***Sinorhizobium fredii* HH103 *rkp-3* Genes Are Required for K-Antigen Polysaccharide Biosynthesis, Affect Lipopolysaccharide Structure and Are Essential for Infection of Legumes Forming Determinate Nodules**

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The *Sinorhizobium fredii* HH103 *rkp-3* region has been isolated and sequenced. Based on the similarities between the *S. fredii* HH103 *rkpL*, *rkpM*, *rkpN*, *rkpO*, *rkpP*, and *rkpQ* genes and their corresponding orthologues in *Helicobacter pylori*, we propose a possible pathway for the biosynthesis of the *S. fredii* HH103 K-antigen polysaccharide (KPS) repeating unit. Three *rkp-3* genes (*rkpM*, *rkpP*, and *rkpQ*) involved in the biosynthesis of the HH103 KPS repeating unit (a derivative of the pseudaminic acid) have been mutated and analyzed. All the *rkp-3* mutants failed to produce KPS and their lipopolysaccharide (LPS) profiles were altered. These mutants showed reduced motility and auto-agglutinated when early-stationary cultures were further incubated under static conditions. *Glycine max*, *Vigna unguiculata* (determinate nodule-forming legumes), and *Cajanus cajan* (indeterminate nodules) plants inoculated with mutants in *rkpM*, *rkpQ*, or *rkpP* only formed pseudonodules that did not fix nitrogen and were devoid of bacteria. In contrast, another indeterminate nodule-forming legume, *Glycyrrhiza uralensis*, was still able to form some nitrogen-fixing nodules with the three *S. fredii* HH103 rifampicin-resistant *rkp-3* mutants tested. Our results suggest that the severe symbiotic impairment of the *S. fredii* *rkp-3* mutants with soybean, *V. unguiculata*, and *C. cajan* is mainly due to the LPS alterations rather than to the incapacity to produce KPS.

Rhizobia are soil bacteria able to establish symbiotic interactions with leguminous plants that lead to the formation of root nodules in which these bacteria fix atmospheric nitrogen. This *Rhizobium*-legume interaction involves a complex set of

molecular interactions that takes place during the development and maintenance of nitrogen-fixing nodules on the plant roots. Inside the nodules, rhizobia fix atmospheric nitrogen that becomes an assimilable source of nitrogen to the plant. This molecular dialogue between the bacteria and the plant begins when flavonoids, naturally exuded by the legume, activate the transcription of bacterial nodulation genes. Some of these genes (*nod*, *nol*, and *noe*) encode proteins responsible for the biosynthesis and secretion of signal molecules called “Nod factors” or lipochitin oligosaccharides, which mediate species specificity and induce the development of root nodules in the plant (Gage 2004; Jones et al. 2007).

In addition to Nod factors, different rhizobial surface polysaccharides are commonly necessary for successful nodulation. Exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS [K-antigen polysaccharides]), and cyclic glucans (CG) are the main rhizobial polysaccharides investigated, because they might act as signal molecules or could prevent plant defense reactions (Breedveld and Miller 1998; Frayssé et al. 2003; Krol and Becker 2009; Mithöfer 2002). KPS and CG but not EPS appear to play important roles in the *S. fredii*–soybean symbiosis (Crespo-Rivas et al. 2009; Parada et al. 2006). The symbiotic properties of *S. fredii* mutants specifically affected in genes responsible for the biosynthesis of LPS have not been reported. In *Bradyrhizobium japonicum*, however, mutants showing deficiencies in structure of their LPS are symbiotically impaired (Puvanesarajah et al. 1987; Quelas et al. 2010).

S. fredii possesses an exceptionally broad host range (Pueppke and Broughton 1999), so that this rhizobial species is especially interesting for the study of the relevance of each bacterial surface polysaccharide in the symbiotic interaction with many different legumes, including both plants forming determinate or indeterminate nodules. In the *S. meliloti*–alfalfa symbiosis, which generates the formation of indeterminate nodules, KPS can substitute for EPS in the nodulation process (Reuhs et al. 1995). In contrast, KPS by itself appears to be relevant in the *S. fredii*–soybean symbiosis, which induces the formation of determinate nodules. In this symbi-

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 5 appears in color online.

osis, EPS cannot substitute for the KPS function (Parada et al. 2006).

Three *rkp* gene clusters (*rkp-1*, *rkp-2*, and *rkp-3*) involved in KPS production were identified in *S. meliloti* Rm41 (Kereszt et al. 1998; Kiss et al. 2001; Putnoky et al. 1990). This strain produced a K antigen of strain-specific structure, designated as the K_{R5} antigen (Kiss et al. 2001). In *S. meliloti* AK631 (an *exoB* mutant derivative of *S. meliloti* Rm41), the repeating unit of the K_{R5} antigen produced is composed of a hexose and a derivative of the pseudaminic acid (PseAc) (Kannenberg et al. 1998). It has also been shown that membrane-anchored 3-deoxy-D-manno-2-octulosonic acid (Kdo) homopolymers (poly-Kdo) are consensus sinorhizobial capsular polysaccharides present in *S. meliloti* Rm41, *S. meliloti* Rm1021, and *S. fredii* HH103 (Chataigné et al. 2008; Krol and Becker 2009). In *S. meliloti* Rm1021, this polyKdo is symbiotically inactive (Frayse et al. 2005; Reuhs et al. 1995).

Most of the *S. meliloti* Rm41 *rkp* mutants are unable to produce wild-type KPS (K_{R5} antigen) and do not infect alfalfa nodule cells. The *rkpA* to *rkpF* genes of the *rkp-1* region show significant sequence similarity to type I fatty acid synthases (Becker et al. 2005; Geiger and López-Lara, 2002; Petrovics et al. 1993), so that they are most probably involved in the synthesis of a specific lipid carrier for KPS biosynthesis. The *rkp-2* region comprises two genes, *lpsL* and *rkpK*. The former is required for wild-type LPS biosynthesis, while the latter appears necessary for the production of wild-type LPS and KPS (Kereszt et al. 1998). The *rkp-3* region contains the *rkpL*, *rkpM*, *rkpN*, *rkpO*, *rkpP*, and *rkpQ* gene cluster, which appears to be involved in the synthesis of the PseAc component of the KPS produced by *S. meliloti* Rm41 (Kiss et al. 2001). Mutations in the *S. meliloti* AK631 *rkpL*, *rkpM*, *rkpN*, *rkpO*, *rkpP*, and *rkpQ* genes abolish K_{R5} antigen production and impair the bacterial symbiotic capacity with *Medicago sativa* (Kiss et al. 2001). The in silico *S. meliloti* Rm41 RkpLMNOPQ predicted proteins show homology to the respective putative products of the *Campylobacter jejuni* and *Helicobacter pylori* *pseBCFGHI* genes, which are involved in the biosynthesis of PseAc (Guerry et al. 2006; Schirm et al. 2003; Schoenhofen et al. 2006).

Rhizobium sp. strain NGR234 is a broad host range endosymbiont able to form nitrogen-fixing symbioses with more than 112 genera of legumes (Pueppke and Broughton 1999). Although NGR234 and *S. fredii* HH103 share the capacity to nodulate many particular legumes, the former is not able to

nodulate soybean. The structure of the KPS produced by *Rhizobium* sp. strain NGR234 also contains PseAc and is very similar to that of the K_{R5} antigen of *S. meliloti* Rm41. A deletion of the *rkpM*, *rkpN*, and part of *rkpO* generated a NGR234 mutant that does not produce the K-antigen and showed reduced nodulation efficiency on different legumes, such as *Vigna unguiculata* (Le Quéré et al. 2006).

S. fredii HH103 constitutively produces two types of KPS. One of them, here called poly-PseAc, is a homopolymer of a derivative of the pseudaminic-acid (Gil-Serrano et al. 1999), while the other is a homopolymer of Kdo (Chataigné et al. 2008). The implication of the *S. fredii* HH103 poly-Kdo in the bacterial symbiotic capacity has not been investigated, but the symbiotic relevance of the *S. fredii* HH103 poly-PseAc appears to be related to each specific bacterium-legume interaction rather than to the type of nodule, determinate or indeterminate, formed by the host plant. The poly-PseAc (hereafter called "KPS") plays a relevant role in the *S. fredii*-soybean symbiosis, since mutants affected in genes of the *rkp-1* region are symbiotically impaired with soybean (Hidalgo et al. 2010; Margaret-Oliver et al. 2012; Parada et al. 2006). However, the *S. fredii* HH103 *rkp* mutants analyzed so far are not impaired for nodulation of cowpea, another determinate-nodule-forming legume (Hidalgo et al. 2010). Instead, *S. fredii* HH103 *rkp-1* mutants are negatively affected to nodulate *Glycyrrhiza uralensis*, an indeterminate nodule-forming legume (Margaret-Oliver et al. 2012). *Glycyrrhiza uralensis* is one of the 50 fundamental herbs used in traditional Chinese medicine and can be used for many different healing purposes, such as stress relief or in asthma and respiratory illnesses.

In this study, we identified and characterized genes of the *rkp-3* locus that appear to be involved in the synthesis of the repeating unit (poly-PseAc) of the *S. fredii* HH103 KPS. We showed that mutations in the *rkpM*, *rkpQ*, and *rkpP* genes not only abolish poly-PseAc KPS production but also alter the bacterial LPS. These mutants showed reduced mobility, auto-agglutinated in tryptone yeast (TY) liquid medium, and failed to form nitrogen-fixing nodules with *Glycine max*, *Vigna unguiculata* (two determinate nodule-forming legumes), and *Cajanus cajan* (indeterminate nodules). In contrast, they were still able to form nitrogen-fixing nodules with *Glycyrrhiza uralensis*, another indeterminate nodule-forming legume, also called Chinese liquorice. We also propose a possible pathway for the biosynthesis of the *S. fredii* HH103 KPS repeating unit.

Table 1. Primers used in polymerase chain reaction (PCR) experiments

Primer	Sequence (5'-3') ^a	Position of priming site	Predicted length of PCR product ^b
Smr k pL-F	GAACGCATGACTCGCTTCATGA	<i>rkpL</i> coding region (from Rm41)	1,426-bp in <i>Sinorhizobium meliloti</i> Rm41
Smr k pM-R	GTATGAACCGGAATATAGTGCA	<i>rkpM</i> coding region (from Rm41)	
HHr k pL-F	AGCTAGTGTGGCACGCCTTTGA	<i>rkpL</i> coding region	1,326-bp in <i>S. fredii</i> HH103.
HHr k pM-R1	TCGAAATCCCGCCGCGCGATT	<i>rkpM</i> coding region	
HHr k pM-F	CCGGCGGAACCTTGACGCAA	<i>rkpM</i> coding region	690 bp in <i>S. fredii</i> HH103 Rif ^r and 2,726 bp in SVQ581
HHr k pM-R2	AGGCTGCGTGTGAACCGGAA		
<i>lacZ</i> int-R	GCCTCTTCGCTATTACGCCA	<i>lacZ</i> coding region	Estimated about 550 bp in SVQ582 (by using HHr k pM-F and <i>lacZ</i> int-R)
HHr k pQF1	CGACGGGACGTTCTACGACA	<i>rkpQ</i> coding region	816 bp in HH103 Rif ^r , 2,852 bp in SVQ592, and estimated about 590 bp in SVQ594 (by using HHr k pQF1 and <i>lacZ</i> int-R)
HHr k pQR1	TCTCGATCAACGAGCCCCCTA		
HHr k pPext-F	GGAAGAGGATCCTGATGTCGA	<i>rkpM</i> coding region	1,180 bp in HH103 Rif ^r
HHr k pPext-R	TATAGCGGATCCCGCCCAA	<i>rkpQ</i> coding region	

^a The underlined sequence (GGATCC) in primers HHr k pPext-F and HHr k pPext-R is the nucleotide sequence recognized by the endonuclease *Bam*HI. This *Bam*HI restriction site, which is not present in the original rhizobial sequences, has been created for cloning purposes. The original sequences are CCA CCG (for HHr k pPext-F) and GTCTTT (for HHr k pPext-R).

^b The pair of primers *rkpM*-F and *rkpM*-R2 was used to check the insertion of the Ω interposon in SVQ581. Primers *rkpM*-F and *lacZ*int-R were used to check the insertion of the *lacZ* Δ p-Gm^r cassette in SVQ582. Primers *rkpQ*F1 and *rkpQ*R1 were used to check the insertion of the Ω interposon in SVQ592. Primers *rkpQ*F1 and *lacZ*int-R were used to check the insertion of the *lacZ* Δ p-Gm^r cassette in SVQ594.

RESULTS

Isolation of the *rkpL*, *rkpM*, *rkpN*, *rkpO*, *rkpP*, and *rkpQ* genes of *S. fredii* HH103.

To isolate genes of the *S. fredii* HH103 *rkp*-3 region, primers *SmrkpL*-F and *SmrkpM*-R were respectively designed from the coding sequences of the *rkpL* and *rkpM* genes of *S. meliloti* Rm41 (Table 1). Using *S. fredii* HH103 genomic DNA as a template, primers *SmrkpL*-F and *SmrkpM*-R led to the polymerase chain reaction (PCR) amplification of a 1.3-kb fragment that showed 80% identity with the 3' end of *rkpL* and the 5' end of *rkpM* of *S. meliloti* Rm41. This fragment was sequenced, and primers *HHrkl*-F and *HHrkm*-R1, respectively, were designed from the *S. fredii* HH103 *rkpL* and *rkpM* coding sequences (Table 1). Then, 1,200 clones of a genomic library of *S. fredii* HH103 were screened for positive PCR amplification with primers *HHrkl*-F and *HHrkm*-R1, resulting in the identification of cosmid pMUS664. DNA sequencing of an 11,274-bp fragment (accession number JN642175) demonstrated the presence of seven open reading frames (ORF) showing the same polarity (Fig. 1). The G+C content of this DNA fragment was 54.4%. Six ORF encode for putative proteins that have a 68 to 93% identity (Table 2) with their respective orthologues (*rkpL*, *rkpM*, *rkpN*, *rkpO*, *rkpP*, and *rkpQ*) of *S. meliloti* Rm41. Five of these *S. fredii* HH103 putative proteins, RkpLMNOQ, have a 74 to 95% and a 38 to 67% identity with the corresponding orthologues of *Rhizobium* sp. strain NGR234 and *Aeromonas caviae*, respectively.

A large ORF of 4,089-bp, coding for a putative protein of 1,362 amino acids (aa), is located close to the 5' end of *rkpL*. The deduced protein, provisionally annotated as SfHH103_b1955 in the *S. fredii* HH103 genome sequence project (Margaret et al. 2011), has two regions that show homology to other bacterial hypothetical proteins in computer (Blast) analyses. The amino terminus of SfHH103_b1955 (442 residues) is homologous (55% identity) to the entire *S. meliloti* Rm1021 RkpZ protein (440 residues), while its carboxyl-terminus (from residues 831 to 1361) shows homology (31% identity) with a hypothetical protein (of 1,043 residues) of *Roseobacter* sp. strain SK209-2-6. The central part of SfHH103_b1955 (from residues 443 to 851) does not show significant homology in Blast analyses.

Two ORF (provisionally annotated as SfHH103_b1962 and SfHH103_b1963) appear in the vicinity of the 3' end of *rkpQ*. They are transcribed in the opposite direction to *rkpQ* and coded for putative transposases. SfHH103_b1963 and SfHH103_b1962 code for putative transposases (of 166 and 140 aa) that are respectively homologous to transposases of the insertion sequences NGRIS-2b (93% identity in 166 aa) and NGRIS-2a (92% identity in 140 aa) of *Rhizobium* sp. strain NGR234. SfHH103_b1962 also showed 98% identity with 419 bp of the 5' end of the 1,248-bp repetitive sequence RFRS9 (U18764.1) of *Sinorhizobium fredii* USDA257 (Krishnan and Pueppke 1993).

The complete genome sequence project of *S. fredii* HH103 (Margaret et al. 2011) has shown the presence of three ORF homologous to the *S. meliloti* Rm41 and *Rhizobium* sp. strain

Fig. 1. Genetic organization of the *rkp*-3 region of *Sinorhizobium fredii* HH103 and a comparison to that of *S. meliloti* Rm41 and *Rhizobium* sp. strain NGR234.

Table 2. Proteins encoded by the *Sinorhizobium fredii* HH103 *rkp*-3 region and identities with the corresponding proteins of *S. meliloti* Rm41 and *Rhizobium* sp. strain NGR234^a

Protein	Orthologue ^b	Putative function ^c	Identity with	
			<i>S. meliloti</i> Rm41 (%)	<i>Rhizobium</i> sp. strain NGR234 (%)
RkpL (334)	PseB	NAD(p)-dependent dehydratase/epimerase	93 RkpL (334)	95 RkpL (334)
RkpM (386)	PseC	Pyridoxal phosphate (PLP)-dependent aminotransferase	87 RkpM (386)	86 RkpM (386)
RkpN (234)	PseF	CMP-pseudaminic acid synthase	80 RkpN (227)	84 RkpN (377)
RkpO (382)	PseG	Nucleotidase	68 RkpO (377)	74 RkpO (377)
RkpP (184)	PseH	N-acetyltransferase	80 RkpP (185)	Not present
RkpQ (350)	PseI	Pseudaminic acid synthase	90 RkpQ (354)	89 RkpQ (350)
RkpR (446)	Not present	Polysaccharide export protein	89 RkpR/KpsE (317)	85 RkpR (443)
RkpS (219)	Not present	Polysaccharide export protein	91 RkpS (219)	92 RkpS (219)
RkpT (256)	Not present	Cell surface polysaccharide export ABC-2 transporter	91 RkpT1 (259)	91 RkpT1 (256)
RkpZ (406)	Not present	Capsule polysaccharide biosynthesis protein	65 RkpZ1 (432)	Two open reading frames (Rkp3_001a and Rkp3_001b)

^a Number of residues of each protein is shown in parentheses.

^b Corresponding orthologue of *Helicobacter pylori* and *Campylobacter jejuni*.

^c Proposed function as defined in *S. meliloti* Rm41 (Kiss et al. 2001) and *H. pylori* and *Campylobacter jejuni* (Schoenhofen et al. 2006).

NGR234 *rkpT*, *rkpS*, and *rkpR* genes (Fig. 1; accession number JN642176). These three genes are putatively involved in polysaccharide export (Kiss et al. 2001). In Rm41 and NGR234 strains, the *rkpT*, *rkpS*, and *rkpR* genes are in the vicinity of the *rkpQ* gene and are transcribed in the opposite direction. In contrast, the *S. fredii* HH103 *rkpT*, *rkpS*, and *rkpR* genes, together with a gene (*rkpZ*) coding for a KPS chain-length determination enzyme, are located approximately 7 kb downstream of *rkpQ* in the 2.1-Mbp plasmid (Margaret et al. 2011; unpublished data).

***S. fredii* HH103 mutants affected in the *rkpM*, *rkpP*, or *rkpQ* genes do not produce KPS and show an altered electrophoretic LPS pattern.**

As described below, *S. fredii* HH103 *rkpM* and *rkpQ* mutant derivatives carrying the Omega interposon were constructed and were named SVQ581 and SVQ592, respectively. *S. fredii* HH103 *rkpM*, *rkpP*, and *rkpQ* mutants carrying the *lacZ*Δ*p*-Gm^r cassette were also constructed and were named SVQ582, SVQ667, and SVQ594, respectively.

HH103 mutants *rkpM*::Ω, *rkpM*::*lacZ*, *rkpP*::*lacZ*, *rkpQ*::Ω, and *rkpQ*::*lacZ* were investigated for the production of KPS by polyacrylamide gel electrophoresis (PAGE) followed by a treatment with Alcian Blue and silver staining. PAGE experiments to visualize KPS were carried out in the absence of sodium dodecyl sulfate (SDS) to avoid interferences of LPS. The silver-stained material that presumably corresponds to the high-molecular weight forms of KPS was not present in crude cell extracts of mutants *rkpM*::Ω, *rkpM*::*lacZ*, *rkpQ*::Ω, *rkpQ*::*lacZ*, and *rkpP*::*lacZ* (Fig. 2, lanes 2, 4, 7, 8, and 11, respectively). The wild-type KPS produced by the parental strain HH103 Rif^r (rifampicin resistant) (Fig. 3; Gil-Serrano et al. 1999) was not detected by ¹H-NMR (nuclear magnetic resonance) analyses in the *rkpM*, *rkpP*, and *rkpQ* mutants investigated, supporting the hypothesis that the *rkpM*, *rkpO*, and *rkpP* genes are required for KPS production in *S. fredii* HH103. ¹H-NMR spectra of the HH103 *rkpM*::Ω and *rkpQ*::Ω derivatives carrying cosmid pMUS664 showed the presence of the wild-type KPS. As expected, CG production was not affected in any of the *rkp-3* mutants analyzed in this study.

In *Aeromonas* spp., the inactivation of the *rkp-3* orthologous genes has been described to affect both LPS production and motility (Tabei et al. 2009). The LPS profiles of the *rkpM*, *rkpQ*, and *rkpP* mutants were analyzed by PAGE experiments performed in the presence of SDS (Fig. 4) as well as by immunostaining experiments using a monoclonal antibody (NB6-228.22) that recognizes the LPS of HH103 (Buendía-Clavería

Fig. 3. Nuclear magnetic resonance (NMR) analysis of K-antigen polysaccharide (KPS) production by *Sinorhizobium fredii* strains. ¹H-NMR spectra (500 MHz) of crude cell extracts containing surface polysaccharides isolated from *S. fredii* HH103 Rif^r, SVQ581 (*rkpM*::Ω), SVQ581 (pMUS664), SVQ592 (*rkpQ*::Ω), SVQ592 (pMUS664), and SVQ667 (*rkpP*::*lacZ*Δ*p*-Gm^r). Signals corresponding to KPS, cyclic glucans (CG), or the solvent (HDO, deuterated water) are indicated.

Fig. 2. Polyacrylamide gel electrophoresis analysis of K-antigen polysaccharide (KPS) production by *Sinorhizobium fredii* HH103 Rif^r and different *rkp* derivatives. Samples were run in the absence of detergent (SDS), were treated with Alcian Blue, and were silver stained. Lanes 1, 5, 6, and 12, HH103 Rif^r; lane 2, SVQ581 (*rkpM*::Ω); lane 3, SVQ581 (pMUS664); lane 4, SVQ582 (*rkpM*::*lacZ*Δ*p*-Gm^r); lane 7, SVQ592 (*rkpQ*::Ω); lane 8, SVQ594 (*rkpP*::*lacZ*Δ*p*-Gm^r); lane 9, SVQ592 (pMUS664); lane 10, SVQ594 (pMUS664); and lane 11, SVQ667 (*rkpP*::*lacZ*Δ*p*-Gm^r). The high- and low-molecular weight forms of KPS are indicated as HMW-KPS and LMW-KPS, respectively.

et al. 2003). The electrophoretic profile of mutants *rkpM*:: Ω , *rkpM*::*lacZ*, *rkpQ*:: Ω , *rkpQ*::*lacZ*, and *rkpP*::*lacZ* (Fig. 4A, lanes 2, 4, 6, 8, and 10, respectively) was altered in comparison with that of HH103 Rif^r (Fig. 4A, lanes 1, 5, 9, and 12).

These altered profiles consisted of an increase in the electrophoretic mobility of both the smooth (slow-migrating bands) and the rough (fast-migrating bands) LPS. The introduction of cosmid pMUS664 in mutants SVQ581, SVQ592, and SVQ667

Fig. 4. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver-staining or **B**, immuno-staining using the monoclonal antibody NB6-228.22 of lipopolysaccharides (LPS) crude extracts from *Sinorhizobium fredii* HH103 Rif^r and its *rkpM*:: Ω (SVQ581), *rkpM*::*lacZ* Δ p-Gm^r (SVQ582), *rkpP*::*lacZ*-Gm^r (SVQ667), *rkpQ*:: Ω (SVQ592), and *rkpQ*::*lacZ* Δ p-Gm^r (SVQ594) mutant derivatives. The rough and smooth forms of LPS are indicated as R-LPS and S-LPS, respectively. A, lanes 1, 5, 9, and 12, HH103 Rif^r; lane 2, SVQ581; lane 3, SVQ581 (pMUS664); lane 4, SVQ582; lane 6, SVQ592; lane 7, SVQ592 (pMUS664); lane 8, SVQ594; lane 10, SVQ667; and lane 11, SVQ667 (pMUS664). B, lane 1, HH103 Rif^r; lane 2, SVQ581; and lane 3, SVQ581 (pMUS664).

Strain	HH103 Rif ^r	HH103 Rif ^r (pLAFR1)	SVQ581	SVQ581 (pMUS664)	SVQ582
Colonized area (cm ²)	5.33 ± 0.38 (100%)	5.02 ± 0.69 (100%)	3.14 ± 0.30 (59%)	4.45 ± 0.38 (88%)	2.67 ± 0.52 (50%)
Strain	SVQ592	SVQ592 (pMUS664)	SVQ594	HH103 Rif ^r	SVQ667
Colonized area (cm ²)	2.97 ± 0.37 (56%)	4.40 ± 0.65 (83%)	2.75 ± 0.51 (52%)	5.10 ± 0.68 (100%)	2.25 ± 0.56 (44%)

Fig. 5. Swimming capacity of *Sinorhizobium fredii* HH103 Rif^r and its mutant derivatives SVQ581 (*rkpM*:: Ω), SVQ582 (*rkpM*::*lacZ* Δ p-Gm^r), SVQ592 (*rkpQ*:: Ω), SVQ594 (*rkpQ*::*lacZ* Δ p-Gm^r), and SVQ667 (*rkpP*::*lacZ* Δ p-Gm^r). Data represent averages of eight replicates. Numbers in brackets are percentages of the area colonized by 3-day-old SVQ81, SVQ582, SVQ592, SVQ594, and SVQ667 cultures in relation to that occupied by the wild-type strain HH103 Rif^r. The percentage of the area colonized by SVQ581 (pMUS664) and SVQ592 (pMUS664) was calculated in relation to that of HH103 Rif^r (pLAFR1). The dotted circles indicate the approximated limits of the bacterial growth. The area colonized by each mutant was pairwise compared with that of the parental strain HH103 Rif^r (or with HH103 Rif^r [pLAFR1] if mutants were carrying cosmid pMUS664) by using the Mann-Whitney nonparametrical test. The areas colonized by mutants SVQ581, SVQ582, SVQ592, SVQ594, and SVQ667 were significantly different ($P < 0.01$) at the level $\alpha = 5\%$. Significant differences (at $\alpha = 5\%$) were not found among HH103 Rif^r (pLAFR1), SVQ581 (pMUS664), and SVQ592 (pMUS664) cultures.

(Fig. 4A, lanes 3, 7, and 11, respectively) restored the wild-type electrophoretic pattern. These results show that the inactivation of the *rkpM*, *rkpQ*, or *rkpP* genes has an effect not only on KPS but also on LPS production. The monoclonal antibody NB6-228.22 recognizes the *S. fredii* HH103 Rif^r LPS (Fig. 4B, lane 1) but failed in the recognition of the altered LPS produced by mutants SVQ581 (Fig. 4B, lane 2), SVQ592 (not shown), and SVQ667 (not shown). The antibody, however, recognized the LPS produced by mutants SVQ581, SVQ592, and SVQ667 carrying cosmid pMUS664 (Fig. 4B, lane 3, and data not shown).

Inactivation of the *S. fredii* HH103 *rkpM*, *rkpP*, or *rkpQ* genes affected the bacterial motility and auto-agglutination.

Mutants affected in *rkpM* (SVQ581 and SVQ582), *rkpQ* (SVQ592 and SVQ594), and *rkpP* (SVQ667) were also investigated for their swimming capacity. Figure 5 shows that the motility of all the mutants tested (expressed as the surface area colonized by the bacteria after three days of incubation) was significantly lower in comparison with that of HH103 Rif^r. The introduction of cosmid pMUS664 in mutants SVQ581 and

SVQ592 increased their motility to values that were close to those scored for the wild-type strain carrying pLAFR1, the empty cosmid vector.

TY liquid cultures of HH103 Rif^r, SVQ582, SVQ594, and SVQ667 at their early stationary-growth phase (optical density at 600 nm [OD₆₀₀] >1.0) were left in static for 24 h to investigate whether the reduction in bacterial motility shown by the *rkp* mutants was also associated to an increase of the speed at which bacteria deposited at the bottom of the tube. The intensity of auto-agglutination of SVQ582, SVQ594, and SVQ667 cultures was clearly higher than that shown by the HH103 Rif^r culture (Fig. 6). Introduction of cosmid pMUS664 into the three *rkp* mutants reduced bacterial auto-agglutination at wild-type levels (Fig. 6; data not shown). Differences in auto-agglutination were not observed when bacteria were grown in liquid yeast extract mannitol (YMB) medium.

Effect of pH, flavonoids, and YMB composition on the expression of *rkpM*.

The flavonoid genistein can be found in soybean root exudates (Kosslak et al. 1987) and is able to activate the transcription of *S. fredii* HH103 *nod* genes (Vinardell et al. 2004a). *S. fredii* SVQ582 (which carry a *rkpM::lacZΔp-Gm^r* fusion) cultures grown in the presence of and the absence of the flavonoid genistein were assayed for β-galactosidase activity. Three independent experiments, each composed of two replicates, showed that the LacZ activity of SVQ582 cultures in the presence of genistein (287.8 ± 56.8 Miller units) was similar to that in the absence of the flavonoid (291.5 ± 41.7 Miller units).

The effect of the pH at which bacteria were grown on *rkpM* expression was investigated by culturing mutant SVQ582 in acidic-, neutral-, and alkaline-buffered YMB media (pH 6.0, 7.0, and 8.0, respectively). 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 SVQ582 cultures was similar in all the conditions tested (data not shown).

The LacZ activity of SVQ582 cultures grown in different formulations of the YMB medium (in which the amounts of yeast extract and mannitol had been altered) was determined, to investigate the effect of C and N availability on the *rkpM* expression. The higher and lower values of SVQ582 LacZ activity were found, respectively, in YMB formulations containing the higher and lower amounts of yeast extract (Table 3). SVQ582 cultures showed the same β-galactosidase activity in formulations that differed only in the concentration of mannitol (Table 3, formulations B and C), indicating a lack of correlation between *rkpM* transcription and mannitol concentration. The presence of 5 or 10 mM KNO₃ in YMB formulation containing the lower amount of yeast extract did not alter the LacZ activity of SVQ582 cultures.

Fig. 6. Auto-agglutination assays of *Sinorhizobium fredii* HH103 Rif^r and its mutant derivatives SVQ581 (*rkpM::Ω*), SVQ592 (*rkpQ::Ω*), and SVQ667 (*rkpP::lacZΔp-Gm^r*). Tubes 1 to 7 show the aspect of liquid cultures after 24 h in static conditions. Tube 1, *S. fredii* HH103 Rif^r; tube 2, SVQ581; tube 3, SVQ581 (pMUS664); tube 4, SVQ592; tube 5, SVQ592 (pMUS664); tube 6, SVQ667; and tube 7, SVQ667 (pMUS664). The arrow indicates the depth in the liquid column at which the optical density was measured. The optical density of the cultures was monitored at 0 (black bar), 1 (dark-gray bar), 4 (light-gray bar), and 24 (white bar) h of incubation at static conditions.

Table 3. Effect of the C/N relation in liquid yeast mannitol (YMB) media on the expression of the *Sinorhizobium fredii* HH103 *rkpM* gene

YMB formulation ^a	Yeast extract (grams per liter)	Mannitol (grams per liter)	β-Galactosidase activity ^b
A	1.0	1	404 ± 40*
B	0.4	10	303 ± 10
C	0.4	1	275 ± 27
D	0.1	10	203 ± 20*
D + 5 mM KNO ₃	0.1	10	219 ± 23*
D + 10 mM KNO ₃	0.1	10	215 ± 39*

^a Formulation "B" corresponds to the standard YMB composition.

^b Shown in Miller units. Data represent averages of three independent experiments, each composed of two replicates. Numbers followed by an asterisk are significantly different ($P < 0.01$) from that of the standard B condition, using the Mann-Whitney nonparametrical test. β-Galactosidase activity of D cultures with and without KNO₃ were not significantly different.

Symbiotic properties of *S. fredii* HH103 *rkpM* (SVQ581), *rkpP* (SVQ667), and *rkpQ* (SVQ592) mutants with determinate and indeterminate nodule-forming legumes.

S. fredii HH103 Rif^r nodulates a broad range of legumes, including determinate and indeterminate nodule-forming plants. The symbiotic phenotype of *S. fredii rkpM*, *rkpP*, and *rkpQ* mutants was investigated with two determinate nodule-forming legumes (*G. max* cv. Williams and *V. unguiculata*) and also with *C. cajan* and *Glycyrrhiza uralensis*, which form indeterminate nodules (Table 4; Fig. 7).

G. max cv. Williams and *V. unguiculata* plants inoculated with mutant SVQ581 (*rkpM*::Ω) showed severe symptoms of nitrogen starvation (Fig. 7B and F). Soybean and cowpea roots inoculated with SVQ581 formed pseudonodules that were devoid of bacteroids (Fig. 7I, L, N, and P) and did not show nitrogenase activity in acetylene reduction assays (data not shown). Inoculation of soybean and cowpea plants with SVQ592 (*rkpQ*::Ω) and SVQ667 (*rkpP*::*lacZ*) also induced the formation of pseudonodules that did not contain bacteroids (Table 4; Fig. 7G). All attempts to isolate bacteria from soybean and cowpea pseudonodules induced by SVQ581, SVQ592, or SVQ667 were unsuccessful.

Mutants SVQ581, SVQ592, and SVQ667 carrying cosmid pMUS664 regained the capacity to induce the formation of nitrogen-fixing nodules in soybean cv. Williams (Table 4; Fig. 7C and J; data not shown).

S. fredii HH103 is fully effective with *C. cajan* (Parada et al. 2006). All the HH103 *rkp*-3 mutants failed to form nitrogen-fixing nodules with *C. cajan*. Most of the pigeon pea roots inoculated with the *S. fredii* HH103 *rkpM*, *rkpP*, or *rkpQ* mutants developed pseudonodules and macroscopic swellings. *S. fredii* HH103 *rkpM*, *rkpP*, and *rkpQ* mutants carrying cosmid

pMUS664 formed nodules similar in shape and color to those formed by the wild type with *C. cajan*.

S. fredii HH103 Rif^r effectively nodulates with *Glycyrrhiza uralensis*, although the number of nodules formed and plant development are highly variable (Table 4; Fig. 7Q and T). Mutants SVQ581 (*rkpM*::Ω) and SVQ592 (*rkpQ*::Ω) were still able to form nitrogen-fixing nodules in about 50% of the inoculated plants (Table 4; Fig. 7R, S, U, and V). *Glycyrrhiza uralensis* plants showing better nodulation were also better developed, indicating that, in the experimental conditions used, the development of each particular plant was positively correlated to the number of nitrogen-fixing nodules formed. Acetylene reduction assays confirmed that *Glycyrrhiza uralensis* nodules induced by mutants SVQ581 and SVQ592 had nitrogenase activity (data not shown). Optical microscopy analyses of *Glycyrrhiza uralensis* nodules induced by *S. fredii* HH103 Rif^r showed plant cells fully invaded by bacteroids (Fig. 7X). Many infected cells had a large vacuole that occupied the center of the cell. In contrast, nodules induced by SVQ581 or SVQ592 showed fewer cells infected (Fig. 7Y and Z). The central vacuole observed in cells of nodules induced by HH103 Rif^r was not so clearly visible in nodules induced by mutants SVQ581 and SVQ592. Similarly, the *rkpP* mutant (SVQ667) was also able to induce the formation of nitrogen-fixing nodules on *Glycyrrhiza uralensis* roots. Microscopy analyses of nodules induced by SVQ667 were not carried out.

Salicylic acid (SA) production in soybean roots inoculated with *S. fredii* HH103 Rif^r and its *rkpM* mutant derivative.

One of the symbiotic functions of rhizobial surface polysaccharides might be the control (suppression) of plant defense responses (Jones et al. 2008; Soto et al. 2009). To test whether

Table 4. Plant responses to inoculation of *Glycine max* cv. Williams, *Vigna unguiculata* cv. Bisbee Red, *Glycyrrhiza uralensis*, and *Cajanus cajan* with *Sinorhizobium fredii* HH103 Rif^r and its *rkpM* (SVQ581 and SVQ582), *rkpQ* (SVQ592 and SVQ594), and *rkpP* (SVQ667) mutant derivatives

Legume tested	Inoculant ^a	Nodulation and nitrogen fixation	Aspect of the plant
<i>G. max</i> cv. Williams ^b	HH103 Rif ^r	Nitrogen-fixing (Fix ⁺) nodules (91 ± 25)	Well developed, green leaves
	SVQ581	Pseudonodules unable to fix nitrogen (Fix ⁻)	Stunted, yellow leaves
	SVQ581 (pMUS664)	Fix ⁺ nodules (91 ± 18)	Well developed, green leaves
	SVQ592	Pseudonodules, Fix ⁻	Stunted, yellow leaves
	SVQ592 (pMUS664)	Fix ⁺ nodules (64 ± 30)	Well developed, green leaves
	SVQ594	Pseudonodules, Fix ⁻	Stunted, yellow leaves
	SVQ667	Pseudonodules Fix ⁻	Stunted, yellow leaves
<i>V. unguiculata</i> ^b	None	Nod ⁻	Stunted, yellow leaves
	HH103 Rif ^r	Fix ⁺ nodules (60 ± 15)	Well developed, green leaves
	SVQ581	Pseudonodules, Fix ⁻	Stunted, yellow leaves
	SVQ592	Pseudonodules, Fix ⁻	Stunted, yellow leaves
	SVQ594	Pseudonodules, Fix ⁻	Stunted, yellow leaves
	SVQ667	Pseudonodules, Fix ⁻	Stunted, yellow leaves
	None	Nod ⁻	Stunted, yellow leaves
<i>Glycyrrhiza uralensis</i> ^c	HH103 Rif ^r	Fix ⁺ nodules (35/38)	Variable
	SVQ581	Fix ⁺ nodules (10/17)	Variable
	SVQ592	Fix ⁺ nodules (9/18)	Variable
	SVQ667	Fix ⁺ nodules (5/9)	Variable
	None	Nod ⁻ (0/41)	Stunted, yellow leaves
<i>C. cajan</i> ^d	HH103 Rif ^r	Fix ⁺ nodules (87 ± 30)	Well developed, green leaves
	SVQ582	Pseudonodules, Fix ⁻	Stunted, yellow leaves
	SVQ594	Pseudonodules, Fix ⁻	Stunted, yellow leaves
	SVQ667	Pseudonodules, Fix ⁻	Stunted, yellow leaves

^a Bacteria were isolated from at least 10 nodules of each inoculant and legume combination that formed Fix⁺ nodules. The antibiotic-resistant markers of all nodule isolates were equal to those of their respective strains used as inoculants. All attempts to isolate bacteria from pseudonodules were unsuccessful.

^b Two independent plant tests were carried out and numbers in parenthesis are the mean ± standard deviation of the number of nodules formed. Determinations were made 7 and 5 weeks after inoculation for *G. max* and *V. unguiculata*, respectively.

^c Data presented here correspond to the total number of plants used in three different experiments. Determinations were made 12 to 17 weeks after inoculation. Numbers in parenthesis represent the fractions representing the number of plants nodulated to the number of plants inoculated. Positive relationship between plant development and nodule number was observed in all inoculation treatments.

^d Data presented here correspond to one experiment. Eight plants (in four jars) were inoculated with each rhizobial inoculant. Determinations were carried out 40 days after inoculation. Two plants inoculated with SVQ667 formed one or two nodules that were not analyzed. *C. cajan* roots showing pseudonodules also formed many root swellings.

Fig. 7. Plant responses to inoculation of A to D, and H to J, *Glycine max* cv. Williams, E to G, and K to L, *Vigna unguiculata*, and Q to Z, *Glycyrrhiza uralensis* with *Sinorhizobium fredii* HH103 Rif^r and different *rkp-3* mutant derivatives. A, Aerial parts of soybean plants inoculated with HH103 Rif^r, B, SVQ581 (*rkpM::Ω*), C, SVQ581 (pMUS664), or D, noninoculated (NI). E, Aerial parts of *V. unguiculata* plants inoculated with HH103 Rif^r, F, SVQ581, or G, SVQ592 (*rkpQ::Ω*). H and M, Macroscopic and microscopic aspect of soybean Williams nodules induced by HH103 Rif^r, I and N, SVQ581, and J, SVQ581 (pMUS664). K and O, Macroscopic and microscopic aspect of *V. unguiculata* nodules induced by HH103 Rif^r and L and P, SVQ581. Q, Aerial parts of *Glycyrrhiza uralensis* plants inoculated with HH103 Rif^r, R, SVQ581, and S, SVQ592. T and X, Macroscopic and microscopic aspect of *Glycyrrhiza uralensis* nodules induced by HH103 Rif^r, U and Y, SVQ581, and V and Z, SVQ592. All uninoculated controls remained nonnodulated. Bar size = 50 μm (M, N, P), 100 μm (X, Y, Z), or 500 μm (O).

alterations in the *S. fredii* LPS and KPS caused by the loss of function of the *rkpM* gene could trigger plant defense responses, SA content was determined in soybean roots at different times after inoculation with either the wild type or its SVQ581 (*rkpM::Ω*) mutant derivative. SA measurements were also performed on noninoculated plants to detect any effect in response to rhizobial inoculation. It has been observed that, regardless of the treatment, SA content in soybean roots 12 h and 1 day after inoculation (dai) is higher compared with the SA content present in older plants, probably reflecting the acclimation of the seedlings to the new growth conditions. Under these experimental conditions, differences in SA levels could be observed only in plants 7 dai (Fig. 8). Whereas soybean roots inoculated with the *rkpM* mutant showed similar SA content to that shown by noninoculated plants, plants inoculated with the wild-type strain showed approximately 50% less SA content. This could indicate that either wild-type LPS or KPS or both produced by *S. fredii* are able to limit the normal synthesis of SA in soybean plants. However, this effect seems to be transient.

DISCUSSION

In *S. fredii* HH103 the *rkp-1* and *rkp-2* regions are in the chromosome, while the *rkp-3* region is located in the 2.1-Mbp megaplasmid (Margaret et al. 2011). This megaplasmid also carries genes for the biosynthesis of EPS (*exo*) and genes involved in LPS production, such as a transporter (homologous to *mbsA*) of the LPS lipid A. Thus *S. meliloti* Rm41, *Rhizobium* sp. strain NGR234, and *S. fredii* HH103 show a similar distribution of their three *rkp* regions. In all cases, only the region (*rkp-3*) that contains genes for PseAc biosynthesis (*rkpL*, *rkpM*, *rkpN*, *rkpO*, *rkpP*, and *rkpQ*) is plasmid borne. This situation resembles that described for other bacteria, such as *A. caviae*, in which the genes required for biosynthesis of PseAc are located in a genetic island that might have been laterally acquired.

The *S. fredii* HH103 *rkpL*, *rkpM*, *rkpN*, *rkpO*, *rkpP*, and *rkpQ* genes are orthologous, respectively, to the *pseB*, *pseC*, *pseF*, *pseG*, *pseH*, and *pseI* genes of *Helicobacter pylori*, which have been identified as encoding the enzymes of the complete biosynthetic pathway of PseAc from UDP-*N*-acetylglucosamine (Schoenhofen et al. 2006). Although early reports indicated that RkpP might act as an acetyltransferase in *S. meliloti* Rm41 (Kiss et al. 2001), later studies suggest that this enzyme might be involved in the 3-hydroxybutyryl acylation of the Rm41 KPS (Le Quéré et al. 2006). The *rkpP* gene is present in *S. meliloti* Rm41 and *S. fredii* HH103 but absent in *Rhizobium* sp. strain NGR234. This difference is in agreement with the presence of a 3-hydroxybutyryl residue in the KPS repeating unit of *S. meliloti* Rm41 and *S. fredii* HH103 but not in *Rhizobium* sp. strain NGR234. Taking all these facts together, we propose that the biosynthesis pathway of the *S. fredii* HH103 KPS repeating units is nearly identical to that described for *H. pylori* (Schoenhofen et al. 2006), but with the difference that the *S. fredii* HH103 RkpP enzyme would add 3-hydroxybutyryl residues instead of the acetyl substituents added by the *H. pylori* PseH enzyme. Figure 9 shows the proposed pathway as well as its putative intermediates, from UDP-*N*-acetylglucosamine to CMP-5-acetamido-3,5,7,9-tetra-deoxy-7-(3-hydroxybutyramido)-*L*-glycero-*L*-manno-nonulosonic acid, which would correspond to the CMP-activated form of the *S. fredii* HH103 KPS repeating unit (Gil-Serrano et al. 1999).

Previous studies performed on *S. fredii* USDA205 and *Rhizobium* sp. strain NGR234 have shown that the presence of flavonoids influences KPS production (Reuhs et al. 1994; Simsek et al. 2009). In *S. fredii* HH103, however, the flavonoid genistein did not significantly alter the transcriptional activity

of the *rkp-1* genes (Hidalgo et al. 2010; Margaret-Oliver et al. 2012). In this work, we show that genistein does not significantly influence the transcriptional activity of the *rkpM* gene (in mutant SVQ582), indicating that this flavonoid, which is a major activator of *S. fredii* HH103 *nod* genes (Vinardell et al. 2004a and b), might not have any significant effect on the transcriptional regulation of HH103 *rkp-1* and *rkp-3* regions. In contrast, this flavonoid represses the mucosity of *S. fredii* HH103 colonies, which indicates an effect over the exopolysaccharide (EPS) produced by this strain (Vinardell et al. 2004b).

We have also tested whether different formulations of the YMB medium (different amounts of mannitol and yeast extract) affected the expression of the *rkpM* gene. The higher LacZ activity of SVQ582 cultures correlated with the higher amount of yeast extract present in the medium, which might indicate that an increase of N availability induces an increase of the *rkpM* transcription. However, the addition of KNO₃ did not increase the LacZ activity of SVQ582, indicating that the yeast extract used might contain a compound that, directly or indirectly, increases the transcription of the *rkpM::lacZΔp-Gm^r* fusion.

The rhizobial KPS expression can also be affected by abiotic factors, such as temperature or pH (Kanneberg et al. 1998). In *S. fredii* HH103, however, the expression of the *rkpM* gene was not significantly affected at acidic (pH 6.0), neutral (pH 7.0), or alkaline (pH 8.0) conditions.

The *S. fredii* HH103 *rkp-1* region appears to be involved in the synthesis of a specific lipid carrier for KPS biosynthesis. All individual mutants affected in each gene of the *rkpAGHIJ* operon and in the *rkpU* gene are unable to produce the wild-

Fig. 8. Total salicylic acid (SA) content in soybean roots. Data represent nanograms of total SA per gram of fresh weight (FW) of soybean roots noninoculated (white bars) and inoculated with the wild-type strain (black bars) or the *rkpM* mutant strains of *Sinorhizobium fredii* (gray bars). Results are averages from at least two independent biological experiments with two technical replicates. At least three plants were used for each extraction. Error bars indicate standard error at 95% confidence, and different letters indicate significant differences according to an analysis of variance test ($P < 0.01$). dai = days after inoculation.

type KPS (poly-PseAc) and show the same symbiotic phenotype, which varies according to the plant inoculated (Hidalgo et al. 2010; Margaret-Oliver et al. 2012; Parada et al. 2006). All *rkp-1* mutants showed reduced nodulation and nitrogen-fixation capabilities with soybean but not with *V. unguiculata*, which indicates that determinate nodule-forming legumes can show differences in their symbiotic capacity with *S. fredii* HH103 *rkp-1* mutants. All these *rkp-1* mutants were also able to fix nitrogen with indeterminate nodule-forming legumes such as *C. cajan* or *Glycyrrhiza uralensis*, although the bacterial symbiotic capacity was reduced in comparison with that of the wild-type *S. fredii* HH103 Rif^r (Margaret-Oliver et al. 2012; Parada et al. 2006).

Although *S. fredii* HH103 *rkp-1* or *rkp-3* mutants share the characteristic of being unable to produce KPS, they differed in other traits. Mutants in the *rkp-1* region showed LPS profiles that apparently were indistinguishable from that of the wild-type HH103 Rif^r. The only exception was found in mutants of the *rkpU* gene (which composes a transcriptional unit separated from the *rkpAGHIJ* operon), which produce altered forms of LPS (Hidalgo et al. 2010). In contrast to most of the *rkp-1* mutants, all mutants of the *rkp-3* region (in the *rkpM*, *rkpP*, or *rkpQ* genes) showed clear alterations of their LPS profiles in PAGE experiments (Fig. 4) and only formed ineffective, noninfected pseudonodules in soybean, *V. unguiculata*, and *C. cajan* (Table 4; Figure 7). Thus, *S. fredii* HH103 mutants in the *rkp-1* region are still able to form some nitrogen-fixing nodules on soybean and *C. cajan*, while *rkp-3* mutants only induce the formation of noninfected, ineffective pseudonodules. This difference between the symbiotic capacity of *rkp-1* and *rkp-3* mutants is even clearer in *V. unguiculata*; *rkp-1* mutants appear to be fully effective, while *rkp-3* mutants only form pseudonodules. Since neither *rkp-1* nor *rkp-3* mutants produce the poly-PseAc KPS, the sym-

biotic differences found might be due to the LPS alterations detected in the *rkp-3* mutants. The fact that the SVQ575 mutant (mutated in *rkpU*, KPS⁻, LPS altered) still forms some Fix⁺ nodules on soybean roots could be justified by the following observations. The SVQ575 LPS profile is more similar to that of HH103 Rif^r than to that of *rkp-3* mutants, and it is still recognized by the monoclonal antibody NB6-228.22 (Hidalgo et al. 2010), which recognizes the *S. fredii* HH103 wild-type LPS but fails to recognize the altered LPS produced by the *rkp-3* mutants.

We have not detected differences between *Glycyrrhiza uralensis* responses to inoculation with *rkp-3* mutants and those previously described with *rkp-1* mutants (Margaret-Oliver et al. 2012). In *C. cajan*, however, *S. fredii* HH103 *rkp-1* mutants are still able to form a few nitrogen-fixing nodules (Parada et al. 2006), while the *rkp-3* mutants only induce the formation of pseudonodules and root swellings. Thus, indeterminate nodule-forming legumes (such as *Glycyrrhiza uralensis* and *C. cajan*) also can show different symbiotic responses to the inoculation with *S. fredii* HH103 *rkp-1* and *rkp-3* mutants. In conclusion, *S. fredii* HH103 LPS not only appear to be more relevant than KPS for the bacterial symbiotic capacity with determinate nodule-forming legumes (soybean or cowpea) but also with *C. cajan* (indeterminate nodules).

Although *S. fredii* HH103 appears closely related to *Rhizobium* sp. strain NGR234 (Margaret et al. 2011), their respective *rkp-3* mutants unable to produce PseAc-containing KPS show clear phenotypic differences. An NGR234 mutant carrying a deletion of *rkpM*, *rkpN*, and *rkpO* was still able to form nitrogen-fixing nodules with *V. unguiculata* and its LPS was apparently unaffected (Le Quéré et al. 2006).

A. caviae Sch3N has a small genomic island that is involved in flagellin glycosylation and in the biosynthesis of PseAc and

Fig. 9. A proposal for the pseudaminic acid pathway in *Sinorhizobium fredii* HH103. This proposal is based on the CMP-pseudaminic acid biosynthetic pathway proposed in *Helicobacter pylori* and *Campylobacter jejuni* (Schoenhofen et al. 2006). Pseudaminic enzymes are included to indicate putative functional similarity with Rkp enzymes, although the reaction products vary from compound IV, which is acetylated in *H. pylori* but would be butyrylated in *S. fredii* HH103.

the LPS O-antigen (Tabei et al. 2009). Five genes of the *A. caviae* island (*flmA*, *flmB*, *neuA*, *flmD*, and *neuB*) are orthologous, respectively, to the *S. fredii* HH103 *rkpL*, *rkpM*, *rkpN*, *rkpO*, and *rkpQ* genes. This island appears to have been laterally acquired as it is flanked by insertion element-like sequences and has a much lower G+C content (42%) than the average *A. caviae* C+G content (60%). Similarly, transposon sequences (b1962 and b1963) appear in the vicinity of the *S. fredii* HH103 *rkpQ* gene and the G+C content (54.4%) of the sequenced fragment containing the *rkpL*, *rkpM*, *rkpN*, *rkpO*, *rkpP*, and *rkpQ* genes (JN642175) was lower than that calculated for the entire 2.1-Mbp plasmid (62.3%) or the chromosome (62.6%). Thus, *S. fredii* HH103 could also have acquired the *rkp-3* genes involved in PseAc biosynthesis by lateral transfer.

A. caviae mutants affected in any of these five genes have lost bacterial motility and flagellar expression and their LPS profile is altered. In *A. hydrophila*, however, mutations in the *flmA*, *flmB*, *neuA*, *flmD*, and *neuB* genes affect only motility and flagellar expression but do not cause any alteration in the LPS (Canals et al. 2007; Gryllos et al. 2001). The major modification identified on flagellin from *Campylobacter jejuni* 81-176 and *A. caviae* is the presence of PseAc (Guerry et al. 2006; Tabei et al. 2009). Mutants that produce flagella lacking PseAc failed to autoagglutinate and showed reduced pathogenicity (Guerry et al. 2006; Power and Jennings 2003; Tabei et al. 2009). Thus, PseAc appears to be involved in LPS biosynthesis and flagellar assembly of *A. caviae*. Similarly to the *A. caviae* *flmB* and *neuB* mutants, *S. fredii* HH103 *rkpM*, *rkpP*, and *rkpQ* mutants also show alteration in their LPS profiles, which might indicate that PseAc or a derivative might be involved in *S. fredii* HH103 LPS biosynthesis. In contrast, *S. fredii* *rkpM* and *rkpQ* mutants differ from *A. caviae* *flmB* and *neuB* mutants in those characteristics that could be attributed to changes in flagella, i.e., they are still able to swim (although their motility is reduced) and show enhanced auto-agglutination. Most probably, the reduced motility exhibited by HH103 *rkp3* mutants is not due to a lower growth capacity, since strains SVQ581, SVQ592, and HH103 Rif^r grew equally well in YMB medium (data not shown). Whether *S. fredii* HH103

flagellin carries PseAc substitutions is an interesting question to further investigate the parallelisms between KPS functions in symbiotic and pathogenic bacteria.

MATERIALS AND METHODS

Molecular and microbiological techniques.

The bacterial strains and plasmids used in this work are listed in Table 5. *S. fredii* strains were grown at 28°C on TY medium (Beringer 1974) or YM medium (Vincent 1970). *Escherichia coli* was cultured on Luria-Bertani medium (Sambrook et al. 1989) at 37°C. When required, the media were supplemented with the appropriate antibiotics as described by Vinardell and associates (2004a). Flavonoids were dissolved in ethanol at a concentration of 1 mg ml⁻¹ and used at 1 µg ml⁻¹. Plasmids were transferred from *E. coli* to rhizobia by conjugation as described by Simon (1984).

Motility assays were performed as previously described (Crespo-Rivas et al. 2009). For bacterial auto-agglutination assays, liquid cultures of *S. fredii* strains were incubated at 28°C on an orbital shaker at 180 rpm until they reached the early-stationary phase (about OD₆₆₀ = 1.0). Then, 4.5-ml aliquots of bacterial cultures were transferred to tubes and were incubated at 28°C under static conditions. Bacterial auto-agglutination was estimated by measuring the OD₆₆₀ of the cultures after 1, 4, and 24 h of incubation.

Assays for β-galactosidase activity in liquid bacterial cultures on YMB were carried out 16 h after induction, as previously described (Vinardell et al. 2004a). At least three independent experiments performed in duplicate were carried out.

Recombinant DNA techniques were performed according to the general protocols of Sambrook and associates (1989). For hybridization, DNA was blotted to Amersham HybondTM-N nylon membranes (GE Healthcare, Piscataway, NJ, U.S.A.), and the DigDNA method of Roche (Mannheim, Germany) was employed according to manufacturer's instructions. PCR amplifications were performed as previously described (Vinardell et al. 2004b). All primers pairs used in this work are listed in Table 1.

Table 5. Bacterial strains and plasmids

Strain or plasmid	Derivation and relevant properties ^a	Source or reference
<i>Sinorhizobium fredii</i>		
HH103 Rif ^r (=SVQ269)	Spontaneous Rif ^r derivative of HH103	Madinabeitia et al. 2002
SVQ581	HH103 Rif ^r <i>rkpM</i> ::Ω	This work
SVQ582	HH103 Rif ^r <i>rkpM</i> :: <i>lacZΔp</i> -Gm ^r	This work
SVQ592	HH103 Rif ^r <i>rkpQ</i> ::Ω	This work
SVQ594	HH103 Rif ^r <i>rkpQ</i> :: <i>lacZΔp</i> -Gm ^r	This work
SVQ667	HH103 Rif ^r <i>rkpP</i> ::Ω	This work
<i>Escherichia coli</i>		
DH5α	<i>supE44 ΔlacU169 hsdR17 racA1 endA1 gyr96 thi-1 relA1</i> , Nx ^r	Stratagene
Plasmids		
pAB2001	Ap ^r vector containing the <i>lacZΔp</i> -Gm ^r cassette	Becker et al. 1995
pBluescript II SK+	Cloning and sequencing vector, Ap ^r	Stratagene
pHP45Ω	Ap ^r vector containing the Ω interposon (Spc ^r Str ^r)	Prentki and Krisch 1984
pK18mob	Cloning vector, Km ^r	Schäfer et al. 1994
pMUS664	Cosmid pLAFR1 carrying the <i>rkpLMNOPQ</i> genes of the <i>rkp-3</i> region of <i>S. fredii</i> HH103	This work
pMUS955	pK18mob derivative containing the <i>rkpMN</i> genes of <i>S. fredii</i> HH103	This work
pMUS960	pMUS955 derivative containing HH103 <i>rkpM</i> ::Ω	This work
pMUS964	pMUS955 derivative containing HH103 <i>rkpM</i> :: <i>lacZΔp</i> -Gm ^r	This work
pMUS893	pBluescript II SK+ containing HH103 <i>rkpQ</i>	This work
pMUS953	pMUS893 derivative containing HH103 <i>rkpQ</i> ::Ω	This work
pMUS951	pMUS893 derivative in which two 30 and 600-bp <i>EcoRI</i> - <i>SalI</i> fragments have been eliminated	This work
pMUS966	pMUS951 derivative containing HH103 <i>rkpQ</i> :: <i>lacZΔp</i> -Gm ^r	This work
pMUS972	pK18mob derivative containing HH103 <i>rkpQ</i> :: <i>lacZΔp</i> -Gm ^r	This work
pMUS1032	pBluescript II SK+ containing HH103 <i>rkpP</i>	This work
pMUS1039	pMUS1032 derivative containing HH103 <i>rkpP</i> :: <i>lacZΔp</i> -Gm ^r	This work
pMUS1040	pK18mob derivative containing HH103 <i>rkpP</i> :: <i>lacZΔp</i> -Gm ^r	This work

^a Rif^r, Nx^r, Ap^r, Gm^r, Spc^r, Str^r, and Km^r = resistant to rifampicin, nalidixic acid, ampicillin, gentamycin, spectinomycin, streptomycin, and kanamycin.

Construction of mutants.

In order to generate *rkp* mutants of *S. fredii* HH103 Rif^r, we constructed derivatives of pK18mob (a kanamycin-resistant [Km^r] suicide vector in rhizobia), carrying the *S. fredii* *rkpM*, *rkpP*, or *rkpQ* coding sequences interrupted by the Ω (spectinomycin resistant [Spc^r]) interposon or the *lacZ* Δ p-Gm^r cassette.

To isolate *S. fredii* HH103 Rif^r *rkpM* mutants, a 2.4-kb *Bgl*II fragment of cosmid pMUS664 that contains the 3' end of *rkpL*, the complete *rkpM* and *rkpN* genes and the 5' end of *rkpO* was subcloned into pK18mob, thus generating plasmid pMUS955. Plasmids pMUS960 and pMUS964 were constructed, respectively, by subcloning the Ω (Spc^r) interposon (as a 2-kb *Bam*HI fragment from pMP45 Ω) or the *lacZ* Δ p-Gm^r cassette (as a 4.5-kb *Bam*HI fragment from pAB2001) into the unique *Bam*HI site of *rkpM* cloned in pMUS955.

To isolate *S. fredii* HH103 Rif^r *rkpQ* mutants, a 2.5-kb *Eco*RI fragment of cosmid pMUS664 containing the complete *rkpQ* gene was subcloned into pBluescript KS, generating plasmid pMUS893. Plasmid pMUS953 was constructed by subcloning the Ω (Spc^r) interposon (as a 2-kb *Bam*HI fragment) into the unique *Bam*HI site of *rkpQ* cloned in pMUS893. To insert the *lacZ* Δ p-Gm^r cassette into the *rkpQ* coding region, plasmid pMUS893 was digested with *Eco*RI and *Sal*I (to remove the *Sph*I site of the cloning vector pBluescriptKS) generating a new plasmid (pMUS951) whose unique *Sph*I site is situated in the *rkpQ* coding region. Then, the *lacZ* Δ p-Gm^r cassette (as a 4.3-kb *Sph*I fragment from pAB2001) was subcloned into the unique *Sph*I site of *rkpQ* cloned in pMUS951, generating plasmid pMUS966. The *rkpQ::lacZ* Δ p-Gm^r fusion was subcloned (as a 3.8-kb *Eco*RI-*Sal*I fragment from pMUS966) into pK18mob, generating plasmid pMUS972.

To isolate a *S. fredii* HH103 Rif^r *rkpP* mutant, primers designed from the *rkpO* and *rkpQ* coding sequences (HH*rkpP*-Pext-F and HH*rkpP*-Pext-R) were used to amplify the complete *rkpP* gene. The nucleotide sequence of primers HH*rkpP*-Pext-F and HH*rkpP*-Pext-R was appropriately modified to generate new *Bam*HI restriction sites (Table 1). Using cosmid pMUS664 as template, primers HH*rkpP*-Pext-F and HH*rkpP*-Pext-R led to the PCR amplification of a 1,180-bp *Bam*HI fragment that was subcloned into pBluescriptKS, generating plasmid pMUS1032. Plasmid pMUS1039 was constructed by subcloning the *lacZ* Δ p-Gm^r cassette (as a 4.3-kb *Sph*I fragment) into the unique *Sph*I site of *rkpP* cloned in pMUS1032. Then, plasmid pMUS1040 was constructed by subcloning a 5.5-kb *Bam*HI fragment from pMUS1032 (containing the *rkpP::lacZ* Δ p-Gm^r fusion) into plasmid pK18mob.

The different pK18mob derivatives carrying the *rkpM*, *rkpO*, or *rkpP* genes interrupted by the Ω (Spc^r) interposon or the *lacZ* Δ p-Gm^r cassette were individually transferred to HH103 Rif^r in triparental conjugations, using plasmid pRK2013 as the helper plasmid. Rif^r Spc^r Km^s (for constructions containing the Ω interposon) or Rif^r Gm^r Km^s (for the *lacZ* Δ p-Gm^r cassette) transconjugants were identified in order to obtain putative double recombinants in which the wild-type *rkp* gene has been substituted by the mutated copy of the gene. Thus, mutants SVQ581 (=HH103 Rif^r *rkpM::* Ω), SVQ582 (=HH103 Rif^r *rkpM::lacZ* Δ p-Gm^r), SVQ592 (=HH103 Rif^r *rkpQ::* Ω), SVQ594 (=HH103 Rif^r *rkpM::lacZ* Δ p-Gm^r), and SVQ667 (=HH103 Rif^r *rkpP::lacZ* Δ p-Gm^r) were obtained. In all cases, homogenization of the mutated version of the *rkp* gene was confirmed by PCR and DNA-DNA hybridization. Primers used for the amplification of *rkpM*, *rkpP*, or *rkpQ* coding sequences as well as for mutant derivatives carrying the Ω interposon or the *lacZ* Δ p-Gm^r cassette are listed in Table 1.

PAGE analysis of LPS and KPS.

LPS extraction from bacterial cultures grown on solid TY medium, separation on SDS-PAGE gels, and silver staining were performed as described previously (Buendía-Clavería et al. 2003). Immuno-staining procedures and the monoclonal antibody NB6-228.22 were as described by Buendía-Clavería and associates (2003). KPS were extracted from bacterial cultures grown on solid TY medium, as described by Hidalgo and associates (2010). Samples were analyzed by PAGE as described by Parada and associates (2006), but absolute ethanol was added to the running buffer as well as to the running and stacking gels at a final concentration of 10% (vol/vol) in all cases. The acrylamide concentration of the running gel was 18% (wt/vol), and the acrylamide/N,N'-methylenebisacrylamide ratio was 29:1. Gels were fixed using Alcian Blue (0.5% in 2% acetic acid) and silver staining.

NMR analysis of KPS.

For the detection of KPS by NMR, *S. fredii* SVQ581 and SVQ592 were cultured as follows. TY liquid medium (3 liters) was inoculated with 100 ml of early stationary-phase cultures of bacteria and were incubated on an orbital shaker at 180 rpm for 3 days at 28°C. After incubation, the cells were harvested by slow-speed centrifugation. Bacterial pellets were washed three times with 0.9% (wt/vol) NaCl, were freeze dried, and were stored in sealed bottles at room temperature. Bacterial polysaccharides were extracted from the freeze-dried bacterial cells (5 g) with a 1:1 hot phenol and water mixture (100 ml), and the two phases were separated (Westphal and Jan 1965). The aqueous phase was dialyzed against water, was treated with an ion-exchange resin IRA400 (AcO⁻), and was freeze dried.

Cell extract samples were deuterium-exchanged several times by freeze drying from ²H₂O and were then examined in solution (5 mg of sample in 750 μ l of 99.98% ²H₂O) by ¹H-NMR. Spectra were recorded at 303 K on a Bruker AV500 spectrometer operating at 500.20 MHz. Chemical shifts are given in parts per million (ppm), using the H²O signal (4.71 ppm) as reference.

Plant assays.

Nodulation assays on *Glycine max* (L.) Merr. cv. Williams, *Vigna unguiculata* cv. Bisbee Red (L.) Walp, *Glycyrrhiza uralensis* Fisch, and *Cajanus cajan* (L.) Millsp. were carried out as previously described (Buendía-Clavería et al. 1989; Crespo-Rivas et al. 2007). Germinated seeds were transferred to Leonard jars containing sterilized vermiculite supplemented with Fåhræus nutrient solution (Vincent 1970). Each plant was inoculated with approximately 10⁸ bacteria and were 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 70°C for 48 h and were weighed. Bacterial isolation from surface-sterilized nodules was carried out as previously described by Buendía-Clavería and associates (2003).

Determination of SA content in soybean roots.

Soybean plants were grown as described above and were inoculated with 1 ml of the appropriate *S. fredii* culture grown in TY broth up to an OD₆₀₀ of 0.6 to 0.8 (approximately 10⁸ bacteria). Control plants were inoculated with 1 ml of TY broth. Plant roots were collected at different times after inoculation, were frozen in liquid nitrogen, and were kept at -80°C until their use for SA measurement.

SA measurements were basically performed as previously described (Stacey et al. 2006). For each sample, 0.3 to 0.6 g of the frozen tissue was extracted and quantitated for total SA, free and SA β -glucoside, essentially as described previously

(Bowling et al. 1994; Enyedi et al. 1992). Root methanolic extracts (2 µl from a total volume of 80 µl) were injected into a 3-µm Waters Spherisorb S3 ODS2 column (4.6 × 150 mm). SA was separated isocratically with 30% (vol/vol) methanol containing 1% (vol/vol) acetic acid at a flow rate of 1.1 ml min⁻¹. The temperature of the oven was 35°C. SA was detected with a Varian Prostar fluorescence detector using an excitation wavelength of 313 nm and an emission wavelength of 405 nm.

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