with SVQ582 was only 44% of that shown by plants inoculated with HH103-Rif^r. Other mutants of the *rkp-3* region affected in *rkpQ* (SVQ592 and SVQ594), which show an LPS electrophoretic

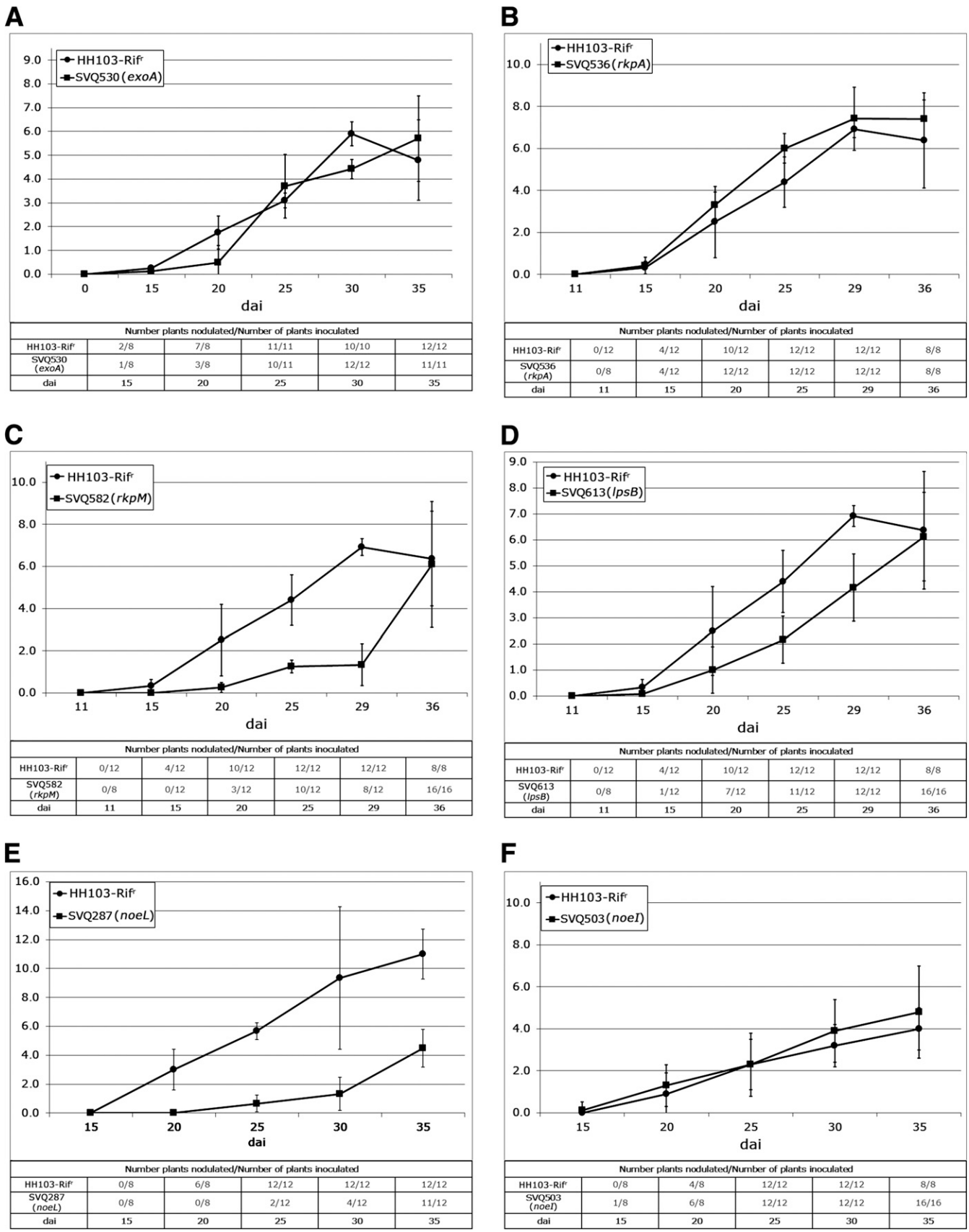
Table 1. *Lotus burttii* and *L. japonicus* Gifu responses to inoculation with *Sinorhizobium fredii* HH103-Rif^r mutants affected in bacterial polysaccharides and *Mesorhizobium loti* NZP2235

Mutant strain and gene mutated[x]	Surface polysaccharide affected[y]	Symbiotic phenotype[z] (number of plants tested)		
			L. japonicus MRO	
		L. burttii	Elongated root outgrowths	Hemispheral structures
HH103-Rif^r	Wild type EPS, KPS, LPS, and CG are produced	Nod$^+$ Fix$^+$ (44)	X	X
SVQ530 *exoA*	EPS$^-$	Nod$^+$ Fix$^+$ (37)	X	x
SVQ536 *rkpA*	KPS$^-$	Nod$^+$ Fix$^+$ (23)	X	X
SVQ575 *rkpU*	KPS$^-$ LPS*	Nod$^+$ Fix$^+$ (10)	X	X
SVQ535 *exoA rkpH*	EPS$^-$ KPS$^-$	Nod$^+$ Fix$^+$ (18)	X	X
SVQ582 *rkpM*	KPS$^-$ LPS*	Nod^R Fix^R (45)	X	x
SVQ594 *rkpQ*	KPS$^-$ LPS*	Nod^R Fix^R (23)	X	x
SVQ703 *rkpK*	EPS$^-$ LPS*	Nod$^+$ Fix$^+$ (37)	X	x
SVQ613 *lpsB*	LPS*	Nod$^+$ Fix^{R*} (27)	X	X
SVQ642 *lpsE*	LPS*	Nod$^+$ Fix$^+$ (11)	X	X
SVQ562 *cgs*	CG$^-$ EPS$^{++§}$	MRO and ineffective nodules (48)	X	x
M. loti NZP2235	Wild type	Nod$^+$ Fix$^+$ (55)	Nod$^+$ Fix$^+$ (56)	

[x] Mutations were induced by insertion of the *lac*ZΔp-Gm^r cassette or the omega interposon.

[y] EPS$^-$ = exopolysaccharide (EPS) is not produced; KPS$^-$ = K-antigen capsular polysaccharide (KPS) is not produced; LPS* = the lipopolysaccharides (LPS) electrophoretic profile is altered in comparison with that of HH103-Rif^r; altered LPS profiles vary among the different mutants analyzed. CG$^-$ = cyclic glucans are not produced. EPS$^{++§}$ = the mutant overproduces EPS of higher molecular weight and bears a higher level of pyruvate and acetate substituents than that of HH103-Rif^r.

[z] MRO = macroscopic root outgrowths. Symbiotic phenotypes with *L. burttii* and *L. japonicus* Gifu are deduced from at least two independent experiments. Nod$^+$ Fix$^+$, nitrogen-fixing nodules are formed and plant development (assessed as plant height or plant-top fresh weight) was similar to those of plants inoculated with *S. fredii* HH103-Rif^r. Nod^R Fix^R, the number of nodules and plant development is significantly lower ($\alpha = 5\%$) than that obtained with HH103-Rif^r. Nod$^+$ Fix^{R*}, the number of nodules is similar but plant-development is significantly reduced and statistical differences (at $\alpha = 5\%$) were found in at least one experiment. Roots forming ineffective (Fix$^-$) nodules can also show macroscopic root outgrowths (MRO) that vary from elongated root zones to hemispherical structures. An upper case X indicates an MRO observed in at least two different experiments; a lower case x indicates an MRO observed in at least one experiment. Many nodules induced in *L. burttii* were pink but green or white nodules can also be observed. Numbers in brackets refer to the total number of plants tested in the different plant assays. Plants were grown in Leonard jars. Nodulation phenotypes were scored 6 to 9 weeks postinoculation.



A	Number plants nodulated/Number of plants inoculated				
HH103-Rif^r	2/8	7/8	11/11	10/10	12/12
SVQ530 (*exoA*)	1/8	3/8	10/11	12/12	11/11
dai	15	20	25	30	35

B	Number plants nodulated/Number of plants inoculated					
HH103-Rif^r	0/12	4/12	10/12	12/12	12/12	8/8
SVQ536 (*rkpA*)	0/8	4/12	12/12	12/12	12/12	8/8
dai	11	15	20	25	29	36

C	Number plants nodulated/Number of plants inoculated					
HH103-Rif^r	0/12	4/12	10/12	12/12	12/12	8/8
SVQ582 (*rkpM*)	0/8	0/12	3/12	10/12	8/12	16/16
dai	11	15	20	25	29	36

D	Number plants nodulated/Number of plants inoculated					
HH103-Rif^r	0/12	4/12	10/12	12/12	12/12	8/8
SVQ613 (*lpsB*)	0/8	1/12	7/12	11/12	12/12	16/16
dai	11	15	20	25	29	36

E	Number plants nodulated/Number of plants inoculated				
HH103-Rif^r	0/8	6/8	12/12	12/12	12/12
SVQ287 (*noeL*)	0/8	0/8	2/12	4/12	11/12
dai	15	20	25	30	35

F	Number plants nodulated/Number of plants inoculated				
HH103-Rif^r	0/8	4/8	12/12	12/12	8/8
SVQ503 (*noeI*)	1/8	6/8	12/12	12/12	16/16
dai	15	20	25	30	35

Fig. 2. Nodulation kinetics of *Lotus burttii* inoculated with *Sinorhizobium fredii* HH103-Rif^r and diverse mutants affected in surface polysaccharides or Nod factors. **A**, SVQ530 (*exoA*); **B**, SVQ536 (*rkpA*); **C**, SVQ582 (*rkpM*); **D**, SVQ613 (*lpsB*); **E**, SVQ287 (*noeL*); and **F**, SVQ503 (*noeI*). Eight to 16 plants were analyzed for determining nodule number per plant at each point of the time course experiments.

profile similar to that of SVQ582, were also significantly (α = 5%) impaired to nodulate *L. burttii*.

Neither the *rkpU* mutant (SVQ575, *rkp-1* region) nor mutants affected in the *rkp-3* region (*rkpM* and *rkpQ* mutants) induced the formation of nitrogen-fixing nodules on *L. japonicus* Gifu roots, although MRO were observed (Table 1). Mutant SVQ667 (*rkpL, rkp-3* region) also showed an altered LPS electrophoretic profile and failed to nodulate *L. japonicus* Gifu.

Mutants affected in KPS and EPS.

Mutant SVQ535 neither produces KPS nor EPS due to mutations in *rkpH* (*rkp-1* region) and *exoA* (Parada et al. 2006). The LPS profile of SVQ535 is similar to that of HH103-Rif^r (Parada et al. 2006). Mutant SVQ535 formed nitrogen-fixing nodules with *L. burttii* and nodulation (30.2 ± 12 nodules per plant) and plant development at 59 dai (13.6 ± 2.4 cm) were similar to those of plants inoculated with HH103-Rif^r (22.2 ± 3.2 nodules per plant and 14.5 ± 1.0 cm). *L. japonicus* Gifu plants inoculated with SVQ535 only formed ineffective root outgrowths.

LPS mutants.

S. fredii lpsB and *lpsE* are two contiguous genes that transcribe in opposite directions (Fig. 1C). The electrophoretic profiles of mutants SVQ613 (*lpsB*) and SVQ642 (*lpsE*) are altered in comparison with that of HH103-Rif^r (Margaret et al. 2013). Both mutants formed nitrogen-fixing nodules with *L. burttii* (Table 1). Further studies were carried out with the *lpsB* mutant. The kinetics of nodulation of SVQ613 was delayed in comparison with that shown by plants inoculated with HH103-Rif^r (Fig. 2D). Shoots developed by plants inoculated with SVQ613 were shorter (9.2 ± 1.8 cm) than those inoculated with HH103-Rif^r (14.5 ± 1.0 cm). In an independent nodulation test, shoot fresh weight of *L. burttii* plants inoculated with SVQ613 were 79% of that scored with the HH103-Rif^r inoculant, at 64 dai. Neither SVQ613 nor SVQ642 formed nitrogen-fixing nodules with *L. japonicus* Gifu plants, but MRO were observed.

CG mutants.

S. fredii SVQ562 is a HH103-Rif^r *cgs* (also called *ndvB*) mutant derivative unable to produce CG (Fig. 1D). It overproduces EPS and only forms ineffective nodules on soybean plants (Crespo-Rivas et al. 2009). As expected, both *L. burttii* and *L. japonicus* Gifu plants inoculated with SVQ562 only formed MRO and ineffective nodules from which bacteria were not isolated (Table 1).

Mutants affected in Nod factors.

We have investigated whether the symbiotic interaction between *S. fredii* HH103-Rif^r and *L. burttii* requires the presence of *S. fredii* HH103-Rif^r Nod factors. The symbiotic importance of the fucosyl residue with or without one or both methyl or acetyl substitutions has also been investigated (Table 2). A *S. fredii* HH103-Rif^r *nodA* mutant (SVQ116) failed to nodulate *L. burttii*, which indicates that bacterial Nod factors are absolutely required in this symbiotic interaction.

Mutant SVQ287 (*noeL*) produces Nod factors that are devoid of the (methyl) fucosyl residue at position C-6 of the reducing end (Fig. 1E). *L. burttii* plants inoculated with SVQ287 showed reduced nodulation in comparison with those inoculated with *S. fredii* HH103-Rif^r at 48 dai (Table 2). Two other independent experiments showed similar results at 42 and 72 dai, since the number of nodules induced by SVQ287 (1.1 ± 0.2 and 8.3 ± 1.2 nodules per plant, respectively) was lower than that of plants inoculated with *S. fredii* HH103-Rif^r (7.0 ± 1.1 and 21.7 ± 2.1 nodules per plant, respectively). Plant-top fresh weight of plants inoculated with SVQ287 was similar to that of uninoculated plants at 48 dai (Table 2) but significantly higher at 72 dai. Kinetics of nodulation experiments showed that *L. burttii* roots inoculated with HH103-Rif^r were nodulated at 20 dai, while those inoculated with SVQ287 had not developed macroscopic nodules yet (Fig. 2E). Thus, nodulation and nitrogen fixation of SVQ287 with *L. burttii* is severely reduced.

Mutant SVQ503 carries a mutation in *noeI*, a gene coding for the transferase that methylates the fucosyl residue linked at the reducing end of the *N*-acetyl-glucosamine oligosaccharide (Fig. 1E). Nod factors produced by mutant SVQ503 were all devoid of the methyl substitution at the fucosyl residue, as it was previously reported (Madinabeitia et al. 2002). SVQ503 effectively nodulated *L. burttii* and the number of pink nodules formed at 48 dai was similar to that found in plants inoculated with *S. fredii* HH103-Rif^r (Table 2). Moreover, nodulation kinetics experiments did not reveal significant differences between plants inoculated with HH103-Rif^r or SVQ503 (Fig. 2F).

S. fredii HH103-Rif^r produces methylated Nod factors (Gil-Serrano et al. 1997), while those produced by *M. loti* R7A are acetylated (Rodpothong et al. 2009). To investigate whether the production of *S. fredii* HH103-Rif^r Nod factors containing acetylated fucosyl residues enhances nodulation on *L. burttii* roots, the *nolL* gene of *M. loti* R7A was polymerase chain reaction (PCR)-amplified and was cloned into plasmid pBBR1MCS5, generating plasmid pMUS1334. Plasmid pMUS1334 was transferred by conjugation to *S. fredii* HH103-Rif^r and SVQ503. Mass spectrometry analyses of HH103-Rif^r pMUS1334 cultures grown in the presence of genistein detected the presence of a mixture of Nod factors only carrying methylfucose, only acetylfucose, and acetylmethylfucose (Supplementary Table S3). Nod factors produced by SVQ503 pMUS1334 cultures mainly contained nonsubstituted or acetylated fucosyl residues. Unexpectedly, a methylated Nod factor (IV [$C_{18:1}$, MeFuc]) was also detected. Nodulation tests on *L. burttii* plants showed that, at 48 dai, the presence of the *M. loti* R7A *nolL* gene in HH103-

Table 2. *Lotus burttii* responses to inoculation with *Sinorhizobium fredii* HH103-Rif^r and mutants SVQ287 (*noeL*) and SVQ503 (*noeI*)[z]

Bacterial strain	Genetic characteristics	Symbiotic properties with *L. burttii*	
		Number of nodules/plant	Plant-top fresh weight (mg)
HH103-Rif^r	Wild type	8.0 ± 0.8 a	47.6 ± 9.0 a
HH103-Rif^r pMUS1334	Wild type carrying the *M. loti* R7A *nolL* gene	7.9 ± 0.6 a	46.5 ± 10.6 a
SVQ503	HH103-Rif^r *noeI*:: Ω	9.4 ± 1.0 a	69.3 ± 16.8 a
SVQ503 pMUS1334	HH103-Rif^r *noeI*:: Ω carrying the *M. loti* R7A *nolL* gene	9.6 ± 0.4 a	57.9 ± 6.6 a
SVQ287	HH103-1 *noeL*::Tn*5*-B20	0.9 ± 0.2 b	11.2 ± 1.4 b
Uninoculated		0.0	13.5 ± 1.8 b
LSD (P = 0.05)		2.0	28.8

[z] Three jars containing four plants were tested per inoculant. Plants were analyzed at 48 days after inoculation. Mean numbers followed by the same letter in the same column are not significantly different (P = 0.05), using the least significant difference (LSD) test. Isolates from nodules showed the expected antibiotic resistant markers.

Rif^r and SVQ503 neither significantly increased the number of nodules nor plant-top fresh weight (Table 2).

Microscopic analyses of *L. burttii* roots inoculated with *S. fredii* HH103-Rif^r.

Previous reports have shown that rhizobial EPS, KPS, or an intact LPS are often necessary for the formation of effective nodules in at least one particular rhizobia-legume interaction (Krol and Becker 2009). Since the symbiotic impairment of *S. fredii* HH103-Rif^r KPS and LPS mutants is more severe with *Glycine max* (soybean) than with *L. burttii* (Table 1), we have investigated how *S. fredii* HH103-Rif^r infects *L. burttii* roots. Light microscopic and confocal microscopic analyses of *L. burttii* roots inoculated with *S. fredii* HH103-Rif^r carrying the pFAJDsRed plasmid (Kelly et al. 2013) were carried out. At 2 to 3 weeks postinoculation, *S. fredii* HH103-Rif^r was found in microcolonies of curled root hairs and ITs were only very rarely initiated (Fig. 3 A and B) and none progressed further. The number of ITs in *L. burttii* and *L. japonicus* Gifu inoculated with *S. fredii* HH103-Rif^r was counted and was practically null (Fig. 4). In contrast, *M. loti* MAFF303099 DsRed strain (Maekawa et al. 2009) invaded *L. burttii* roots by the formation of root hair ITs (Figs. 3J and K and 4). In vibratome sections of root nodules, we could observe infection pockets associated with crack entry in the nodules (Fig. 3D and E). Additionally, *S. fredii* HH103-Rif^r was detected between cells in the nodule (Fig. 3E and G). Therefore, the successful infection of *L. burttii* roots by *S. fredii* HH103-Rif^r occurs by crack entry rather than via root hair ITs (Figs. 3H to K and 4).

Purine and pyrimidine auxotrophic mutants.

Previous reports have shown that purine- and pyrimidine-auxotrophic rhizobial mutants are not only impaired for infecting legumes through root-hair ITs (Buendía-Clavería et al. 2003; Crespo-Rivas et al. 2007; Noel et al. 1988) but, also, for the infection of legumes through cracks in the root epidermis (Giraud et al. 2007). Therefore, we have investigated a *S. fredii* HH103-Rif^r auxotrophic mutant (SVQ295, mutated in

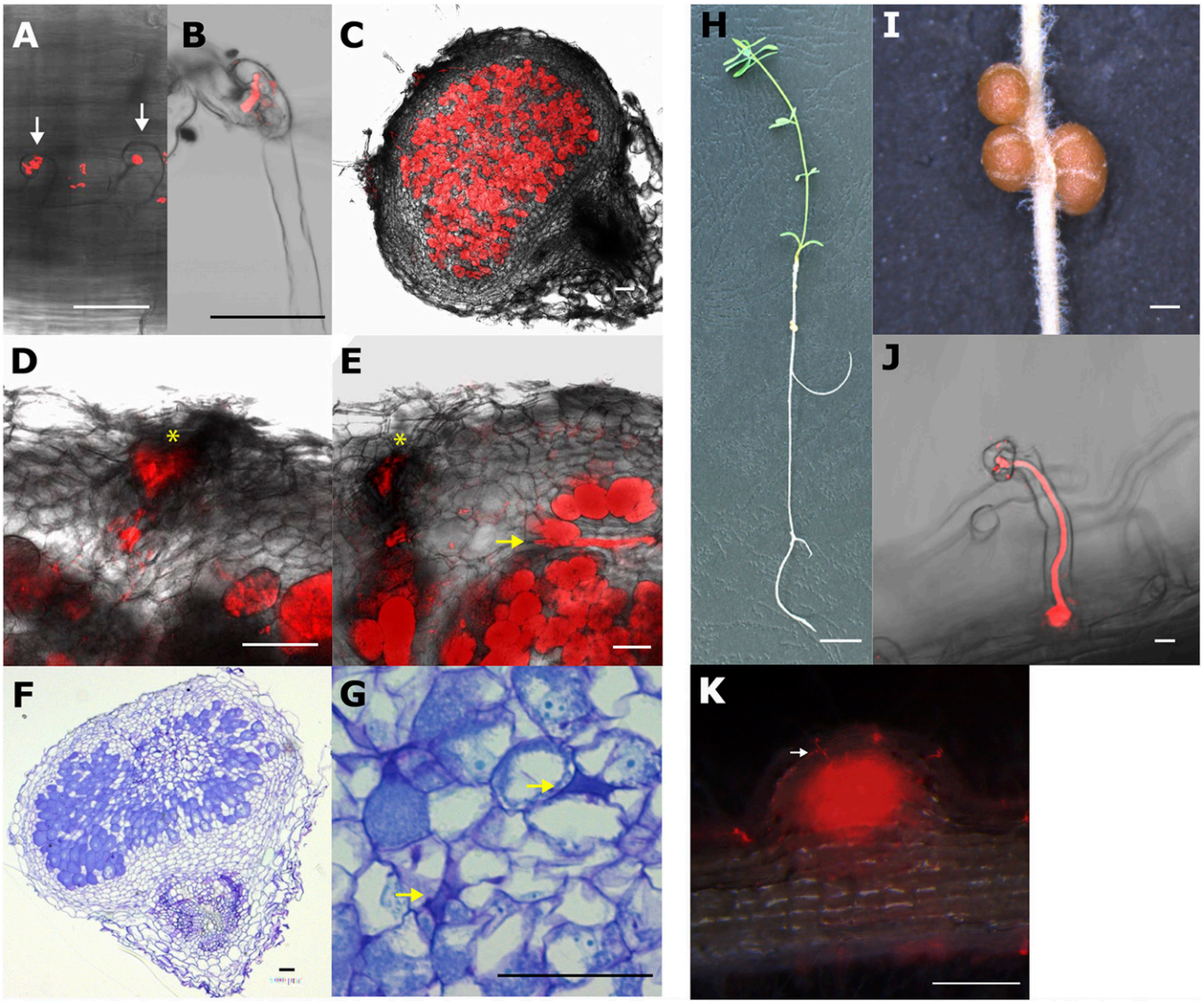


Fig. 3. The symbiotic phenotype of *Lotus burttii* inoculated with *Sinorhizobium fredii* HH103-Rif^r and *Mesorhizobium loti* MAFF303099. **A,** *S. fredii* HH103-Rif^r DsRed colonies in curled root hairs at 2 weeks postinoculation (wpi) and **B,** in early infection threads (IT) at 3 wpi. **C** to **G,** Sections of *L. burttii* nodules at 2 to 4 wpi with S. *fredii* HH103-Rif^r DsRed. White arrows show microcolonies of *S. fredii* HH103-Rif^r. Yellow stars show the place for crack entry. Yellow arrows show bacteria that colonize between the cells in the nodule. Bars in A to G are 50 µm. **H** to **K,** *L. burttii* plants at 3 wpi with *M. loti* MAFF303099 DsRed. **H,** *L. burttii* whole plant, **I,** nodules, **J,** IT, and **K,** IT elongated from the root hair to the nodule of *L. burttii* inoculated with *M. loti* MAFF303099 DsRed at 9 days after inoculation. Scale bars are 1 cm in H, 1 mm in I, 10 µm in J, and 500 µm in K.

purL) that requires the exogenous addition of adenine and thiamine for their capacity to nodulate *L. burttii* plants. Sixteen *L. burttii* plants inoculated with SVQ295 failed to nodulate at 49 dai. We also tested whether SVQ295, which produces Nod factors (Buendía-Clavería et al. 2003), could capacitate mutant SVQ116 (*nodA*) for nodulating *L. burttii* roots in coinoculation experiments. None of the 22 *L. burttii* plants coinoculated with SVQ295 and SVQ116 formed nodules at 49 dai. SVQ295 also failed to nodulate *L. burttii* plants grown for 35 days in petri dishes containing agar-solidified plant nutrient solution supplemented with adenine (12 µg/ml) and thiamine (0.8 µg/ml). Neither the presence of adenine or adenine and thiamine together appeared to cause any negative impact on uninoculated roots.

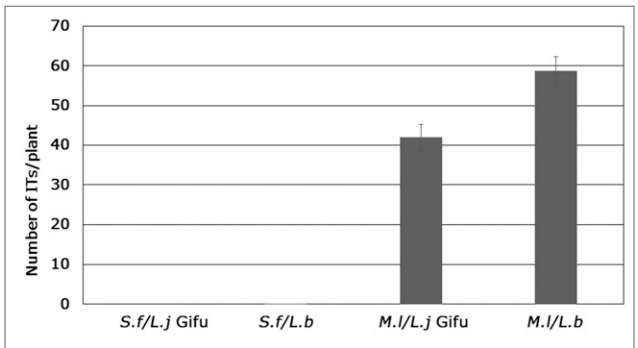
Mutant SVQ292 is a *S. fredii* HH103-Rif^r pyrimidine auxotrophic mutant (mutated in *pyrF*) that requires the addition of uracil. *L. burttii* plants inoculated with SVQ292 only formed ineffective nodules in which nitrogenase activity was not detected by acetylene-reduction assays. The presence of uracil (12 µg/ml) in the plant nutritive solution did not alleviate the symbiotic impairment of SVQ292 with *L. burttii*, in petri-dish nodulation assays.

Bacterial flagella are not required for crack infection.

To investigate whether flagella-dependent mobility was required by *S. fredii* HH103-Rif^r to invade *L. burttii* roots through cracks, we constructed a *S. fredii* HH103-Rif^r mutant (SVQ705) devoid of flagella by deletion of the *flaCBAD* genes (discussed below). Mutant SVQ705 did not show any swimming mobility in Bromfield's semisolid media (Supplementary Fig. S5). Nodulation assays revealed that *L. burttii* plants inoculated with SVQ705 formed nitrogen-fixing nodules.

DISCUSSION

L. burttii and the model legume *L. japonicus* are mainly nodulated by rhizobial strains classified as *Mesorhizobium loti*. *S. fredii* HH103-Rif^r nodulates *L. burttii* and, also, a broad range of herbaceous, shrub, and tree legumes (Margaret et al. 2011; Sandal et al. 2012). Some of them form determinate nodules, while others induce the formation of indeterminate nodules. Because of this broad symbiotic capacity, *S. fredii* HH103-Rif^r can be used in studies aimed at determining which bacterial structures or chemical signals or combination thereof are specifically relevant for the symbiosis with a particular legume.



Plants/Inoculum	Number of Infection Threads (ITs) at 9 dai	
	HH103-Rif^r	*M. loti* MAFF303099
L. burttii	0.06±0.04	58.8±3.62
L. japonicus Gifu	0±0	41.93±3.28

Fig. 4. Number of root hair infection threads (ITs) of *Lotus japonicus* Gifu and *L. burttii* 9 days after inoculation with *Sinorhizobium fredii* HH103-Rif^r DsRed (columns 1 and 2, respectively) or *Mesorhizobium loti* MAFF303099 DsRed (columns 3 and 4, respectively). The number of *L. japonicus* Gifu and *L. burttii* plants inoculated with *S. fredii* HH103-Rif^r DsRed was 50 and 54. Fifteen *L. japonicus* Gifu and *L. burttii* plants were inoculated with *M. loti* MAFF303099 DsRed.

We have investigated the symbiotic properties of *S. fredii* HH103-Rif^r mutants affected in the biosynthesis of surface polysaccharides and Nod factors with *L. burttii* and *L. japonicus*. The studies on *L. burttii* were aimed at determining which particular *S. fredii* HH103-Rif^r surface polysaccharide and which Nod factor structure or structures are important or even indispensable for a successful *L. burttii–S. fredii* HH103-Rif^r symbiosis. In contrast, studies on *L. japonicus* Gifu were carried out to investigate whether elimination or alteration of any particular surface polysaccharide or Nod factor structure enabled *S. fredii* HH103-Rif^r to nodulate this legume. All *S. fredii* HH103-Rif^r surface polysaccharide mutants tested here have been previously investigated for their symbiotic capacity with *Glycine max* (López-Baena et al. 2016) so that the comparisons with their symbiotic phenotype with *L. burttii* can be established.

Mutant SVQ530 (*exoA*) does not produce EPS and is not impaired in nodulation of soybeans (Parada et al. 2006). Instead, SVQ530 shows enhanced competitiveness for soybean nodulation (Rodríguez-Navarro et al. 2014). *S. fredii* HH103-Rif^r and SVQ530 appear to have the same symbiotic capability with *L. burttii*, which indicates that EPS might not play any significant role in this symbiotic interaction. However, a recent report has shown that *L. japonicus* possesses a receptor-like kinase that binds to *M. loti* R7A EPS. This receptor, EPR3, distinguishes symbiotically compatible EPS forms from those that determine symbiotic incompatibility (Kawaharada et al. 2015). Truncated *M. loti* R7A EPS forms impaired nodulation with *L. japonicus*, while an R7A *exoA* mutant, unable to produce EPS, formed nitrogen-fixing nodules (Kelly et al. 2013). The *S. fredii* HH103-EPS is composed of glucose, galactose, and glucuronic acid (Rodríguez-Navarro et al. 2014), while that of *M. loti* R7A also contains riburonic acid (Kelly et al. 2013; Muszyński et al. 2016). *L. japonicus* EPR3 might recognize the *S. fredii* HH103-EPS as nonsymbiotic, resulting in the formation of noninfected nodules. However, a hypothetical EPS-mediated incompatibility would not entirely account for the symbiotic incompatibility between *S. fredii* HH103-Rif^r and *L. japonicus*, because SVQ530 does not produce EPS and has not gained nodulation capacity with this legume.

Two KPS mutants affected in the *rkp-1* region (SVQ536 and SVQ575) did not show any significant symbiotic impairment with *L. burttii*. *rkpA* belongs to an operon of five genes (*rkpA*, *rkpG*, *rkpH*, *rkpI*, and *rkpJ*) that are contiguous and transcribe in the opposite direction of *rkpU* (Fig. 1A). The *rkpA* gene encodes a putative 2-amino-3-ketobutyrateCoA-ligase, while *rkpU* codes for a protein related to the export of capsular polysaccharides (Hidalgo et al. 2010; Margaret-Oliver et al. 2012). SVQ536 does not show any appreciable alteration in its LPS electrophoretic profile (Margaret-Oliver et al. 2012). Thus, the absence of only KPS in SVQ536 does not provoke any severe symbiotic impairment with this legume.

Mutant SVQ575 shows slight variations in the LPS profile but only when bacterial cultures become 5 days old (Hidalgo et al. 2010), which corresponds to the late stationary phase. No detectable SVQ575 LPS alterations are visualized in the early stationary phase (Hidalgo et al. 2010), which is the normal age (2 to 3 days) of the *S. fredii* HH103-Rif^r inoculum used in nodulation tests. All these results suggest that the LPS profile alterations shown by SVQ575 are not relevant for the symbiotic interaction between *S. fredii* HH103-Rif^r and *L. burttii* or that they are not present in the 2- to 3-day-old bacterial inoculant. In summary, *S. fredii* HH103-Rif^r mutants in the *rkp-1* region are symbiotically impaired with soybeans (Hidalgo et al. 2010;

Margaret et al. 2012; Parada et al. 2006) but not with *L. burttii* (Table 1).

In contrast, mutant SVQ582 (affected in the *rkp-3* region, unable to produce KPS) showed reduced nodulation with *L. burttii* (Table 1) and its kinetics of nodulation was delayed in comparison with its parental strain (Fig. 2C). The LPS electrophoretic pattern of *S. fredii* SVQ582 is clearly altered in comparison with that of HH103-Rif^r (Margaret al. 2012), and these alterations are different to that exhibited by SVQ575. In comparison with their respective HH103-Rif^r control, SVQ582 LPS silver-stained bands migrate faster than those of SVQ575. Thus, the differential symbiotic capacity of *rkp-1* mutants and SVQ582 with *L. burttii* could be due to the specific LPS structural alterations shown by SVQ582. The *S. fredii* HH103-Rif^r *rkpM* mutation appears more deleterious for the bacterial symbiotic capacity with soybean than with *L. burttii*, since only the latter can form nitrogen-fixing nodules. None of the tested KPS mutants belonging to the *rpk-1* region (*rkpA* and *rkpU*) or *rkp*-3 region (*rkpM* and two other mutants affected in *rkpL* and *rkpQ*) has gained nodulation capacity with *L. japonicus* Gifu, which indicates that the presence of KPS might not be responsible for the absence of effective nodules on *L. japonicus* roots inoculated with *S. fredii* HH103-Rif^r.

KPS can replace EPS for a successful nodulation of *S. meliloti* with *Medicago sativa* (Pellock et al. 2000). Due to this symbiotic equivalence, *S. meliloti* mutants that only produce EPS or KPS are still symbiotically effective. In the soybean–*S. fredii* HH103-Rif^r symbiosis, mutant SVQ536 (EPS$^+$ KPS$^-$) is impaired, while mutant SVQ530 (EPS$^-$ KPS$^+$) is not negatively affected (Parada et al. 2006). The *S. fredii* mutant (SVQ535) unable to produce EPS and KPS was still able to form nitrogen-fixing nodules with *L. burttii* and failed to nodulate *L. japonicus* Gifu. Hence, the simultaneous absence of EPS and KPS production neither abolishes nodulation with *L. burttii* nor enhances nodule formation on *L. japonicus* roots.

Mutations affecting rhizobial LPS usually cause severe negative impacts on symbiosis forming determinate nodules, such as those constituted by *S. fredii*–soybean (Margaret et al. 2013), *Bradyrhizobium* spp.–soybean (Stacey et al. 1991), or *Rhizobium etli–Phaseolus vulgaris* (Noel et al. 1986). Although the LPS electrophoretic profiles of *S. fredii* mutants SVQ613 (*lpsB*) and SVQ642 (*lpsE*) are clearly altered due to mutations in genes involved in LPS synthesis (Fig. 1) (Margaret et al. 2013), only the former showed moderate symbiotic impairment with *L. burttii* (Table 1). The negative impact caused by the *lpsB* mutation on the *S. fredii* HH103-Rif^r symbiotic capacity is much stronger in soybean than in *L. burttii*, suggesting that the requirement of an intact HH103-Rif^r LPS structure might be more important in soybean and *P. vulgaris* than in the *Lotus* sp. This absence of severe symbiotic impairments associated to LPS mutations is in line with previous results showing that a *M. loti* mutant totally devoid of O-antigen was still able to induce nitrogen-fixing nodules on *L. tenuis* (D'Antuono et al. 2005). Further studies including more *Lotus* spp. should be carried out to elucidate this question.

L. burttii plants inoculated with mutant SVQ562 (*ndvB*) formed ineffective nodules, which is the same symbiotic phenotype previously observed with soybean (Crespo-Rivas et al. 2009). This result is also in line to those previously reported for a diversity of rhizobial mutants unable to produce CG (Breedveld and Miller 1998). To our knowledge, the symbiotic capacity of all the *ndvB* mutants (such as *S. meliloti*, *S. fredii*, *M. loti*, or *B. japonicum*) investigated fail in the formation of nitrogen-fixing nodules with their respective host legumes (Crespo-Rivas et al. 2009; D'Antuono et al. 2005; Dylan et al. 1986).

Nodulation factors produced by *S. fredii* HH103-Rif^r and *M. loti* R7A share some structural characteristics, such as the presence of C16 and C18 saturated or mono-unsaturated acyl groups at the nonreducing terminal *N*-acetyl-glucosamine residue and the presence of fucosyl decorations linked to the terminal reducing end. Examples of structural differences are the presence of carbamoyl substitutions only in *M. loti* R7A and that the fucosyl residue has 2-*O*-methyl substitutions in *S. fredii* HH103-Rif^r but 4-*O*-acetyl (or 3-*O*-acetyl) decorations in *M. loti* R7A (Bek et al. 2010; Gil-Serrano et al. 1997; López-Lara et al. 1995). *S. fredii* SVQ287 is a *noeL* mutant that produces Nod factor devoid of fucosyl residues (Lamrabet et al. 1999). Now, we have identified new Nod factors bearing saturated C_{10}, C_{12}, and C_{14} acyl chains as well as a Nod factor carrying an *N*-methylation at the nonreducing *N*-acetyl-glucosamine (IV [$C_{14:0}$, NMe]). Nod factor bearing C_{12}, and C_{14} fatty acids have previously been reported in *M. loti* (Rodpothong et al. 2009) and Nod factors carrying C_{10} acyl chains in *Rhizobium tropici* (Morón et al. 2005).

SVQ287 is outcompeted by its wild-type HH103-1 to nodulate soybean cv. Williams (Lamrabet et al. 1999). The reduction in the number of nodules formed with *L. burttii* (89 and 84% in two independent experiments) was similar to that (80%) previously reported in soybean cv. Williams (Lamrabet et al. 1999). Mutant SVQ287 shows delayed nodulation with both *L. burttii* and soybean, although this delay appears more pronounced with the former. Previous reports have shown that a *M. loti* R7A *nodZ* mutant has a host-specific phenotype: normal effective nodulation with *L. burttii*, delayed effective nodulation with *L. japonicus* Gifu and the formation of uninfected nodules on *L. filicaulis* and *L. corniculatus* (Rodpothong et al. 2009). Pacios-Bras and associates (2000) demonstrated that a *Rhizobium leguminosarum* strain showing constitutive (flavonoid-independent) transcription of *nod* genes and also carrying the *M. loti nodZ* and *noeL* genes has gained the capacity to nodulate *L. japonicus* but not *L. filicaulis*. This host-specific phenomenon was due to differences in the *Nfr5* receptor genes of *L. japonicus* and *L. filicaulis* (Radutoiu et al. 2007). Thus, the symbiotic impairment caused by the absence of fucosyl residues is very evident in the symbiosis *L. burttii–S. fredii* HH103-1 but negligible in the symbiosis *L. burttii–M. loti* R7A. Whether the differential symbiotic impact caused by the absence of the fucosyl residue is related to the fact that *S. fredii* HH103-Rif^r infects *L. burttii* roots through cracks while *M. loti* NZP2235 invades through ITs remains to be elucidated.

S. fredii SVQ503 is a *noeI* mutant devoid of the symbiotic methyl transferase that adds methyl substituents to the fucosyl residue (Fig. 1E). Mass spectrometry analysis showed that Nod factors produced by SVQ503 do not carry methyl substitutions in the fucosyl residue, as we previously reported (Madinabeitia et al. 2002). No significant differences were found between the number of nodules, plant-top fresh weight, and kinetics of nodulation of *L. burttii* plants inoculated with HH103-Rif^r or SVQ503 (Table 2; Fig. 2F). Thus the presence of a methyl substitution in the fucosyl residue does not apparently play any significant role in the *L. burttii–S. fredii* HH103-Rif^r symbiotic interaction. In the *L. burttii–M. loti* R7A symbiosis, however, the absence of the acetyl substitution in the fucosyl residue causes a delay in nodule formation (Rodpothong et al. 2009).

Because *S. fredii* HH103-Rif^r Nod factors do not contain acetylfucosyl residues, we have transferred the *M. loti* R7A *nolL* gene (cloned in plasmid pMUS1334) to *S. fredii* HH103-Rif^r and to its *noeI* mutant derivative SVQ503. The production of Nod factors carrying acetyl substituents in the fucosyl residue did not significantly improve bacterial capacity to nodulate *L. burttii* (Table 2), regardless whether the methyl substitution is also present (as in HH103-Rif^r pMUS1334) or absent (as in SVQ503 pMUS1334). In conclusion, the loss of the fucosyl residue is very damaging for the *S. fredii* HH103 symbiotic capacity with *L. burttii*, while the presence or absence of one or

more methyl and acetyl substitutions does not cause a significant impact.

A single Nod factor carrying methylfucose (IV [$C_{18:1}$, MeFuc]), which might originate from the activity of an unspecific methyl transferase that can functionally complement the NoeI enzyme was unexpectedly present in *S. fredii* SVQ503 pMUS1334 cultures. It was not detected in previous works (Madinabeitia et al. 2002). This finding is in line with previous reports showing that a *M. loti* R7A *nolL* mutant still produces some acetylated Nod factors, probably due to the activity of other acetyltransferases (Rodpothong et al. 2009).

None of the *S. fredii* HH103-Rif^r surface polysaccharide and Nod factor mutants investigated has gained nodulation capacity with *L. japonicus*. This fact does not exclude that HH103-Rif^r surface polysaccharides could act as incompatible signals for *L. japonicus*, because it is still possible that more than one HH103-Rif^r surface polysaccharide is blocking the nodulation process. Should it be the case, *S. fredii* HH103-Rif^r mutants unable to produce a single particular wild-type surface polysaccharide would still be blocked by the presence of another incompatible surface polysaccharide. The double EPS$^-$ KPS$^-$ mutant (SVQ535) has not gained nodulation capacity with *L. japonicus*, revealing that these two surface polysaccharides are not cooperating to block nodulation. The loss of CG production leads to the loss of effective nodulation in *L. burttii* and does not cause any symbiotic improvement with *L. japonicus*. Thus, if two different HH103-Rif^r surface polysaccharides were responsible for the failure to nodulate *L. japonicus*, LPS might be one of the candidates.

Up to now, three different rhizobial entry modes have been described in the different legumes investigated: intercellular single infection, crack entry, and IT invasion (Sprent 2007). Intercellular single infection is suggested to be the most primitive way by which rhizobia invade legume roots, followed by the crack entry and root hair–infection modes (Madsen et al. 2010). Our confocal microscopy studies, together with the inability of the HH103-Rif^r *nodA* mutant to nodulate *L. burttii*, demonstrate that the way *S. fredii* HH103-Rif^r invades *L. burttii* roots can be classified in the intermediate-evolutionary stage, i.e., Nod factor–dependent crack entry mode. At least in the *L. burttii*–*S. fredii* HH103-Rif^r symbiosis, this mode of infection does not strictly require the production of surface polysaccharides released by the bacteria (such as EPS) or linked to the external leaflet of the outer membrane (such as KPS). Neither does it require the wild-type HH103-Rif^r LPS forms, since none of the different LPS alterations shown by mutants SVQ613 (*lpsB*), SVQ642 (*lpsE*), SVQ575 (*rkpU*), and SVQ582 (*rkpM*) have totally abolished nodule formation in *L. burttii* roots (Table 1). Mutant SVQ562 was the only *S. fredii* HH103-Rif^r surface polysaccharide mutant that failed to form nitrogen-fixing nodules with *L. burttii*. Occasionally, *L. burttii* roots inoculated with SVQ562 formed nitrogen-fixing nodules, but isolates from these nodules did not carry the *ndvB* mutation. Thus, CG are not only necessary for invasion through ITs, such as in the *S. fredii* HH103-Rif^r–soybean or *S. meliloti*–alfalfa symbioses but, also, in the less-evolved *L. burttii*–*S. fredii* HH103-Rif^r crack-entry mode.

A *S. fredii* HH103-Rif^r *purL* mutant (SVQ295) also failed to nodulate *L. burttii*, indicating that the purine metabolic pathway is important for this symbiotic interaction. Coinoculation of soybean roots with SVQ295 (auxotrophic for adenine and thiamine but producing Nod factor) and SVQ116 (prototrophic but unable to produce Nod factors) results in the formation of nitrogen-fixing nodules on soybean roots in which only SVQ116 is detected inside the nodules (Buendía-Clavería et al. 2003). Physiological complementation has also been observed in coinoculation experiments in which *S. meliloti* Nod$^-$ mutants are complemented by Exo$^-$ mutants, resulting in the formation of nitrogen-fixing nodules on alfalfa roots (Müller et al. 1988). In contrast, the pair of *S. fredii* SVQ295 and SVQ116 coinoculants does not induce effective nodules on *L. burttii* roots, which suggest that plant requirements of bacterial signals (quantity and, possibly, localization) are not the same in the symbioses mentioned above. Elucidating whether the different modes of root infection, through root hair ITs (soybean–*S. fredii* HH103-Rif^r) or through cracks (*L. burttii*–*S. fredii* HH103-Rif^r), actually accounts for the different results observed in coinoculation experiments remains to be investigated.

L. burttii roots are infected by *M. loti* through root-hair ITs, while *S. fredii* HH103-Rif^r invades this legume through epidermal cracks (Fig. 3). Similarly, *L. japonicus* Nepal can switch from intercellular bacterial entry with *Rhizobium leguminosarum* Norway to root-hair IT infection with *M. loti* (Gossmann et al. 2012). All these results clearly indicate that the roots of a particular legume that can be infected by different rhizobia might be invaded by different modes of infection.

MATERIALS AND METHODS

Molecular and microbiological techniques.

Bacterial strains and plasmids used in this work are listed in Supplementary Table S4. *S. fredii* strains were grown at 28°C on tryptone yeast (TY) medium (Beringer 1974) or yeast mannitol medium (Vincent 1970). *Escherichia coli* was cultured on Luria-Bertani medium (Sambrook et al. 1989). When required, the media were supplemented with antibiotics (concentrations in micrograms per milliliter): rifampicin, 25; streptomycin, 400; kamamycin, 50 (25 for *E. coli*); neomycin, 100; tetracycline, 4 (10 for *E. coli*); gentamycin, 5 (10 for *E. coli*); and spectinomycin, 100. Plasmids were transferred from *E. coli* to rhizobia by conjugation, as described by Simon (1984), with plasmid pRK2013 as helper. Recombinant DNA techniques were performed according to the general protocols of Sambrook and associates (1989). PCR amplifications were performed as previously described (Vinardell et al. 2004). The sets of DNA primers used in this work are listed in Supplementary Table S5. LPS extraction from bacterial cultures grown on solid TY medium, separation on sodium dodecyl sulfate PAGE gels, and silver staining were performed as described previously (Buendía-Clavería et al. 2003).

Cloning of the *M. loti* R7A *nolL* gene.

A 1.5-kb fragment containing the *nolL* gene was PCR-amplified using primers MlnolLEco-F and MlnolLBam-R and *M. loti* R7A genomic DNA as template. The 1.5-kb amplified fragment was digested with *Eco*RI and *Bam*HI and was subcloned into plasmid PBBR1MCS-5, generating plasmid pMUS1334. Plasmid pMUS1334 was transferred to HH103-Rif^r and SVQ503 by triparental conjugations, using pRK2013 as a helper.

Construction of a *S. fredii* HH103-Rif^r mutant carrying a deletion of the *flaC*, *flaB*, *flaA*, and *flaD* genes.

An in-frame *flaC*, *flaB*, *flaA*, and *flaD* gene deletion (4,438 bp) was constructed by the overlap extension PCR technique (Griffitts and Long 2008), using the pairs of primers flaA/flaB and flaC/flaD. The resulting fragment (1,401 bp) was digested with *Hin*dIII and *Bam*HI and was subcloned into pK18mobsac, generating plasmid pMUS1141. The homogenotization was carried out by triparental conjugation, using pRK2013 as a helper plasmid. A rifampicin- and sucrose-resistant (12.5% wt/vol) HH103-Rif^r transconjugant, called SVQ705, was investigated for the absence of the *flaC*, *flaB*, *flaA*, and *flaD* genes by PCR assays using the flaA/flaD primers. Swimming assays were carried out to investigate the flagella-dependent motility capacity of SVQ705. Petri plates

containing Bromfield's medium solidified with 0.24% (wt/vol) agar were inoculated with 3-µl aliquots of rhizobial cultures grown in TY until early stationary phase (optical density at 600 nm = 1.0). Bacterial mobility was scored 4 days after inoculation.

Identification of lipo-chitooligosaccharides (LCOs).

Purification and liquid chromatography-tandem mass spectrometry (MS/MS) analyses of LCOs produced by *Sinorhizobium fredii* HH103-Rif^r and its *noeI* (SVQ503) mutant carrying the *M. loti* R7A *nolL* gene grown in B minimal medium (van Brussel et al. 1977) supplemented with 3.6 µM genistein were performed as described previously (Crespo-Rivas et al. 2009). Briefly, the samples were redissolved in 1 ml of 50% acetonitrile/water (vol/vol) and 50-µl aliquots were injected onto the high-pressure liquid chromatography (HPLC)-electrospray ionization-MS/MS system. Chromatographic separation was performed using a PerkinElmer Series 200 HPLC system (Wellesley, MA, U.S.A.) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, CA, U.S.A.) consisting of a hybrid triple quadrupole linear ion trap (QqQlit) mass spectrometer equipped with an electrospray ion source. HPLC analyses were performed on a 200 × 2.1 mm Tracer Excel 120 ODSB C18 reversed-phase column with a particle size of 5 mm (Teknokroma, Barcelona, Spain). Chromatographic separation was performed using a binary gradient consisting of (A) water and (B) acetonitrile, both components containing 0.1% formic acid (vol/vol). The elution profile was 30% B (30 min), linear up to 30% B (15 min), 100% B (2 min), linear up to 30% B (3 min), and isocratic for 5 min (30% B). The flow rate was 0.3 ml min^{-1}. The mass spectrometer was set to the following optimized tune parameters: curtain gas, 35 psi; ion spray voltage, 5,500 V; source temperature, 300°C; source gas, 20 psi; declustering potential, 70 V; and entrance potential, 10 V; and, for each MS/MS spectrum, the collision energy setting was 35 V. In MS mode, ions over the m/z range of 700 to 1,600 were registered. For MS/MS acquisition, the information data-dependent function was used, where each MS scan was followed by product ion acquisition of the two most abundant ions in the mass spectrum. Product ions of m/z 150 to 1,600 were recorded in MS/MS mode.

Plant tests.

Nodulation tests were carried out on *Lotus arenarius* Brot, *L. corniculatus* L. cv. Leo, *L. creticus* L., *L. cytisoides* L., *L. filicaulis* Durieu, *L. glaber* Mill., *L. krylovii* Schischkin & Serg., *L. japonicus* (Regel) K. Larsen ecotypes Gifu and Miyakojima MG-20, *L. burttii* Borsos and *L. uliginosus* Schkuhr (Degtjareva et al. 2008; Handberg and Stougaard 1992; Kawaguchi et al. 2001, 2005). *Lotus* seeds were surface-sterilized as previously described (Heckmann et al. 2011). Four germinated seeds were transferred to each Leonard jar assembly. Leonard jars were composed of an upper vessel containing 220 ml of sterilized vermiculite supplemented with Rigaud and Puppo's nutrient solution (Rigaud and Puppo 1975) and a lower recipient filled with 180 ml (pH 7.0) of the plant nutrient solution. Each Leonard jar was inoculated with at least 10^8 bacteria. Inoculated *Lotus* plants were grown for 6 to 10 weeks in a greenhouse or in plant-growth chambers. The detection of nitrogenase activity in *Lotus* nodules was carried out by acetylene reduction assays as described previously for clovers (Buendía-Clavería et al. 1986). To identify bacteria occupying the nodules, *Lotus* nodules were surface-sterilized by immersing them in bleach (1/5 dilution of a stock solution containing 14% [wt/vol] sodium hypochlorite] for 2 min, followed by 5 washing steps in Vincent's mineral salt solution [VMS] [Vincent 1970]). The effectiveness of the surface-sterilizing treatment was checked by inoculating TY plates with 20-µl aliquots of the last washing step. Individual surface-sterilized nodules were crushed in 30 µl of VMS and 20-µl aliquots were used to inoculate TY plates. Bacterial isolates were tested on appropriately supplemented medium to determine whether they retained the antibiotic-resistance markers of the bacteria used to inoculate the plants. *L. burttii* nodulation tests were also carried out in square petri dishes containing the plant nutrient solution solidified with 10 g per liter of agar supplemented with adenine and thiamine at final concentrations of 12 and 0.8 µg/ml, respectively.

Kinetics of nodulation experiments were carried out as follows: Leonard jars containing four *L. burttii* seedlings were inoculated with 1 ml of midlog phase bacterial cultures. Inoculated *L. burttii* plants with *S. fredii* HH103-Rif^r (positive control) or with mutants affected in surface polysaccharides or Nod factors were grown in a plant-growth chamber with a 16 h photoperiod at 25°C (light) and 18°C (darkness). Nodulation of 8 to 16 plants was scored at 15, 20, 25, 29 to 30, and 35 to 36 days after inoculation.

Competition experiments between *S. fredii* HH103-Rif^r and *M. loti* NZP2235 strains on *Lotus burttii* and the determination of nodule occupancy were carried out as follows: bacteria were grown to midlog phase and five Leonard jar assemblies containing four *L. burttii* seedlings were inoculated with 1 ml of a mixture of bacterial competitors in a 1:1 ratio. Plants were grown for 6 weeks under greenhouse conditions. To determine nodule occupancy, *L. burttii* nodules were surface-sterilized and were crushed on yeast mannitol agar media. Because *S. fredii* HH103-Rif^r and *M. loti* NZP2235 are both resistant to rifampicin, melanin production capacity and the aspect (color and mucosity) of bacterial colonies were used to determine nodule occupancy in competition experiments between these two strains. *S. fredii* HH103-Rif^r produces melanin while *M. loti* NZP2235 fails to produce the pigment, following the protocol for melanin detection previously described (Cubo et al. 1988).

Nodule sectioning.

Two- to six-week-old *L. burttii* nodules induced by *S. fredii* HH103-Rif^r DsRed strain were embedded in 3% agarose in water and were sliced in thick layer sections (50 to 100 µm), using a Leica VT 1000S vibratome. Sections of nodule were observed using a Zeiss LSM510 META confocal microscope.

For semithin layer sections, nodules were fixed in a mixture of 4% (wt/vol) paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Samples were then embedded using a Technovit 7100 (Heraeus Kulzer), were sliced in semithin layer sections (7 µm) using a Leica RM2045 microtome, and were then mounted onto microscope slides. Sections were stained with 0.1% toluidine blue in water and were observed using a Zeiss Axioplan 2 image microscope. For counting ITs, *L. burtii* and *L. japonicus* Gifu seedlings were inoculated with *S. fredii* HH103-Rif^r DsRed or *M. loti* MAFF303099 DsRed, 6 days after germination. Nine days after inoculation, the ITs were counted by Zeiss Axioplan 2 imaging and were observed using a Leica M165FC microscope.

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