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e-Xtra*

Sinorhizobium fredii HH103 *cgs* Mutants Are Unable to Nodulate Determinate- and Indeterminate Nodule–Forming Legumes and Overproduce an Altered EPS

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***Sinorhizobium fredii* HH103 produces cyclic β glucans (CG) composed of 18 to 24 glucose residues without or with 1-phosphoglycerol as the only substituent. The *S. fredii* HH103-Rif^r *cgs* gene (formerly known as *ndvB*) was sequenced and mutated with the *lacZ*-gentamicin resistance cassette. Mutant SVQ562 did not produce CG, was immobile, and grew more slowly in the hypoosmotic GYM medium, but its survival in distilled water was equal to that of HH103-Rif^r. Lipopolysaccharides and K-antigen polysaccharides produced by SVQ562 were not apparently altered. SVQ562 overproduced exopolysaccharides (EPS) and its *exoA* gene was transcribed at higher levels than in HH103-Rif^r. In GYM medium, the EPS produced by SVQ562 was of higher molecular weight and carried higher levels of substituents than that produced by HH103-Rif^r. The expression of the SVQ562 *cgs::lacZ* fusion was influenced by the pH and the osmolarity of the growth medium. The *S. fredii cgs* mutants SVQ561 (carrying *cgs*::Ω) and SVQ562 only formed pseudonodules on *Glycine max* (determinate nodules) and on *Glycyrrhiza uralensis* (indeterminate nodules). Although nodulation factors were detected in SVQ561 cultures, none of the *cgs* mutants induced any macroscopic response in *Vigna unguiculata* roots. Thus, the nodulation process induced by *S. fredii cgs* mutants is aborted at earlier stages in *V. unguiculata* than in *Glycine max*.**

Nucleotide and amino acid sequence data is available in the EMBL database under accession number EU622805.

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Rhizobia are soil α-proteobacteria able to establish symbiotic associations with many leguminous plants. This symbiosis leads to the formation of nodules on roots of leguminous plants where the bacteria fix nitrogen. Nodule development requires the exchange of symbiotic signals between the two partners (Gage 2004, Jones et al. 2007). Plant flavonoids and bacterial lipochitooligosaccharides (also called Nod factors or LCO) are two well-known signals acting at the very early stages of nodule formation (Gage 2004).

In addition to Nod factors, different rhizobial surface polysaccharides are required for successful nodulation, during which they might act as signal molecules or could prevent plant defense reactions (Becker and Pühler 1998; Breedveld and Miller 1998; Fraysse et al. 2003; Mathis et al. 2005). Exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS or K-antigens), and cyclic β-glucans (CG) are the main rhizobial polysaccharides investigated for their roles in nodulation (Fraysse et al. 2003; Parada et al. 2006). The importance of each particular polysaccharide in the nodulation process varies according to the type of nodule (determinate or indeterminate) that each particular legume is able to develop. For instance, EPS is required for the formation of nitrogen-fixing nodules on plants forming indeterminate nodules, such as *Medicago sativa* (alfalfa), but apparently it is dispensable in plants that form determinate nodules, such as *Glycine max* (soybean). In contrast, the symbiotic impairment of rhizobial mutants affected in LPS production appears to be more severe in determinate-nodule forming legumes than in those forming indeterminate nodules (Fraysse et al. 2003; Gage 2004).

Rhizobial mutants unable to form CG are severely impaired in their ability to nodulate determinate and indeterminate nodule-forming legumes (Bhagwat et al. 1992; Breedveld and Miller 1998; D'Antuono et al. 2005; Fraysse et al. 2003; Geremia et al. 1987). In *Sinohizobium meliloti*, two genes, *ndvA* and *ndvB*,

are necessary for CG production (Breedveld and Miller 1998). The *ndvA* gene is involved in CG transport while *ndvB* (renamed *cgs* [cyclic glucan synthase]) is responsible for the biosynthesis of CG. *M. sativa* and *G. max* plants inoculated with rhizobial mutants unable to produce CG only form ineffective pseudonodules. Bacteroids cannot be isolated from alfalfa pseudonodules, which contain a small number of infection threads that abort at an early stage (Breedveld and Miller 1998; Dickstein et al. 1988). In soybean, however, *cgs*-like mutants of *Bradyrhizobium japonicum* form ineffective but bacteroid-containing nodules (Bhagwat and Keister 1995). cDNA array technology has been used to compare the transcriptome profiles of *Lotus japonicus* roots inoculated with a *Mesorhizobium loti cgs* mutant. The expression of *L. japonicus* genes associated with the development of a fully functional nodule was significantly affected in plants inoculated with the *cgs* mutant (D'Antuono et al. 2008).

CG are important not only for the symbiotic relationship between rhizobia and legumes but also for the microbial capacity to develop phytopathogenic interactions (Jones et al. 2007). Frequently, mutants of phytopathogenic bacterial strains (such as *Agrobacterium tumefaciens* and *Xanthomonas campestris*) unable to produce CG are also impaired in their capacity to develop plant diseases (Breedveld and Miller 1994, 1998; Rigano et al. 2007). The requirement for CG for bacterial pathogenic capacity has also been demonstrated in *Brucella abortus*, a cattle pathogen that can also infect humans (Arellano-Reynoso et al. 2005; Ugalde 1999). *S. meliloti cgs* mutants are severely impaired for motility and attachment and invasion of alfalfa roots, so that nitrogen-fixing nodules are not formed (Dylan et al. 1990b). In spite of the dramatic effects observed in bacterial mutants unable to form CG, studies using *S. meliloti* pseudorevertants indicated that these polymers, by themselves, might not be strictly required for the successful interaction of symbiotic bacteria with their eukaryotic hosts. Pseudorevertants selected for restoration of motility showed enhanced attachment capacity to alfalfa roots but were only slightly restored symbiotically. On the other hand, pseudorevertants that have regained nodulation capacity showed little or no attachment capability, indicating that the level of attachment capacity exhibited by the wild-type strain is not strictly required for the invasion of alfalfa roots. Neither motile nor symbiotic revertants regained the capacity to produce CG, which indicates that this polymer is not strictly required for nodule development, even though *S. meliloti cgs* mutants only form pseudonodules that do not fix nitrogen (Dylan et al. 1990b). Mutants unable to produce CG also show alterations in a variety of cell-surface properties when grown in media of low osmolarity. These properties include i) a loss of motility with reduced numbers of flagella, ii) increased resistance to particular bacteriophages, iii) increased sensitivity to some antibiotics, iv) increased production of EPS, and v) modified cell-surface protein composition (Breedveld and Miller 1998). Thus, the symbiotic impairment observed in *cgs* mutants might be due to a dramatic alteration in the bacterial surface properties.

CG produced by *A. tumefaciens*, all biovars of *Rhizobium leguminosarum*, *S. meliloti*, and *S. fredii* HH303 only contain glucosyl residues that are solely linked by β-(1,2) glycosidic bonds. The *Xanthomonas* CG has 16 glucosyl residues, and contains one (1,6) glycosidic bond and one α linkage (Amemura and Cabrera-Crespo 1986). *R. leguminosarum* CG are not substituted while those produced by *A. tumefaciens*, *S. meliloti*, and *S. fredii* HH303 carry *sn*-1-phosphoglycerol substituents (Breedveld and Miller 1994). *Bradyrhizobium japonicum*, another soybean symbiont, also requires CG for effective nodulation of soybean roots (Bhagwat and Keister 1995). This bacterium, however, forms a different CG, in which glucosyl residues are linked by β-(1,3)- and β-(1,6)-linkages (Inon de Iannino and Ugalde 1993).

The nucleotide sequence of the *S. fredii cgs* (*ndvB*) gene is not available in databases and it is not clear whether, in all cases, *S. fredii cgs* mutants are unable to form nitrogen-fixing nodules with soybean plants. *S. fredii* Rf19, an *ndvB::*Tn*5* mutant derivative of *S. fredii* HH303, induced ineffective pseudonodules on soybean (*G. max* cv. Williams) and *Vigna unguiculata* cv. California Black-eye (Bhagwat et al. 1992). Another report showed, however, that this mutant was able to form nitrogen-fixing nodules with the American soybean cv. McCall and the Asiatic cv. Peking (Inon de Iannino et al. 1996), and an earlier report described that *S. fredii* USDA191 mutants unable to produce EPS and CG induced some functional nodules on *G. max* cv. Peking (Ko and Gayda 1990). These discrepancies, and the fact that *S. fredii* strains show marked cultivar-strain specificity, prompted us to investigate the symbiotic capacity of *cgs* mutants of *S. fredii* HH103 with different soybean cultivars, including those used in the above-mentioned reports. The structure of the CG produced by *S. fredii* HH103 was also determined. *S. fredii* HH103 was isolated from a Chinese soil sample (Dowdle and Bohlool 1985) and was able to form nitrogen-fixing nodules with Asiatic and American soybean cultivars (Buendía-Clavería et al. 1989; Dowdle and Bohlool 1985).

RESULTS

Structural characterization of the CG of *S. fredii* HH103.

S. fredii HH103 cells were treated with hot water-phenol (a treatment that allows the isolation of rhizobial LPS and periplasmic CG) and the aqueous phase was submitted to dialysis, enzyme treatments, and size-exclusion chromatography (SEC). CG were located in a low molecular-weight fraction mixed with the KPS. Subsequent SEC on Biogel P6 allowed the isolation of periplasmic CG, which were submitted to structural analysis. Monosaccharide composition analysis by gas-liquid chromatography (GLC) of trimethylsilyl methyl glycosides showed that only glucose and its absolute configuration was determined as D. Methylation analysis identified only 1,2,5-tri-*O*-acetyl-1-deutero-3,4,6-tri-*O*-methylglucitol, derived from units of →2)-D-Glc*p*. The ^{1}H-nuclear magnetic resonance (NMR) spectrum (Supplementary Fig. 1A) is very similar to that reported for anionic cyclic β-glucans from *S. meliloti* 1021 (Breedveld et al. 1995). Correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) (Supplementary Fig. 2), and ^{1}H-^{31}P heteronuclear multiple-bond correlation (HMBC) NMR experiments allowed the assignment of NMR signals (Supplementary Table 1) and the determination of the structure of this polysaccharide. Thus, the COSY spectrum allows the assignment of monosaccharide protons H-2 to H-6a,b starting from the broad signal of H-1 at δ_H 4.9 ppm; consequently, the HSQC spectrum allows the assignment of the corresponding ^{13}C signals. The chemical shifts of the anomeric signal (δ_H = 4.9 ppm, δ_C = 102.2 ppm) correspond to β-Glc*p* units, and the chemical shift of C-2 (approximately 83 ppm) confirms the linkage position. The HSQC spectrum also shows minor signals for methylene groups at δ_C 66.4, 63.9, and 62.2 ppm, together with an additional crosspeak at δ_H = 3.87 ppm and δ_C = 70.7 ppm. These sets of signals correspond to a phosphoglycerol residue linked to some of the glucose units via *O*-6 and the corresponding *C*-6 methylenic protons of the substituted monosaccharide residue. The presence of a phosphate group was confirmed from a ^{31}P NMR spectrum, which shows a signal at δ_P 0.9 ppm. In addition, the ^{1}H-^{31}P HMBC experiment correlates the phosphorus signal with the methylene signals at δ_H = 4.15 and 4.05 ppm (H-6a and H-6b

of a 6-*O*-substituted glucose) and 3.91 and 3.84 ppm (H-1a and H-1b of a glycerol unit), which agrees with a glucose unit bearing a phosphoglycerol residue at *O*-6. We can conclude that the structure of this polysaccharide consists of units of →2)-β-D-Glc*p*-(1→, partially substituted with glycerol-1-phosphate at the C-6 position of some of the glucose units, and it is similar to that found in other *Rhizobium* spp. (Batley et al. 1987; Breedveld et al. 1995). The ratio of substituted to unsubstituted units (1:7) was estimated from the relative areas of H-6 crosspeaks in HSQC spectrum. Other possible substituents, such as succinyl groups, were not found.

Negative-ion mode matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry (using the linear ToF mode) was used to determine the degree of polymerization (DP) of the cyclic β-glucans (Fig. 1A). The peaks can be assigned as deprotonated molecules having DP values between 20 and 23 glucose units, with the major components bearing three phosphoglycerol substituents, although there is also evidence for the presence of molecular species carrying four substituents (Fig. 1A, *m/z* 4383 and 4407). Dephosphorylation of CG isolated from HH103-Rif^r (rifampicin-resistant) cells with 1 M NaOH (100°C, 4 h) yielded a fraction com-

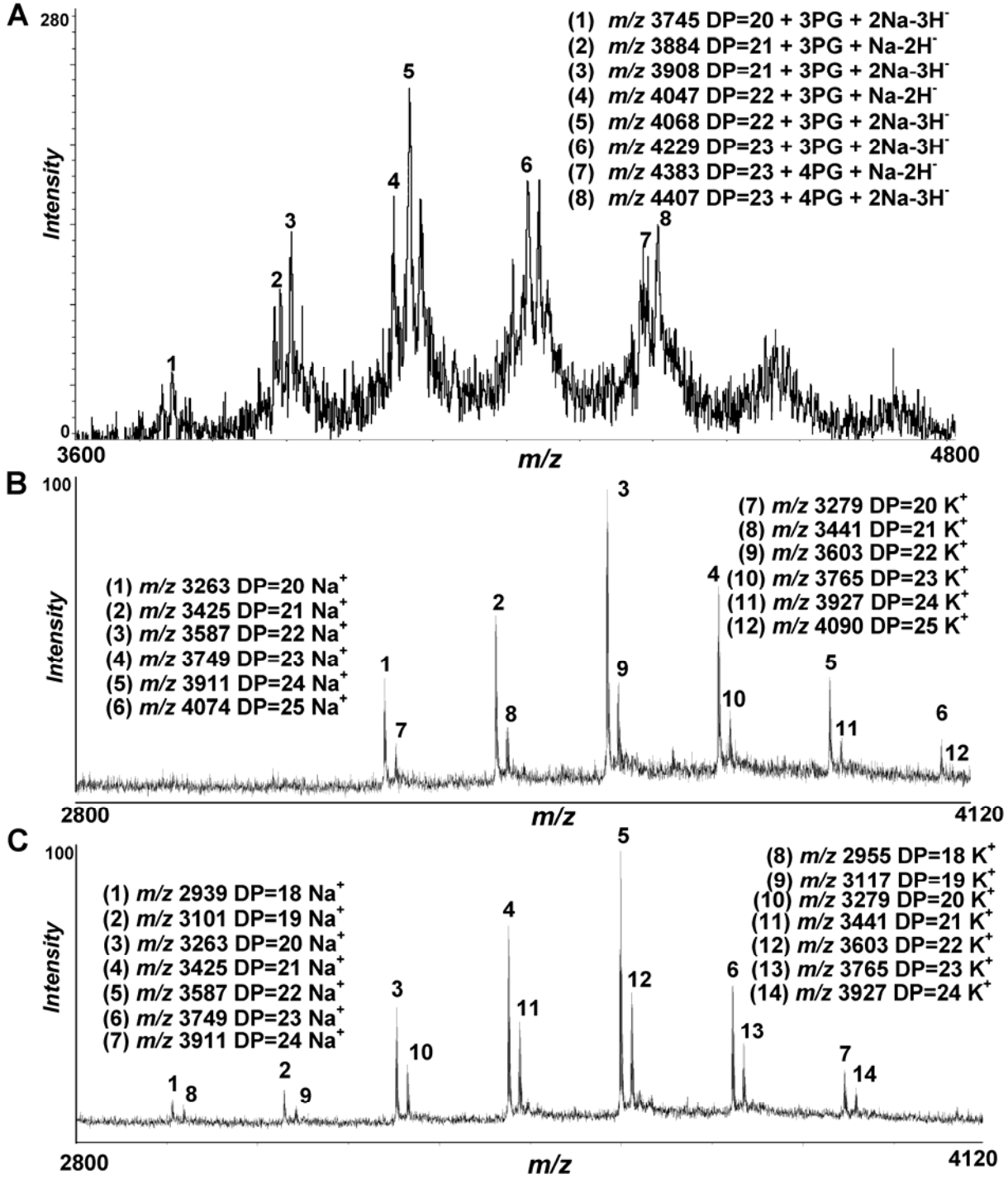

Fig. 1. Matrix-assisted laser desorption ionization time-of-flight analysis of fractions containing the cyclic glucans isolated from **A,** cells, negative-mode spectrum; **B,** chemically dephosphorylated periplasmic cyclic glucan, positive-mode spectrum; and **C,** culture medium of *S. fredii* HH103, positive-mode spectrum. DP = degree of polymerization; PG = phosphoglycerol.

posed of neutral cyclic β-glucans. The positive-ion MALDI reflectron mode ToF mass spectrum of dephosphorylated CG is shown in Figure 1B. It allows the identification of species with DP ranging from 20 to 25 glucose units.

Extracellular CG were isolated from culture media by precipitation with ethanol and further purification by SEC (Gil Serrano et al. 1993). The positive-ion mode MALDI reflectron mode ToF mass spectrum (Fig. 1C) of the glucans obtained is very similar to that of the dephosphorylated periplasmic CG, and indicates that the secreted CG isolated do not bear phosphorylated substituents. The set of peaks separated by 162 *m/z* units indicates species with DP between 18 and 24 glucose units.

Isolation of the *cgs* gene of *S. fredii* HH103.

Although *cgs* mutants of *S. fredii* HH303 have been isolated and studied (Bhagwat et al. 1992; Inon de Iannino et al. 1996), the nucleotide sequence of the *S. fredii cgs* (*ndvB*) gene is not available. Thus, to isolate the *cgs* gene of *S. fredii* HH103, primers *cgs*-F and *cgs*-R were designed from the coding sequence of the *ndvB* gene of *S. meliloti* 1021 (accession number AL591793). Using *S. fredii* HH103 genomic DNA as a template, primers *cgs*-F and *cgs*-R led to the polymerase chain reaction (PCR) amplification of a 2.3-kb fragment whose partial sequencing revealed that it had a high level of identity (87%) with the *ndvB* gene from *S. meliloti* 1021. From this HH103 sequence, a new pair of primers, called *cgs*HH-F and *cgs*HH-R, was designed and used to amplify a 1,230-bp internal fragment of the *S. fredii* HH103 *cgs* gene. Then, 1,200 clones of a genomic library of *S. fredii* HH103 were screened for positive PCR amplification with primers *cgs*HH-F and *cgs*HH-R, resulting in the identification of cosmid pMUS909.

Hybridization analysis of cosmid pMUS909, using the 1,230-bp HH103 *cgs* internal fragment as a probe, led us to sequence a 13,293-bp segment (accession number EU622805) which covered the complete *S. fredii* HH103 *cgs* gene (between positions 2,610 and 11,237). This gene encodes a predicted protein of 2,875 residues, with a molecular weight of 320.6 kDa, that is 90 and 89% identical to the *S. meliloti* 1021 NdvB protein and the *S. medicae* WSM419 glycosyl transferase 36, respectively (accession nos. CAC47865 and ABR61979). High levels of identity were also found with the Cgs proteins of other rhizobia such as *R. etli* CFN42 (69%, ABC92741), *R. leguminosarum* bv. *viciae* 3841 (68%, CAK10127), *M. loti* MAFF303099 (57%, BAB53905), and the ChvB protein of *A. tumefaciens* C58 (67%, AAL43711). No significant homologies were found between the *S. fredii* HH103 Cgs protein and those involved in *B. japonicum* CG production.

In addition to *cgs*, the *S. fredii* HH103 segment sequenced contains three other complete and one partial open reading frame (ORF) (Fig. 2). The homologies of the predicted encoded products of the different ORF found in the sequenced fragment are summarized in Table 1. The *cgs* gene is flanked by two ORF showing the same polarity. An ORF (positions 1,374 to 2,267) homologous to *SMc04882*, a putative transcriptional regulator of the LysR family, is located upstream of *cgs* (and in the same frame as this gene) and its deduced encoded product is 91 and 90% identical to the corresponding proteins from *S. meliloti* and *S. medicae*, respectively. An ORF (positions 11,429 to 12,592) situated downstream of *cgs* is homologous to *S. meliloti SMc04381* (80% identity) and *S. medicae* (79% identity), whose deduced encoded proteins are putative glucan succinyl transferases.

At the 5′ terminus of the sequenced fragment (1,299 to 1), there is an incomplete ORF homologous to *S. meliloti* 1021 *SMc04383* which encodes a hypothetical protein of unknown function. The nucleotide homology of the whole *S. fredii*-sequenced fragment with that of *S. meliloti* 1021 (85 to 86% identity) extends between positions 1 and 2,262 and 2,441 and 12,582 in the former, and 204,156 and 201,895 and 201,722 and 191,630 in the latter (AL591793). Thus, the homologous zones between these two rhizobia covered four of the five ORF located in the *S. fredii* HH103-sequenced fragment, from *SMc04383* to *SMc04381*. Downstream of *SMc04381*, the *S. fredii*-sequenced fragment did not show significant homology to *S. meliloti* sequences. Instead, the sequenced fragment from position 12,649 to the end was 75% identical to a segment of the *R. leguminosarum* bv. *viciae* 3841 chromosome fragment (accession number AM236080) that contains a putative ORF (*RL2635*) encoding a lysine exporter family protein (RhtB/LysE). The corresponding *S. fredii* HH103 ORF, here called *lysE*, extends between positions 13,262 and 12,630 and it codes for a predicted protein that is 75% identical to RL2365 and 38% identical to putative *S. meliloti* and *S. medicae* RhtB proteins, whose chromosomal encoding ORF are located approximately 440 kb away from the *cgs* gene. The transcriptional direction of *lysE* and *SMc04383* is opposite to that of *cgs*.

The *S. meliloti* 1021 genetic organization of the *ndv* genes (*ndvB-Smc043381-Smc03901-ndvA*) is not conserved in *S. fredii* HH103 because of the presence of *lysE* just after

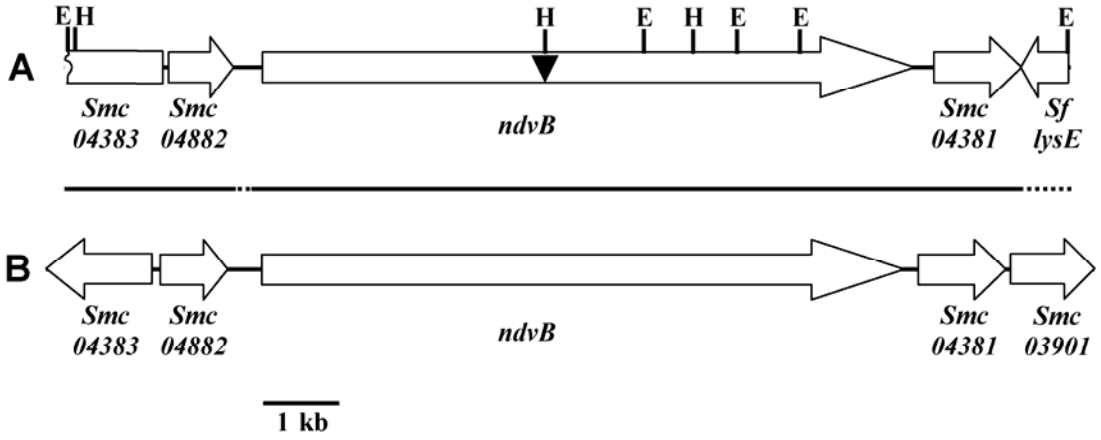

Fig. 2. Genetic organization of the *cgs* region of **A,** *Sinorhizobium fredii* HH103 (accession number EU622805) and **B,** *S. meliloti* 1021 (a segment of 13,850 bp between positions 204,297 and 190,447 of the sequence available at the accession number AL591791). The line drawn between the two nucleotide segments represents the presence (continuous) or absence (dotted) of homology between them. The black inverted triangle indicates the position (3,736 of the *cgs* coding sequence) at which either the Ω interposon or the *lacZ*-Gm^r cassette have been inserted to generate mutants SVQ561 and SVQ562 respectively. E = *Eco*RI and H = *Hin*dIII.

Smc04381. Oligonucleotide primers homologous to the 3′ ends of the *cgs* (*ndvB*) and *lysE* genes amplified a band showing the expected size (approximately 2.1 kb), regardless of whether plasmid pMUS909 or total genomic DNA from HH103 was used as the template (data not shown). Thus, the differences observed between *S. meliloti* 1021 and *S. fredii* HH103 are not due to DNA reorganizations in cosmid pMUS909. The same pair of primers also amplified the 2.1-kb DNA band when genomic DNA from *S. fredii* strains USDA192 and USDA205 were used as DNA templates. Thus, this *S. fredii* HH103 genetic organization (*cgs* and *lysE* are closely located) also appears in other *S. fredii* strains.

Isolation and characterization of *S. fredii* HH103 *cgs* mutants.

A *cgs* mutant (named SVQ562) of *S. fredii* HH103 was generated by inserting the *lacZ*-Gm^r (gentamycin-resistant) cassette into the *cgs* coding sequence. SVQ562 was analyzed for the production of different surface polysaccharides. As expected, CG were not detected in the cellular fraction of mutant SVQ562 using ^{1}H-NMR analyses (Fig. 3) or in supernatants of SVQ562 cultures (Fig. 4). The K-antigen KPS produced by SVQ562 appears to be identical to that produced by the wild-type strain (Fig. 3), because the ^{1}H-NMR spectrum corresponding to the KPS produced by the mutant was identical to that previously described for the KPS of *S. fredii* HH103 (Gil-Serrano et al. 1999). The *cgs* mutation did not apparently affect bacterial LPS because *S. fredii* HH103-Rif^r and SVQ562 showed the same LPS electrophoretic profile and the same pattern of LPS bands recognized by the monoclonal antibody NB6-228.22 (data not shown). This monoclonal antibody specifically recognizes the LPS of *S. fredii* HH103 (Buendía-Clavería et al. 2003).

Mobility in hypoosmotic GYM medium (Dylan et al. 1990a) was examined as described below. The SVQ562 mutant did not show mobility in comparison with the parental strain HH103 and the presence of 100 mM NaCl in GYM media did not restore the mobility of the *cgs* mutant (Fig. 5B and C). Moreover, the parental strain showed lower mobility in GYM supplemented with NaCl than in GYM medium.

Mutant SVQ562 was also examined for the production of EPS. On solid yeast extract–mannitol agar (YMA), SVQ562 was more mucous than HH103-Rif^r (Fig. 5A). In liquid YM broth (YMB) medium, the amount of EPS recovered with a glass rod from SVQ562 culture supernatants treated with two volumes of acetone was much higher than that collected from HH103-Rif^r (Table 2). In the hypoosmotic GYM medium, EPS was not recoverable with a glass rod either from HH103-Rif^r cultures or from SVQ562 although, in both cases, the addition of acetone provoked the formation of a fibroid material. The addition of 100 mM NaCl to GYM medium allowed the production of large (rod-recoverable) quantities of EPS by SVQ562 cultures and a slight increase in EPS production by HH103-Rif^r cultures.

However, EPS was recovered from the extracellular medium of all HH103-Rif^r and SVQ562 cultures when culture supernatants were subjected to a fivefold concentration process, followed by the addition of three volumes of ethanol (discussed below). Under these conditions, ^{1}H-NMR spectra corresponding to the EPS present in the supernatants of *S. fredii* HH103-Rif^r cultures were quite similar regardless of whether the bacteria were grown in liquid YM, GYM, or GYM supplemented with 100 mM NaCl (Fig. 6). ^{1}H-NMR spectra of EPS produced by HH103-Rif^r and SVQ562 in YMB cultures were almost identical (Fig. 6C). However, the ^{1}H-NMR spectra of EPS produced by SVQ562 in GYM and GYM + 100 mM NaCl indicated that the EPS produced by the mutant bears a higher level of substitution (assigned as acetate and pyruvate substituents) than that produced by the parental strain HH103-Rif^r, based on the increase in relative areas of signals at approxi-

Table 1. Proteins encoded by the *Sinorhizobium fredii* HH103-sequenced fragment, (accession number EU622805)

Gene position	Protein[a]	Putative function	Identity[b]
1,299–1[c]	SMc04383 (432)[d]	Conserved hypothetical protein, unknown function	90% SMc04383 *S. meliloti* 1021 (466; CAC47867) 86% DUF1338 *S. medicae* WSM419 (472; ABR61981) 74% DUF1338 *Ochrobactrum anthropi* ATCC 49188 (488; ABS14177)
1,374–2,267	SMc04882 (297)[d]	Probable transcription regulator protein, LysR family	91% SMc04882 *S. meliloti* 1021 (297; CAC47866) 90% Smed_3156 *S. medicae* WSM419 (297; ABR61980) 72% BRA0906 *Brucella suis* 1330 (310; AAN34078)
2,610–11,237	NdvB (2875)	β-(1,2)-Glucan production-associated transmembrane protein	90% NdvB *S. meliloti* 1021 (2,832; CAC47865) 89% Glycosyltransferase 36 *S. medicae* WSM419 (2870; ABR61979) 69% NdvB *R. etli* CFN42 (2,825; ABC92741)
11,429–12,592	SMc04381 (387)[d]	Hypothetical transmembrane protein, glucan succinyl transferase	80% SMc04381 *S. meliloti* 1021 (385; CAC47864) 79% Smed_3156 *S. medicae* WSM419 (385; ABR61978) 54%RHE_CH03998 *R. etli* CFN42 (412; ABC92742)
13,262–12,630	LysE (210)	Lysine exporter family protein (RhtB/LysE)	75% RL2635 *R. leguminosarum* bv. *viciae* 3841 (203; CAK08123) 75% SIAM614_25766 *Stappia aggregata* IAM 12614 (222; EAV41606) 67% BamMC406DRAFT_5884 *Burkholderia ambifaria* MC40-6 (290; EAV51594)

[a] Number of residues of each protein is shown in parentheses.
[b] Number of residues and the accession number are shown in parentheses.
[c] Stop codon of this open reading frame is not found in the sequenced fragment.
[d] As annotated for the highest identical protein found in the databases.

mately 2 and 1.5 ppm (Fig. 6A and B). The molecular weight of the EPS produced by HH103-Rif^r and SVQ562 was estimated by SEC high-pressure liquid chromatography (HPLC) (discussed below). Most of the EPS produced by HH103-Rif^r appeared in a region that corresponds to a polysaccharide with a molecular weight of approximately 50 to 60 kDa (Fig. 7, region B). The size exclusion chromatogram of the EPS produced by SVQ562 showed two peaks, one in region B, the

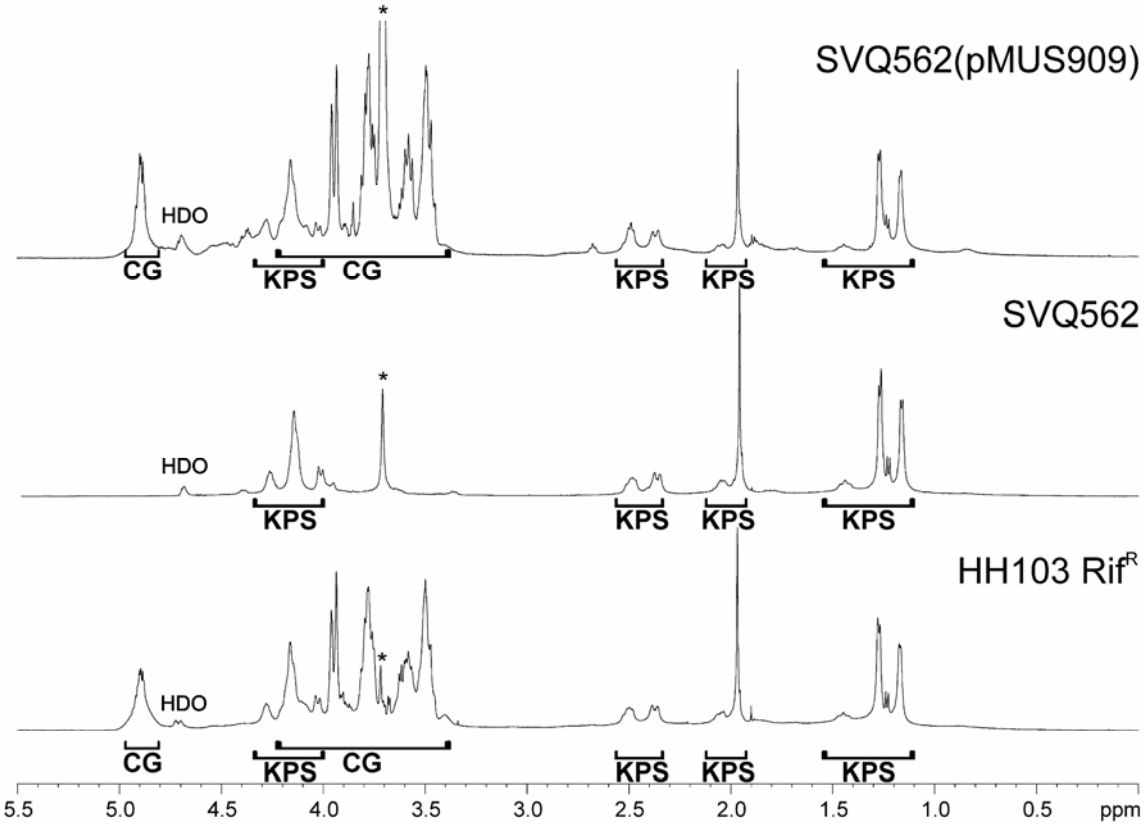

Fig. 3. [1]H nuclear magnetic resonance spectra of phenol-water extracts isolated from *Sinorhizobium fredii* HH103-Rif^r, SVQ562, and SVQ562 (pMUS909). Signals corresponding to capsular polysaccharides (KPS) and cyclic glucans (CG) are indicated. Asterisks show signals from Tris buffer.

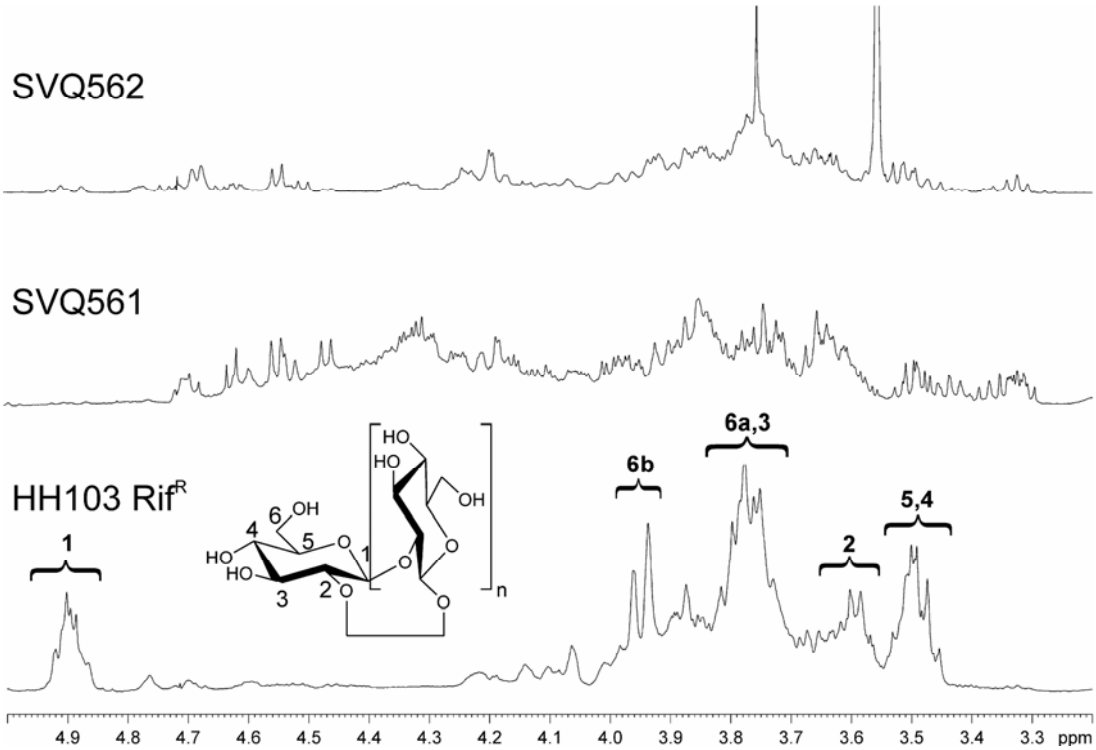

Fig. 4. [1]H nuclear magnetic resonance (500 MHz) of fractions isolated from culture supernatants of *Sinorhizobium fredii* HH103-Rif^r , SVQ561, and SVQ562. Signals corresponding to cyclic glucans were found only in the wild-type strain HH103-Rif^r.

other in the region corresponding to at least 2,000 kDa (Fig. 7, region A).

CG have been described as playing a defensive role against hypoosmotic conditions (Breedveld and Miller 1998). We have examined the growth rates of strains HH103-Rif^r and SVQ562 in the three different media mentioned above. HH103-Rif^r and SVQ562 showed similar generation times in YMB (204 ± 8 and 228 ± 12 min, respectively) and in GYM media supplemented with NaCl (216 ± 8 and 222 ± 9 min, respectively). In the hypoosmotic GYM medium, however, the generation time exhibited by the *cgs* mutant (approximately 324 ± 19 min) was clearly higher than that of *S. fredii* HH103 (240 ± 10 min). This difference in the speed with which bacteria grew in the different media assayed did not result in significant differences in the total bacterial population reached by the cultures at stationary phase, which was approximately 10^8 cells ml^{-1}. We have also investigated whether the absence of CG affects the bacterial capacity to survive under severe hypoosmotic conditions. For this purpose, HH103-Rif^r and SVQ562 were resuspended in distilled water (at a cell density of 3 to 8 × 10^5 cells ml^{-1}) and incubated at 28°C. After incubation for 72 h, the number of viable cells of SVQ562 (10^5 cells ml^{-1}) was similar to that of strain HH103-Rif^r (0.8 × 10^5 cells ml^{-1}). HH103-Rif^r

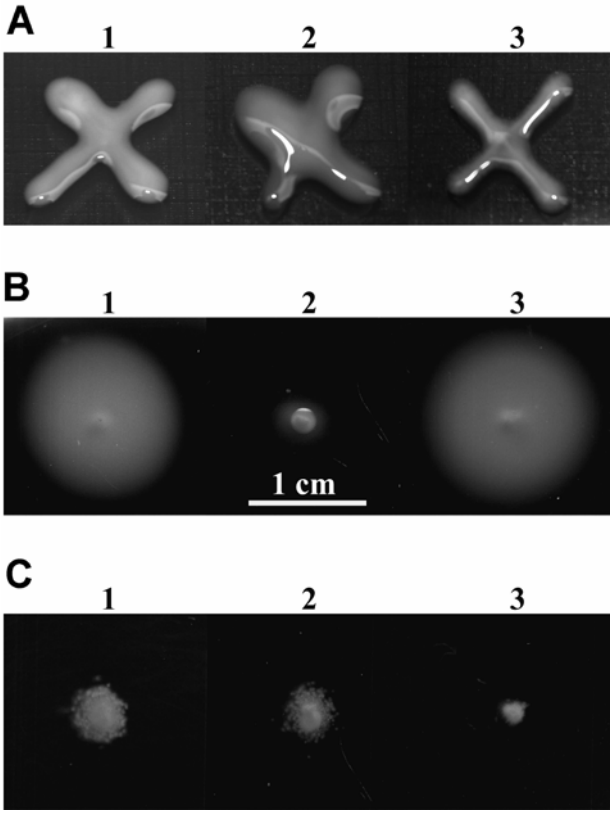

Fig. 5. Appearance of *Sinorhizobium fredii* HH103-Rif^r and its *cgs* derivative SVQ562 growing on **A,** yeast extract–mannitol agar medium and **B,** their mobility on GYM (Dylan et al. 1990a) and **C,** GYM supplemented with 100 mM NaCl. Column 1, HH103-Rif^r ; column 2, SVQ562; column 3, SVQ562 (pMUS909).

Table 2. Exopolysaccharides produced by *Sinorhizobium fredii* HH103-Rif^r and SVQ562[a]

Media	Bacterial strain	Cells/ml (×10^8)	Exopolysaccharide (mg) released into 100 ml of medium
YMB	HH103-Rif^r	2.7	6.3 ± 0.8
	SVQ562	7.0	17.7 ± 1.2
GYM	HH103-Rif^r	1.5	0.0
	SVQ562	1.3	0.0
GYM + NaCl	HH103-Rif^r	2.1	0.6 ± 0.1
	SVQ562	2.2	19.8 ± 1.0

[a] In yeast extract–mannitol broth (YMB), GYM (Dylan et al. 1990a), and GYM supplemented with 100 mM NaCl. Numbers are mean values of three different experiments. Bacterial cultures were gown for 7 days before exopolysaccharides released into the cells milieu were precipitated by adding two volumes of acetone.

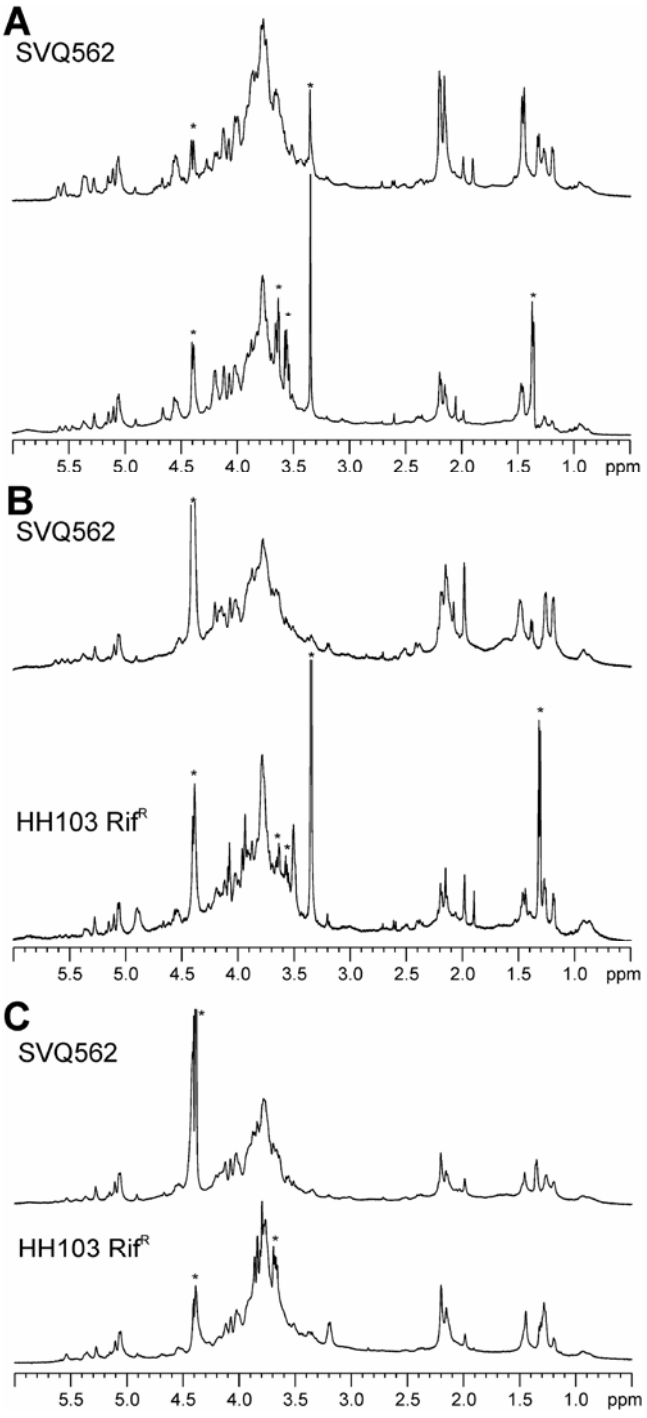

Fig. 6. ^{1}H nuclear magnetic resonance (500 MHz; 333 K) of exopolysaccharide produced by *Sinorhizobium fredii* HH103-Rif^r and SVQ562 cultures grown in **A,** the hypoosmotic GYM (Dylan et al. 1990a) medium; **B,** GYM supplemented with 100 mM NaCl; and **C,** yeast extract–mannitol broth medium. Asterisks indicate signals of HDO (4.40 ppm) and glycerol.

and SVQ562 were able to grow in YMB media containing 0.07% (wt/vol) of the detergent sodium dodecyl sulfate (SDS). However, the *cgs* mutant was delayed in reaching the stationary growth phase: approximately 72 and 96 h (absence and presence of SDS, respectively) for HH103-Rif^r and 96 and 168 h (absence and presence of SDS, respectively) for mutant SVQ562.

S. fredii HH103 *cgs* expression is affected by the presence of flavonoids, pH, and osmolarity.

S. fredii SVQ562 (it carries a *cgs::lacZ*-Gm^r fusion) cultures grown in the presence and absence of the flavonoids genistein and coumestrol were assayed for β-galactosidase activity. Three independent experiments, each composed of two replicates, showed that LacZ activity of SVQ562 cultures in the presence of genistein (202 ± 35 Miller units) or coumestrol (185 ± 39) was significantly lower (α= 5%) than that in the absence of flavonoids (252 ± 23).

The effect of the pH at which bacteria were grown on *cgs* expression was investigated by culturing mutant SVQ562 in acidic- (pH 6.0), neutral- (pH 7.0), and alkaline-buffered (pH 8.0) YMB media. Nonbuffered YMB media, with a pH value of 7.0 at the time of inoculation, was also included. Three independent experiments, each composed of two replicates, showed that the β-galactosidase activity of SVQ562 cultures grown at pH 8 (146 ± 25 Miller units) was significantly lower (α= 5%) than that of cultures at pH 6 (315 ± 73) and pH 7 (237 ± 28). The pH of cultures in nonbuffered media shifted from an initial pH value of 7.0 to 6.75 at the time β-galactosidase assays were carried out, and their β-galactosidase activity was 257 ± 24. Three independent experiments, each composed of at least three replicates, showed that β-galactosidase activity of SVQ562 in YMB and GYM media (258 ± 77 and 291 ± 62, respectively) were similar but significantly higher than in GYM media supplemented with 100 mM NaCl (185 ± 43).

Effect of the *cgs* mutation on the *S. fredii* HH103 *exoA* gene expression.

Mutant SVQ562 overproduces EPS, thus forming colonies that are more mucous than those of the parental strain HH103-Rif^r. Four independent real-time reverse-transcription polymerase chain reaction (*rt*-RT-PCR) experiments were carried out to investigate whether the *exoA* gene, encoding a glucosyltransferase that adds the first glucosyl residue to the nascent EPS repeating unit, showed higher expression in the *cgs* mutant (SVQ562) than in the wild-type strain HH103-Rif^r. Expression of the *exoA* genes was clearly higher (7.4- ± 1.3-fold) in mutant SVQ562 than in HH103-Rif^r.

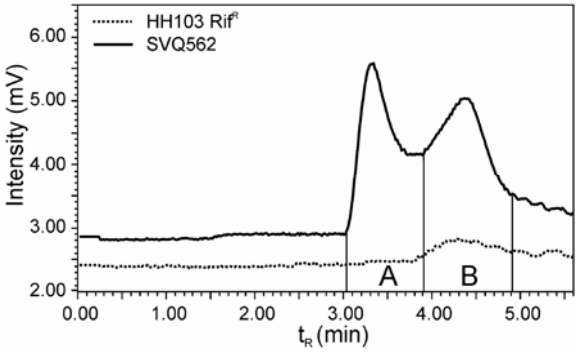

Fig. 7. Size-exclusion chromatogram of exopolysaccharide produced by HH103-Rif^r (dotted line) and SVQ562 (solid line). Region A corresponds to molecular weights equal to or higher than 2,000 kDa (void volume). Region B corresponds to polysaccharides with molecular weights of approximately 50 to 60 kDa.

Symbiotic characterization of *cgs* mutants of *S. fredii* HH103.

Previous studies describing the symbiotic phenotype of an *S. fredii* HH303 *cgs* mutant reported contradictory results (Bhagwat et al. 1992; Inon de Iannino et al. 1996). Because of this, in addition to SVQ562, we have included in our studies a second independent *S. fredii* HH103 *cgs* mutant as a way to reinforce the conclusions derived from plant test results. This mutant, called SVQ561, was produced by inserting the Ω cassette into the *cgs* gene of *S. fredii* HH103-Rif^r. As expected, ^{1}H-NMR spectra showed that supernatants of SVQ561 cultures were devoid of CG (Fig. 4). The symbiotic phenotypes of the *S. fredii* HH103 *cgs* mutant derivatives (SVQ561 and SVQ562) were investigated with several host plants: *G. max* (cvs. McCall, Osumi, Peking, and Williams), *V. unguiculata* cv. Bisbee Red (cowpea), and *Glycyrrhiza uralensis*. These three legumes form nitrogen-fixing nodules with *S. fredii* HH103-Rif^r, although *G. uralensis* plants inoculated with strain HH103-Rif^r show very high variability in the number of nodules formed. Mutants SVQ561 and SVQ562 were unable to induce the formation of nitrogen-fixing nodules in any of the legumes tested, regardless of whether they formed determinate (*Glycine max* and *V. unguiculata*) or indeterminate (*Glycyrrhiza uralensis*) nodules.

G. uralensis inoculated with SVQ561 and SVQ562 only formed pseudonodules devoid of bacteria. However, the two determinate nodule-forming legumes tested showed differences in their plant responses to inoculation with the *cgs* mutants: *Glycine max* plants induced the formation of pseudonodules while no macroscopic root responses were observed in *V. unguiculata* roots. This observation prompted us to investigate whether the lack of bacterial CG could somehow affect the exchange of early symbiotic signals between the *S. fredii* HH103 *cgs* mutants and *V. unguiculata*, such as the capacity of *V. unguiculata* flavonoids to activate the transcription of *nod* genes or the bacterial capacity to produce Nod factors.

We first investigated whether co-inoculation of *V. unguiculata* (cowpea) roots with mutants SVQ295 and SVQ562 results in the formation of nitrogen-fixing nodules occupied by the mutant unable to synthesize CG. SVQ295 is an auxotrophic *purL* mutant unable to invade soybean roots (it secretes LCO but only pseudonodules are formed) that, in co-inoculation experiments, can complement *S. fredii* SVQ116 (a *nodA* mutant, unable to produce LCO) to form nitrogen-fixing nodules on soybean plants (Buendía-Clavería et al. 2003). Mutant SVQ562 was chosen for these studies because any nodule formed could be investigated for its β-galactosidase activity (presence of SVQ562). Cowpea plants inoculated with the co-inoculants SVQ295 (*purL* mutant) and SVQ562 (*cgs* mutant) only formed a few pseudonodules, indicating that CG produced by the *purL* mutant (which produces a ^{1}H-NMR spectrum identical to that observed for wild-type CG) cannot complement the symbiotic deficiency of mutant SVQ562. Cowpea roots inoculated with the co-inoculants used as a control (SVQ295 and SVQ116) formed nitrogen-fixing nodules that were solely occupied by SVQ116, the mutant unable to synthesize LCO.

Plasmid pMP240 was introduced into strains SVQ561 and HH103-Rif^r to investigate whether flavonoids or *G. max* or *V. unguiculata* root exudates were able to activate the transcription of nodulation genes in the *cgs* mutant. Plasmid pMP240 contains the *lacZ* gene under the control of the *R. leguminosarum nodA* promoter (Spaink et al. 1987). The presence of the flavonoid genistein (50 nM) as well as root exudates of *G. max* or *V. unguiculata* in HH103-Rif^r (pMP240) and SVQ561 (pMP240) cultures provoked at least an eightfold increase in lacZ activity. Nod factors produced by SVQ561 cultures in the

presence of the *nod* gene inducer genistein were also investigated. Nineteen different LCO were detected in the SVQ561 culture grown in the presence of genistein: III(C16:0, MeFuc); III(C16:1); III(C16:1, MeFuc); III(C18:1); III(C18:0, MeFuc); III(C18:1, Fuc); III(C18:1, MeFuc); III(C20:1, MeFuc); IV(C16:0, MeFuc); IV(C16:1); IV(C16:1, MeFuc); IV(C18:1); IV(C18:1, Fuc); IV(C18:1, MeFuc); IV(C18:0, MeFuc); IV(C20:1, MeFuc); V(C16:0, MeFuc); V(C16:1, MeFuc); and V(C18:1, MeFuc).

Although *V. unguiculata* roots inoculated with *cgs* mutants of *S. fredii* HH103 did not show macroscopic responses (such as root outgrowths), microscopy studies showed that mutant SVQ561 was indeed able to form nodule primordia on *Vigna* roots (Fig. 8). One possibility that might explain why these microscopic nodule primordia do not further develop into a macroscopic nodule is that, for whatever reason, the survival of *S. fredii* HH103 *cgs* mutants in the rhizosphere of *V. unguiculata* might be very poor. Liquid samples from *V. unguiculata* plants growing in hydroponic cultures and inoculated with *S. fredii* HH103-Rif^r or SVQ561 (10^6 cells/ml of plant nutritive solution) contained similar bacterial populations (approximately 5×10^6 cells ml^{-1}) 10 days after inoculation, indicating that the survival of the *cgs* mutant is not compromised in the rhizosphere of *V. unguiculata* plants.

Complementation of the *S. fredii* HH103 cgs mutant.

Cosmid pMUS909 (carrying the complete *cgs* gene) was transferred by conjugation to mutant SVQ562. SVQ562 transconjugants carrying pMUS909 regained the capacity to produce wild-type CG in view of the fact that the ^{1}H-NMR spectrum corresponding to the CG produced by SVQ562 (pMUS909) was identical to that of *S. fredii* HH103-Rif^r (Fig. 3).

The presence of pMUS909 in SVQ562 reduced EPS production to a level similar to that observed in the wild-type strain HH103-Rif^r (Fig. 5A) and restored bacterial mobility on GYM medium (Fig. 5B). HH103-Rif^r and SVQ562 (pMUS909) showed similar mobility in GYM supplemented with 100 mM NaCl (Fig. 5C).

We have previously mentioned that the expression level of the *exoA* gene was higher (7.4- ± 1.3-fold) in mutant SVQ562 than in the parental strain. However, the expression of the *exoA* gene in SVQ562 carrying pMUS909 was only 50% (0.49 ± 0.02) of that in HH103-Rif^r.

SVQ562 (pMUS909) was able to induce the formation of nitrogen-fixing nodules in soybean cv. Williams, *V. unguiculata*, and *Glycyrrhiza uralensis.* As expected, isolates from nodules were Gm^r (presence of the *lacZ*-Gm^r cassette) and tetracycline resistant (presence of cosmid pMUS909).

DISCUSSION

CG produced by *S. fredii* HH103 can be detected in the cellular fraction as well as in the cell's milieu. The structure consists of a homopolysaccharide of →2)-β-D-Glc*p*-(1→ residues, partially substituted with glycerol-1-phosphate at the *C-6* position of some of the glucose units. Thus, the structure of the CG produced by *S. fredii* HH103 is nearly identical to that of *S. meliloti* strains, which are phylogenetically closely related to *S. fredii*. The only difference between *S. fredii* HH103 CG and those produced by some *S. meliloti* strains, such as Rm1021 or 102F34, is that the former does not carry succinyl substitutions (Breedveld and Miller 1998).

In *S. meliloti*, it has been previously shown that the addition of phosphoglycerol substituents to the cyclic β-(1,2)-glucans occurs primarily during early and mid-logarithmic growth and it is greatly reduced in stationary cells (Geiger et al. 1991), while neutral cyclic β-glucan biosynthesis may continue through the stationary phase (Breedveld et al. 1990). These previous results are in accordance with the fact that supernatants of *S. fredii* HH103 cultures grown for 3 days (stationary phase) only contain neutral CG, whereas bacterial cells still contain a mixture of neutral and anionic CG. The fact that the ^{1}H-NMR spectrum and the MALDI mass spectrum (Figure 1) of extracellular CG are almost identical to that of chemically dephosphorylated cellular CG indicates that the presence of phosphoglycerol substituents is the only difference between the two types of CG.

Although the results of studies of CG produced by another *S. fredii* strain (called HH303) have been previously reported, to our knowledge, the nucleotide sequence of the *S. fredii cgs* (*ndvB*) gene is not available in the databases. We have sequenced the *cgs* gene of *S. fredii* HH103 and showed that its encoded product (Cgs) has a high level of identity to that (NdvB) of *S. meliloti* and *S. medicae*. The 13,293-bp fragment sequenced shows the same genetic organization of the two ORF (*Smc04383* and *Smc04882*) upstream of *cgs* and for that (*Smc04381*) immediately downstream of *cgs*. However, the homology is disrupted just after *Smc04381*, because the next ORF (here called *lysE*) shows homology to an *S. meliloti* ORF that is situated approximately 440 kb away from *cgs*. In *S. meliloti* 1021, the *ndvB* and *ndvA* genes are separated by *Smc04381* and *Smc03901*. In *S. fredii* HH103, the position of *Smc03901* is replaced by *lysE*, which indicates that the *S. fredii* HH103 fragment comprising *cgs* (*ndvB*) and *ndvA* is different from that of *S. meliloti.* This alteration is not specific to strain HH103 because positive PCR amplification is obtained in *S. fredii* USDA192 and USDA205 when primers are designed from the 3′ ends of *cgs* and *lysE*. We have not determined the exact position of the *ndvA* gene but we know that this gene is present in cosmid pMUS909 because PCR experiments (using primers designed from the *S. meliloti* 1021 *ndvA* gene) amplifies a band of the expected size (data not shown). All these results indicate that, in some *S. fredii* strains isolated from different regions of China, the DNA stretch containing the *ndvA* and *ndvB* is different from that in *S. meliloti* 1021. Although databases suggest that the ORF immediately downstream of *cgs* might code for a hypothetical glucan succinyl transferase protein (SMc04381), the enzymatic activity of this gene in *S. fredii* HH103 would be expected to be different, because the presence of succinyl substituents has not been detected in the CG produced by this bacterium.

The *cgs* mutant SVQ562 constructed in this work is unable to produce CG, has lost its swimming capacity, and grows more slowly in GYM hypoosmotic media, as previously reported for other *cgs* (*ndvB*) rhizobial mutants (Breedveld and

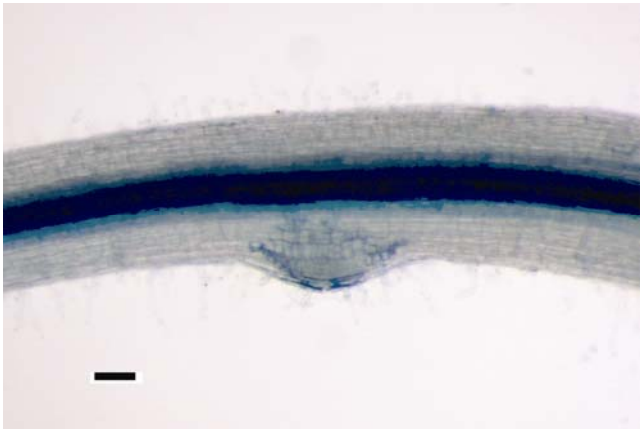

Fig. 8. Nodule primordium formed by mutant SVQ561 on *Vigna unguiculata* plants. Bar = 0.1 mm.

Miller 1998). Although the hypoosmotic sensitivity of rhizobial mutants unable to produce CG is well documented (Dylan et al. 1990a) and *S. meliloti* 1021 *ndvB* mutants do not survive in distilled water, our results show that, although SVQ562 grows more slowly in GYM media, its capacity to withstand severe hypoosmotic conditions (72 h in distilled water) is no different from that of its wild-type strain. This fact suggests that the symbiotic impairment shown by SVQ562 might not be due to an inability of the mutant to adapt to changes in osmotic conditions.

Rhizobial *cgs* (*ndvB*) mutants show a pleiotropic phenotype, which indicates that the absence of CG provokes other alterations, most probably in the bacterial cell surface. We have searched for possible alterations in the other rhizobial surface polysaccharides that are known to play a role in symbiosis (LPS, KPS, and EPS). Apparently, the *cgs* mutation in SVQ562 does not cause alterations in the production of LPS or KPS. However, SVQ562 produced more EPS in YMB and GYM + NaCl media than the parental strain HH103-Rif^r. In YMB medium, the increase in EPS production observed in mutant SVQ562 correlates with the observed higher expression of the *exoA* gene, which is involved in EPS biosynthesis. In the hypoosmotic GYM medium, the EPS produced by SVQ562 showed higher levels of noncarbohydrate substitutions and its molecular weight reached much higher values. Thus, there are clear differences between the EPS produced by HH103-Rif^r and SVQ562 under hypoosmotic conditions. Transcription of *Smc04381*, which appears after *cgs* and in the same transcriptional direction, was detected in HH103-Rif^r and SVQ562 (data not shown), indicating that this gene might not be responsible for the differences observed between the EPS produced by HH103-Rif^r and SVQ562. The fact that mutation of the *cgs* gene can also affect the degree of EPS polymerization appears as a striking coincidence with the previous unexplained and intriguing observation that the oligosaccharide repeating unit of the EPS of *S. meliloti* cannot be detected in culture supernatants of *ndvA* mutants (Stanfield et al. 1988). Thus, all these results indicate that *ndvA* and *cgs* (*ndvB*) not only influence the amount of EPS produced but also its degree of polymerization. In fact, Becker and associates (1995a) suggested that NdvA might participate in EPS export. Differences in the relative production of high molecular weight EPS depending on the environmental osmolarity has been reported for *S. meliloti* SU47 but not for *A. tumefaciens* (Breedveld and Miller 1998; Breedveld et al. 1990).

In the hypoosmotic GYM medium, the EPS produced by SVQ562 carries more substitutions than that produced by HH103-Rif^r cultures, indicating that, in this medium, the mutation abolishing CG production also provokes an increase in the degree of EPS substitutions. This increase in EPS substitutions also appears in SVQ562 cultures in GYM supplemented with 100 mM NaCl, indicating that increasing the osmolarity of the growth medium by adding this ionic solute did not restore the wild-type levels of EPS substitution. The *cgs* mutation does not provoke a clear increase in EPS substitution if the bacteria are grown in YMB medium, which contains large amounts (10 g/liter) of mannitol. Thus, apparently, the composition and osmolarity of the growth medium differentially affect the structural characteristics of the EPS produced by the wild-type strain and its *cgs* mutant. In *S. meliloti* Rm102F34, however, the *ndvA* and *ndvB* mutants produce EPS that is indistinguishable by NMR from that of the wild type (Nagpal et al. 1992).

The symbiotic properties of *S. fredii cgs* (*ndvB*) mutants are contradictory. Some reports have shown that soybean cv. Williams and *V. unguiculata* cv. California Black-eye plants inoculated with a *cgs* mutant (Rf19) of *S. fredii* HH303 only form small knot-like structures (pseudonodules) unable to fix nitrogen and devoid of rhizobial cells (Bhagwat et al. 1992). However, other reports have shown that this mutant is able to form nitrogen-fixing nodules with American (McCall) and Asiatic (Peking) soybean cultivars (Inon de Iannino et al. 1996). Here, we show that two independent *cgs* mutants of another *S. fredii* strain (HH103) only form pseudonodules on American and Asiatic soybean cultivars. Thus, the fact that a *cgs* mutant of another *S. fredii* strain (HH303) is able to form nitrogen-fixing nodules on soybean plants appears as an exceptional case in which rhizobial mutants unable to produce CG are still able to nodulate.

The fact that an *S. fredii* HH103 *exoA* mutant forms nitrogen-fixing nodules on soybean roots (Parada et al. 2006) and that a double *cgs exoA* mutant only forms pseudonodules (*unpublished data*) suggests that EPS may not play a relevant role in the bacterial symbiotic interaction with soybean.

In our experimental conditions, *V. unguiculata* roots did not show macroscopic responses to inoculation, although the formation of microscopic nodule primordia could be observed. This failure to produce clearly visible pseudonodules is not due to any incapacity of the *S. fredii* HH103 *cgs* mutants to produce LCO in the presence of flavonoids or *V. unguiculata* root exudates. These results suggest that the nodulation process induced by *S. fredii* HH103 *cgs* mutants is blocked in *V. unguiculata* plants at earlier stages than in soybean. *S. fredii cgs* mutants only formed ineffective pseudonodules with *G. uralensis*, an indeterminate nodule-forming legume. Thus, we conclude that, as far as we know, mutations abolishing CG production are the most deleterious mutations for symbiosis among those affecting the production of rhizobial surface polysaccharides (EPS, LPS, KPS, and CG) because the bacterial symbiotic capacity is severely impaired regardless of whether the host plant forms determinate or indeterminate nodules. Earlier reports, however, have shown that, apparently, the production of CG is not strictly required by the bacterium to nodulate (Dylan et al. 1990b; Ko and Gayda 1990; Nagpal et al. 1992) because, for instance, spontaneous symbiotic pseudorevertants of *S. meliloti cgs* mutants do not produce CG but have regained the capacity to form nitrogen-fixing nodules with alfalfa. It might be possible that the inability to produce CG together with alterations in the EPS produced could account for the symbiotic incapability of *cgs* mutants. In fact, an *S. meliloti* Rm102F34 cosmid containing *exo* genes restores the symbiotic capacity of Rm102F34 *ndvA* or *ndvB* mutants (Nagpal et al. 1992).

The pleiotropic phenotype of the *S. fredii* SVQ562 mutant is only due to the mutation in the *cgs* gene because SVQ562 (pMUS909) produces CG, its mobility is restored, and it effectively nodulates *Glycine max*, *V. unguiculata*, and *Glycyrrhiza uralensis*. SVQ562 produces more EPS in YMB medium than the parental strain HH103-Rif^r, but the introduction of cosmid pMUS909 into SVQ562 lowers EPS production to, approximately, that of the wild-type strain.

We have shown that the expression level of the *exoA* gene was higher (7.4- $\pm$ 1.3-fold) in mutant SVQ562 than in the parental strain. This overexpression of the *exoA* gene in SVQ562 disappears in the presence of cosmid pMUS909. The fact that the expression level of the *exoA* gene in SVQ562 (pMUS909) was only 50% of that in HH103-Rif^r can be explained as a gene-dosage effect because cosmid pMUS909 (which carries the *cgs* gene) should be in more than one copy.

Although the addition of purified CG increases nodulation of alfalfa roots inoculated with the wild-type *S. meliloti* 102F34 strain, the exogenous application of CG at the time of inoculation with an *S. meliloti* 102F34 *ndvB* mutant was ineffective in correcting the symbiotic impairment (Dylan et al. 1990b). We show here that the presence of the co-inoculant SVQ295 (that

produces LCO and CG) enables an *S. fredii* HH103 *nodA* mutant (unable to produce LCO) to effectively nodulate *V. unguiculata* but it does not enhance the ability of mutant SVQ562 to nodulate this legume. Nodules produced by *V. unguiculata* plants inoculated with co-inoculants SVQ116 and SVQ295 were only occupied by the former, indicating that SVQ295 does not invade *V. unguiculata* roots. These results indicate that, although the external addition of purified GC can enhance nodulation induced by wild-type strains, neither the exogenous addition of purified CG (Dylan et al. 1990b) nor the CG produced by the accompanying co-inoculant can complement in trans the *cgs* co-inoculant in nodulating cowpea plants.

The fact that *cgs* mutants show a pleiotropic phenotype makes it difficult to determine the relative symbiotic importance of each particular phenotypic change observed. Nevertheless, the recent finding that the application of purified *X. campestris* CG to *Nicotiana benthamiana* leaves provokes the suppression of different plant defense mechanisms (Rigano et al. 2007) clearly indicates that CG, by themselves, play an important role in plant–microbe interactions.

MATERIALS AND METHODS

Molecular and microbiological techniques.

Sinorhizobium strains were grown at 28°C on TY medium (Beringer 1974), yeast extract/mannitol (YM) medium (Vincent 1970), or GYM medium (Dylan et al. 1990a). *Escherichia coli* was cultured on Luria-Bertani (LB) medium (Sambrook et al. 1989) at 37°C. When required, the media were supplemented with the appropriate antibiotics as described by Lamrabet and associates (1999). The number of viable cells of liquid cultures was estimated by plate counting. Cell density of liquid cultures was estimated by measuring the absorbance at 600 nm.

Assays for β-galactosidase activity in liquid bacterial cultures on YMB were carried out 16 h after induction as described by Vinardell and associates (2004a). At least three independent experiments performed in duplicate were carried out. When necessary, the pH of the YM medium was buffered to pH 6 with MES (20 mM) or to pH 7 or 8 with HEPES (20 mM).

For mobility assays, GYM medium was solidified with 0.24% (wt/vol) agar and inoculated (by puncturing) with 3 μl of early-exponential phase cultures of *S. fredii* strains. Plates were incubated at 28°C for 72 h.

Sensitivity to SDS was studied on YMB supplemented with 0.03 to 0.07% SDS (wt/vol). For this purpose, late stationary-phase cultures grown in YMB medium were 1,000-fold diluted in YMB containing SDS and incubated in an orbital shaker (180 rpm) at 28°C. The optical density at 600 nm (OD_{600}) was observed for 96 h upon inoculation.

Plasmids were transferred from *E. coli* to rhizobia by conjugation as described by Simon (1984). Recombinant DNA techniques were performed according to the general protocols of Sambrook and associates (1989). For hybridization, DNA was blotted onto Amersham (Tokyo) Hybond-N nylon membranes, and the DigDNA method of Roche Diagnostics (Mannheim, Germany) was employed according to the manufacturer's instructions. PCR amplifications were performed as previously described (Vinardell et al. 2004b). For amplifying internal *cgs* fragments, primer pairs used were *cgs*-F (5′ TGGGAGCGCAAGC GCGGCAA)/*cgs*-R (5′ CGAACATCGAGCCCGACCA) and *cgs*HH-F (5′ GACCCGCTACGAGGTCGAGA)/*cgs*HH-R (5′ CCGGCAATGCGCT CTTCGA). Primers were designed by using the GeneFisher2 utility. The UWGCG program was used for basic DNA sequence analysis and assembly. The National Center for Biotechnology Information ORF-Finder and BLAST programs were used for ORF identification and homology searches, respectively.

In order to generate *cgs* mutants of *S. fredii* HH103, we constructed derivatives of pK18mob (Schafer et al. 1994), a suicide vector in rhizobia, carrying an internal fragment of the HH103 *cgs* gene (the 2.3-kb fragment amplified with primers *cgs*-F and *cgs*-R) interrupted by the *lacZ*::Gm^r cassette (Becker et al. 1995b) or the Ω interposon (Prentki and Krisch 1984) subcloned as a 4.5- or a 2-kb *Hin*dIII fragment into the unique *Hin*dIII site of the HH103 *cgs* internal fragment. The plasmids generated, named pMUS868 (*cgs*:: Ω) and pMUS871 (*cgs*::*lacZ*-Gm^r), were individually transferred to HH103-Rif^r, and Rif^r spectinomycin-resistant Km^s (kanamycin-sensitive) or Rif^r Gm^r Km^s transconjugants, respectively, were identified in order to isolate double recombinants in which the wild-type *cgs* gene had been substituted by the mutated copy of the gene. Thus, mutants SVQ561 (=HH103-Rif^r *cgs*:: Ω) and SVQ562 (=HH103-Rif^r *cgs*::*lacZ*-Gm^r) were obtained. In both cases, homogenotization of the mutated version of the *cgs* gene was confirmed by DNA-DNA hybridization.

***rt*-PCR analysis.**

S. fredii strains HH103-Rif^r and SVQ562 were incubated in YMB in an orbital shaker (180 rpm) at 28°C. When the cultures reached an OD_{660} of 0.3, cells were harvested and RNA was extracted by using the RNAprotect Bacteria Reagent and the RNAeasy mini kit (both provided by Qiagen, Basel, Switzerland) following the manufacturer's instructions. Retrotranscription of the RNA was carried out using the Quantitect kit (Qiagen). Quantitative *rt*-RT-PCR were performed in a 20-μl final volume containing 1 μl of cDNA, 0.6 pmol of each primer, and 10 μl of FastStart SYBR Green Master Mix (Roche Diagnostics). PCR was conducted on the iCycler IQ (Bio-Rad Laboratories SA, Marnes La Coquette, France), and the threshold cycles were determined with the iCycler software. Primers used for amplification of a 197-bp internal fragment of the *S. fredii* HH103 *exoA* gene (accession number AY882558) were rt*exoA*-F (5′-CGCGCGTCCTTTTCCTTGACA) and rt*exoA*-R (5′-GGTTTGCATGGCGACGACCA). To normalize the data, a 196-bp internal fragment of the *S. fredii* HH103 16S rRNA (accession number AY260145) was employed as an internal control in each sample by using primers HH16S-F (5′-GGA TCGGAGACAGGTGCTGCA) and HH16S-R (5′-CGTGTGTAGCCCAG CCCGTA). Three independent experiments performed in triplicate were carried out.

Studies of bacterial polysaccharides.

For extraction of EPS from liquid cultures, *S. fredii* strains were grown in 50 ml of the appropriate medium on an orbital shaker (180 rpm) for 96 h (approximately 5 to 7×10^9 bacteria ml^{-1}). When required, the media were supplemented with genistein at a final concentration of 1 μg ml^{-1}. After centrifugation at 10,000 × *g* for 10 min, supernatants were mixed with three volumes of acetone and the EPS was recovered with a glass rod, dried at 60°C for 3 h, and weighed. To investigate EPS production on solid YM medium, rhizobial strains were grown for 120 h at 28°C followed by 48 h at room temperature. LPS extraction, separation on SDS polyacrylamide gel electrophoresis, and silver staining were performed as previously described (Buendía-Clavería et al. 2003). Immunostaining procedures and the monoclonal antibody NB6-228.22 were described by Buendía-Clavería and associates (2003).

Isolation of bacterial surface polysaccharides for chemical analyses.

S. fredii HH103 or its mutant derivatives were grown in 7 liters of TY liquid medium for 3 days on an orbital shaker (160 rpm) at 28°C. Early stationary phase cells were harvested by slow-speed centrifugation.

Isolation of periplasmic CG. Bacterial pellets (approximately 4.38 g) were washed three times with 0.9% (wt/vol) NaCl and freeze dried. The polysaccharide was extracted from the freeze-dried bacterial cells with 1:1 hot phenol-water mixture (100 ml) (Westphal and Jann 1965), and the two phases were separated. The aqueous phase was dialyzed against water, freeze dried, and redissolved in 10 mM $MgSO_4$ and 50 mM Tris-HCl solution (100 ml, pH 7.0); DNase (1 mg) and RNase (1 mg) were added, and the solution was stirred overnight at 5°C. Proteinase K (2 mg) was added, and the solution was shaken for 24 h at 37°C, dialyzed, and then freeze dried. The polysaccharide was chromatographed on Sephacryl S-500 (60 by 2.6 cm), using 0.05 M EDTA/triethylamine, pH 7.0, 0.02% NaN_3 as eluent, and carbohydrates were detected using a refractive-index detector and the orcinol-sulfuric acid method on thin-layer chromatography plates. Fractions containing carbohydrates were dialyzed and freeze dried. Chromatography on Biogel P6 (60 by 2.6 cm) was carried out using 0.02 M $HCOONH_4$ as eluent, and carbohydrates were detected as above.

Isolation of EPS and extracellular CG. Culture supernatant (1.2 liters) was concentrated to 20% of its original volume and mixed with ethanol (3 vol). The resulting precipitate contained the EPS, which was removed by centrifugation, resuspended, and purified by dialysis. Supernatants were concentrated to 5% of their original volume, mixed with ethanol (10 vol), and centrifuged to precipitate the low molecular weight fraction that contained CG (Gil Serrano et al. 1993). This fraction was dialyzed against water with a 10-kDa JumboSep device (Pall Corporation, New York) and the diffusate was chromatographed on Sephadex G-25 (30 by 5 cm) with water as eluent. Carbohydrates were detected using a refractive-index detector and the orcinol-sulfuric acid method on thin-layer chromatography plates.

Lipo-chitin-oligosaccharide analysis.

Genistein-induced culture medium (2 liters) was extracted with *n*-butanol (1 liter). The butanol extract was evaporated to dryness under vacuum and the residue was suspended in 10 ml of 50% acetonitrile (ACN)-water by overnight shaking. Water (15 ml) was added and the crude extract was prepurificated by solid-phase extraction on a C18 cartridge (6 ml of SPE per gram of Resprep; Restek Corp., Bellefonte, PA, U.S.A.). The SPE cartridge was previously conditioned with ACN (10 ml) and 10 ml of 20% ACN-water. Then, the crude extract was passed through the cartridge. Salts and polar compounds were eluted by passage of 5 ml of 20% ACN-water. Nod factors were eluted using methanol (10 ml). This fraction was concentrated, freeze dried, and dissolved in 1 ml of 50% ACN-water to be analyzed by HPLC-dual mass spectrometry (MS/MS).

Chromatographic separation was performed using a Perkin-Elmer Series 200 HPLC system (Wellesley, MA, U.S.A.) coupled to an Applied Biosystem QTRAP LC-MS/MS system (Foster City, CA, U.S.A.) consisting of a quadrupole-linear ion trap mass spectrometer equipped with an electrospray ion source.

HPLC analyses were performed on a 250-by-2.1-mm Tracer Spherisorb ODS2 C18 reversed-phase column with a particle size of 5 μm (Teknokroma, Barcelona, Spain). The flow rate was 0.3 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of water and ACN. Both components contained 0.1% formic acid (vol/vol). The elution profile was isocratic for 5 min with 30% ACN, linear for 30 min up to 100% ACN, and isocratic for 2 min. Mass spectrometric detection was performed in the positive-ion mode after electrospray ionization. For HPLC-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas, 35 psi; ionspray voltage, 5,500 V; source temperature, 300°C; source gas, 20 psi; declustering potential, 50 V; and entrance potential, 10 V. Collision-induced dissociation (CID) was performed with the following collision energy: 35 V. Only those precursor ions with *m/z* between 800 and 1,600 and intensities higher than 1,000 cps were selected for CID-MS/MS analysis.

Monosaccharide analysis.

Monosaccharides were identified on GLC-MS separation of their trimethylsilylated methyl glycosides obtained as described (Gil-Serrano et al. 1998). The absolute configuration of monosaccharides was assigned following GLC-MS analysis of their trimethylsilylated (*S*)- and (*R,S*)-2-butyl glycosides, which were prepared as described (Gerwig et al. 1978). Derivatives of authentic standard monosaccharides were prepared for comparison.

GLC-MS was performed on a Micromass AutoSpec-Q instrument fitted with an OV-1 column (25 m by 0.25 mm). The temperature program for separating the trimethylsilylated methyl glycosides was isothermal at 150°C for 2 min followed by a 10°C min^{-1} gradient up to 250°C. The temperature program for separating the trimethylsilylated 2-butyl glycosides was isothermal at 130°C for 3 min followed by a 3°C min^{-1} gradient up to 150°C and then a new 10°C min^{-1} gradient up to 250°C. In all cases, the ionization potential was 70 eV and spectra were recorded in low-resolution mode.

Methylation analysis.

The vacuum-desiccated polysaccharide sample was methylated by the method of Ciucanu and Costello (2003). Then, the sample was hydrolyzed, reduced with NaB^2H_4, and acetylated as described (Kim et al. 2006). GLC-MS was performed on a Micromass AutoSpec-Q instrument fitted with an OV-1 column (25 m by 0.25 mm). The temperature program for separating the partially methylated alditol acetates was isothermal at 120°C for 1 min followed by an 8°C min^{-1} gradient up to 250°C. The ionization potential was 70 eV and spectra were recorded in low resolution mode.

Molecular weight determination.

Molecular weight estimation was made by SEC in an HPLC system. Separation was carried out on a μBondagel E-Linear (Waters Corp., Milford, MA, U.S.A.). As the mobile phase, 0.1 M $NaNO_3$ was used with an operating temperature of 50°C, a flow rate of 0.5 ml min^{-1}, and an injection volume of 20 μl. The polymers were detected by refractive index using a 410 Differential Refractrometer (Waters Corp.). Solutions (5 mg ml^{-1}) of standard dextrans of 39.1, 70, 110, 252, and 2,000 kDa (Sigma-Aldrich, St. Louis) were used to estimate the molecular weights of the eluting peaks according to the method of Granath and Kvist (1967).

Samples containing EPS (4 to 7 mg) were resuspended in 0.1 M $NaNO_3$ (1 to 2 ml) and, after stirring overnight, centrifuged at 4,000 rpm. Supernatant was further microfiltered and analyzed by SEC-HPLC.

NMR spectroscopy.

Samples were deuterium exchanged several times by freeze drying from ^2H_2O, and then examined in solution (5 mg per 750 μl) in 99.98% ^2H_2O. Spectra were recorded at 303 K on a Bruker AV500 spectrometer operating at 500.13 MHz (^1H), 125.75 MHz (^{13}C), and 202.46 MHz (^{31}P). Chemical shifts are given in ppm, using the H^2HO signal (4.75 ppm) (^1H), external dimethylsulfoxide (39.5 ppm) (^{13}C), and external phosphoric acid (0 ppm, ^{31}P) as references. The two-dimensional (2D) homonuclear COSY was performed using the Bruker standard

pulse sequence. A data matrix of 256-by-1K points was used to digitize a spectral width of 4,845 Hz; 32 scans were used per increment. The 2D heteronuclear one-bond proton-carbon correlation experiment was registered in the ^{1}H-detection mode via HSQC. A data matrix of 256-by-1K points was used to digitize a spectral width of 4,845 and 22,522 Hz in F_2 and F_1, respectively; 64 scans were used per increment. ^{13}C decoupling was achieved by the GARP scheme. Squared-cosine-bell functions were applied in both dimensions, and zero-filling was used to expand the data to 1K by 1K. The ^{1}H-^{31}P HMBC experiment was performed using the Bruker standard sequence with 256 increments of 1K real points to digitize a spectral width of 4,807 by 20,243 Hz; eight scans were acquired per increment with a delay of 33 ms for evolution of long-range couplings.

MALDI-MS.

Positive-ion MALDI mass spectrometric data were acquired from the neutral CG on an Applied Biosystems 4700 Proteomics analyzer with ToF/ToF optics (Applied Biosystems, Foster City, CA, U.S.A.), operated in reflectron mode. The 2,5-dihydroxybenzoic acid matrix was prepared as a saturated solution in 50% ACN, 0.1% trifluoroacetic acid (TFA), and then diluted 1:1 in the same solvent. Sample solutions were mixed with an equal volume of matrix solution and 1 µl of the resultant mixture was deposited onto the MALDI target plate and allowed to dry by evaporation. The MALDI source was equipped with a 200-Hz frequency-tripled Nd:YAG laser operating at a wavelength of 355 nm. Spectra were recorded over the *m/z* range of approximately 2,500 to 4,500, with a "focus mass" of *m/z* 3,000, using a total of 2,500 laser shots at an intensity of 5,000.

Negative-ion MALDI mass spectra of substituted CG were acquired on an Autoflex II ToF/ToF MS (Bruker Daltonik GmbH) fitted with a 337-nm nitrogen laser and operated in linear mode, summing a total of 400 laser shots. A 1-mg/ml solution of sample was mixed on the MALDI target in a 1:1 ratio with matrix. The matrix, a 50-mg/ml solution of 2,5-dihydroxybenzoic acid (Bruker Daltonik GmbH, Barcelona, Spain), was dissolved in 30% ACN (Sigma, Poole, U.K.), 70% water (18 MΩ) containing 0.1% TFA (Sigma). Raw data were processed using the FlexAnalysis (Bruker Daltonik, 3.0 GmbH) software using the SavitzkyGolay smoothing algorithm.

Plant assays.

Nodulation assays on *Glycine max* (L.) Merr. cvs. Williams, McCall, Osumi, and Peking; *V. unguiculata*, and *Glycyrrhiza uralensis* were carried out as described by Crespo-Rivas and associates (2007). Germinated seeds were transferred to Leonard jars containing sterilized vermiculite supplemented with Fåhraeus nutrient solution (Vincent 1970). Each plant was inoculated with approximately 10^8 bacteria and then grown for at least 6 weeks with a 16-h photoperiod at 25°C in the light and 18°C in the dark. Plant tops were dried at 80°C for 48 h and weighed. Bacterial isolation from surface-sterilized nodules and coinoculation experiments were carried out as described by Buendía-Clavería and associates (2003).

The survival of *S. fredii* HH103-Rif^{r} and SVQ561 in the presence of *V. unguiculata* (cowpea) roots was estimated as follows: surface-sterilized cowpea seed were germinated and transferred to tubes (25 cm high and 3 cm wide) containing 100 ml of Fåhraeus nutrient solution. Each tube containing one cowpea seedling was inoculated with 10^8 bacteria of *S. fredii* HH103-Rif^{r} or SVQ561. The bacterial population sizes were estimated by plate counting 10 days after inoculation.

Plant test and microscopy studies aimed at determining the formation of nodule primordia in *V. unguiculata* roots were carried out as previously described (López–Lara et al. 1995; van Brussel et al. 2002).

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LITERATURE CITED

Amemura, A., and Cabrera-Crespo, J. 1986. Extracellular and low-M_r polysaccharides containing (1,2)-β-D-glucosidic linkages from strains of *Xanthomonas*, *Escherichia coli* and *Klebsiella pneumonie.* J. Gen. Microbiol. 132:2443-2452.

Arellano-Reynoso, B., Lapaque, N., Salcedo, S., Briones, G., Ciocchini, A. E., Ugalde, R., Moreno, E., Morrión, I., and Gorvel, J.P. 2005. Cyclic beta-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. Nat. Immunol. 6:618-625.

Batley, M., Redmond, J. W., Djordjevic, S. P., and Rolfe, B. G. 1987. Characterisation of glycerophosphorylated cyclic β-1,2-glucans from a fast-growing *Rhizobium* species. Biochem. Biophys. Acta 901:119-126.

Becker, A., and Pühler, A. 1998. Production of exopolysaccharides. Pages 97-118 in: *The Rhizobiaceae*. Molecular Biology of Model Plant-Associated Bacteria. H. P. Spaink, A. Kondorosi, and P: J. J. Hooykaas, eds. Kluwer Academic Publisher, Dordrecht, The Netherlands.

Becker, A., Küster, H., Niehaus, K., and Pühler, A. 1995a. Extension of the *Rhizobium meliloti* succinoglycan biosynthesis gene cluster: Identification of the *exsA* gene encoding an ABC transporter protein, and the *exsB* gene which probably codes for a regulator of succinoglycan biosynthesis. Mol. Gen. Genet. 249:487-497.

Becker, A., Schmidt, M., Jäger, W., and Pühler, A. 1995b. New gentamicin-resistance and *lacZ* promoter-probe cassettes suitable for insertion mutagenesis and generation of transcriptional fusions. Gene 162:37-39.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84:188-198.

Bhagwat, A. A., and Keister, D. L. 1995. Site directed mutagenesis of the α(1>3), α(1>6)-D-glucan synthesis locus of *Bradyrhizobium japonicum*. Mol. Plant-Microbe Interact. 8:366-370.

Bhagwat, A. A., Tully, R. E., and Keister, D. L. 1992. Isolation and characterization of an *ndvB* locus from *Rhizobium fredii*. Mol. Microbiol. 6:2159-2165.

Breedveld, M. W., and Miller, K. J. 1994. Cyclic β-glucans of members of the family *Rhizobiaceae*. Microbiol. Rev. 58:145-161.

Breedveld, M. W., and Miller, K. J. 1998. Cell-surface β-glucans. Pages 81-96 in: *The Rhizobiaceae*. Molecular Biology of Model Plant-Associated Bacteria. H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas, eds. Kluwer Academic Publisher, Dordrecht, The Netherlands.

Breedveld, M. W., Zevenhuizen, L. P., and Zehnder, A. J. 1990. Excessive excretion of cyclic beta-(1,2)-glucan by *Rhizobium trifolii* TA-1. Appl. Environ. Microbiol. 56:2080-2086.

Breedveld, M. W., Benesi, A. J., Marco, M. L., and Miller, K. J. 1995. Effect of phosphate limitation on synthesis of periplasmic cyclic (beta)-(1,2)-glucans. Appl. Environ. Microbiol. 61:1045-1053.

Buendía-Clavería, A. M., Chamber, M., and Ruiz-Sainz, J. E. 1989. A comparative study of the physiological characteristics, plasmid content and symbiotic properties of different *Rhizobium fredii* strains. Syst. Appl. Microbiol. 12:203-209.

Buendía-Clavería, A. M., Moussaid, A., Ollero, F. J., Vinardell, J. M., Torres, A., Moreno, J., Gil-Serrano, A. M., Rodríguez-Carvajal, M. A., Tejero-Mateo, P., Peart, J. L., Brewin, N. J., and Ruiz-Sainz, J. E. 2003. A *purL* mutant of *Sinorhizobium fredii* HH103 is symbiotically defective and altered in its lipopolysaccharide. Microbiology 149:1807-1818.

Ciucanu, I., and Costello, C. E. 2003. Elimination of oxidative degradation during the per-O-methylation of carbohydrates. J. Am. Chem. Soc. 125:16213-16219.

Crespo-Rivas, J. C., Margaret, I., Pérez-Montaño, F., López-Baena, F. J., Vinardell, J. M., Ollero, F. J., Moreno, J., Ruiz-Sainz, J. E., and Buendía-Clavería, A. M. 2007. A pyrF auxothrophic mutant of Sinorhizobium fredii HH103 impaired in its symbiotic interactions with soybean and other legumes. Int. Microbiol. 10:169-176.

D'Antuono, A. L., Casabuono, A., Couto, A., Ugalde, R. A., and Lepek, V. C. 2005. Nodule development induced by *Mesorhizobium loti* mutant strains affected in polysaccharide synthesis. Mol. Plant-Microbe Interact. 18:446-457.

D'Antuono, A. L., Ott, T., Krussel, L., Voroshilova, V., Ugalde, R. A., Udvardi, M., and Lepek, V. C. 2008. Defects in rhizobial cyclic glucan and lipopolysaccharide synthesis alter legume gene expresion during nodule development. Mol. Plant-Microbe Interact. 21:50-60.

Dickstein, R., Bisseling, T., Reinhold, V. N., and Ausubel, F. M. 1988. Expression of nodule-specific genes in alfalfa root nodules blocked at an early stage of nodule development. Genes Dev. 2:677-687.

Dowdle, S. F., and Bohlool, B. B. 1985. Predominance of fast-growing *Rhizobium japonicum* in a soybean field in the People's Republic of China. Appl. Environ. Microbiol. 50:1171-1176.

Dylan T., Helinski, D. R., and Ditta, G. 1990a. Hypoosmotic adaptation in *Rhizobium meliloti* requires β-(1,2)-glucan. J. Bacteriol. 172:1400-1408.

Dylan, T., Nagpal, P., Helinski, D. R., and Ditta G. S. 1990b. Symbiotic pseudorevertants of *Rhizobium ndv* mutants. J. Bacteriol. 172:1409-1417.

Fraysse, N., Couderc, F., and Poinsot, V. 2003. Surface polysaccharide involvement in establishing the *Rhizobium*-legume symbiosis. Eur. J. Biochem. 270:1365-1380.

Gage, D. J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Mol. Biol. Rev. 68:280-300.

Geiger, O, Weissborn, A. C., and Kennedy, E. P. 1991. Biosynthesis and excretion of cyclic glucans by *Rhizobium meliloti* 1021. J. Bacteriol. 173:3021-3024.

Geremia, R. A., Cavaignac, A., Zorreguieta, A., Toro, N., Olivares, J., and Ugalde, R. A. 1987. A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharides but fails to form β-(1,2)-glucan. J. Bacteriol. 169:880-884.

Gerwig, G. J., Kamerling, J. P., and Vliegenthart, J. F. G. 1978. Determination of D and L configuration of neutral monosaccharides by high-resolution capillary g.l.c. Carbohydr. Res. 62:349-357.

Gil-Serrano, A. M., Franco-Rodriguez, G., Gonzalez-Jiménez, I., Tejero-Mateo, P., Molina, J. M., Dobado, J. A., Megias, M., and Romero, M. J. 1993. The structure and molecular mechanics calculations of the cyclic (1-2)-β-D-glucan secreted by *Rhizobium tropici* CIAT 899. J. Mol. Struct. 301:211-226.

Gil-Serrano, A. M., Rodríguez-Carvajal, M. A., Tejero-Mateo, P., Espartero, J. L., Thomas-Oates, J., Ruiz-Sainz, J. E., and Buendía-Clavería, A. M. 1998. Structural determination of a 5-O-methyl-deaminated neuraminic acid (Kdn)-containing polysaccharide isolated from *Sinorhizobium fredii*. Biochem. J. 334:585-594.

Gil-Serrano, A. M., Rodríguez-Carvajal, M. A., Tejero-Mateo, P., Espartero J. L., Menéndez, M., Corzo, J., Ruiz-Sainz, J. E., and Buendía-Clavería, A. M. 1999. Structural determination of a 5-acetamido-3,5,7,9-tetradeoxy-7-(3-hydroxybutyramido)-L-glycero-L-manno-nonulosonic acid-containing homopolysaccharide isolated from *Sinorhizobium fredii* HH103. Biochem. J. 342:527-535.

Granath, K. A., and Kvist, B. E. 1967. Molecular weight distribution analysis by gel chromatography on Sephadex. J. Chromatogr. 280:69-81.

Inon de Iannino, N., and Ugalde, R. A. 1993. Biosynthesis of cyclic beta-(1-3), beta-(1-6) glucan in *Bradyrhizobium* spp. Arch. Microbiol. 159:30-38.

Inon de Iannino, N., Briones, G., Kreiman, G., and Ugalde, R. 1996. Characterization of the biosíntesis of β(1-2) cyclic glucan in *R. fredii*. β(1-2) glucan has no apparent role in nodule invasion of McCall and Peking soybean cultivars. Cell. Mol. Biol. 42:617-629.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., and Walker, G. C. 2007. How rhizobial symbionts invade plants: The *Sinorhizobium–Medicago* model. Nature 5:619-633.

Kim, J. S., Reuhs, B. L., Michon, F., Kaiser; R. E., and Arumugham, R. G. 2006. Addition of glycerol for improved methylation linkage analysis of polysaccharides. Carbohydr. Res. 341:1061-1064.

Ko, Y. H., and Gayda, R. 1990. Nodule formation in soybeans by exoplysaccharide mutants of *Rhizobium fredii* USDA191. J. Gen. Microbiol. 136:105-113.

Lamrabet, Y., Bellogín, R. A., Cubo, T., Espuny, R., Gil, A., Krishnan, H. B., Megias, M., Ollero, F. J., Pueppke, S. G., Ruiz-Sainz, J. E., Spaink, H. P., Tejero-Mateo, P., Thomas-Oates, J., and Vinardell, J. M. 1999. Mutation in GDP-Fucose synthesis genes of *Sinorhizobium fredii* alters Nod factors and significantly decreases competitiveness to nodulate soybeans. Mol. Plant-Microbe Interact. 12:207-217.

López-Lara, I. M., van Der Drift, K. M. G. M., van Brussel, A. A. N., Haverkamp, J., Lugtenberg, B. J. J., Thomas-Oates, J., and Spaink, H. P. 1995. Induction of nodule primordia on *Phaseolus* and *Acacia* by lipo-chitin oligosaccharide nodulation signals from broad-host-range *Rhizobium* strain GRH2. Plant Mol. Biol. 29:465-477.

Mathis, R., van Gijsegem, F., De Ryche, R., D'Haeze, W., Van Maelsaeke, E., Antonio, E., van Montagu, M., Holsters, M., and Vereecke, D. 2005. Lipopolysaccharides as a communication signal for progression of legume endosymbionts. Proc. Natl. Acad. Sci. U.S.A. 102:2655-2660.

Nagpal, P., Khanuja, S. P., and Stanfield, S. W. 1992. Suppression of the ndv mutant phenotype of *Rhizobium meliloti* by cloned *exo* genes. Mol. Microbiol. 6:479-488.

Parada, M., Vinardell, J. M., Ollero, F. J., Hidalgo, A., Guitiérrez, R., Buendía-Clavería, A. M., Lei, W., Margaret, I., López-Baena, F. J., Gil-Serrano, A. M., Rodríguez-Carvajal, M. A., Moreno, J., and Ruiz-Sainz, J. E. 2006. *Sinorhizobium fredii* HH103 mutants affected in capsular polysaccharide (KPS) are impaired for nodulation with soybean and *Cajanus cajan*. Mol. Plant-Microbe Interact. 19:43-52.

Prentki, P., and Krisch, H. M. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29:303-313.

Rigano, L. A., Payette, C., Brouillard, G., Marano, M. R., Abramowicz, L., Torres, P. S., Yun, M., Castagnaro, A. P., El Oirdi, M., Dufour, V., Malamud, F., Dow, J. M., Bouarab, K., and Vojnov, A. A. 2007. Bacterial cyclic β-(1,2)-glucan acts in systemic suppression of plant immune responses. Plant Cell 19:2077-2089.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69-73.

Simon, R. 1984. High frequency mobilization of gram-negative bacterial replicons by the in vivo constructed Tn5-Mob transposon. Mol. Gen. Genet. 196:413-420.

Spaink, H. P., Okker, R. J., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. 1987. Promoters in nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. 9:27-39.

Stanfield, S. W., Ielpi, L., O'Brochta, D., Helinski, D. R., and Ditta, G. S. 1988. The *ndvA* gene product of *Rhizobium meliloti* is required for beta-(1----2)glucan production and has homology to the ATP-binding export protein HlyB. J. Bacteriol. 170:1523-1530.

Ugalde, R. A. 1999. Intracellular lifestyle of *Brucella* spp. common genes with other animal pathogens, plant pathogens, and endosymbionts. Microbes Infect. 1:1211-1219.

van Brussel, A. A. N., Tak, T., Boot, K. J. M., and Kijne, J. W. 2002. Autoregulation of root nodule formation: Signals of both symbiotic partners studied in a split-root system of *Vicia sativa* subsp. *nigra*. Mol. Plant-Microbe Interact. 15:341-349.

Vinardell, J. M., López-Baena, F. J., Hidalgo, A., Ollero, F. J., Bellogín, R., Espuny, M. R., Temprano, F., Romero, F., Krishnan, H. B., Pueppke, S. G., and Ruiz-Sainz J. E. 2004a. The effect of FITA mutations on the symbiotic properties of *Sinorhizobium fredii* varies in a chromosomal-background-dependent manner. Arch. Microbiol. 181:144-154.

Vinardell, J. M., Ollero, F. J., Hidalgo, A., López-Baena, F. J., Medina, C., Ivanov-Vangelov, K., Parada, M., Madinabeitia, N., Espuny, M. R.., Bellogín, R. A., Camacho, M., Rodríguez-Navarro, D. N., Soria-Díaz, M. E., Gil-Serrano, A. M., and Ruiz-Sainz, J. E. 2004b. NolR regulates diverse symbiotic signals of *Sinorhizobium fredii* HH103. Mol. Plant-Microbe Interact. 17:676-685.

Vincent, J. M. 1970. Appendix III. The modified Fåhraeus slide technique. Pages 144-145 in: A Manual for the Practical Study of Root Nodule Bacteria. J. M. Vincent, ed. Blackwell Scientific Publications, Oxford.

Westphal, O., and Jann, K. 1965. Bacterial lipopolysaccharides, extraction with phenol-water and further application of the procedure. Methods Carbohydr. Chem. 5:83-91.

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