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# The *rkpU* gene of *Sinorhizobium fredii* HH103 is required for bacterial K-antigen polysaccharide production and for efficient nodulation with soybean but not with cowpea

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In this work, the role of the *rkpU* and *rkpJ* genes in the production of the K-antigen polysaccharides (KPS) and in the symbiotic capacity of *Sinorhizobium fredii* HH103, a broad host-range rhizobial strain able to nodulate soybean and many other legumes, was studied. The *rkpJ*- and *rkpU*-encoded products are orthologous to *Escherichia coli* proteins involved in capsule export. *S. fredii* HH103 mutant derivatives were contructed in both genes. To our knowledge, this is the first time that the role of *rkpU* in KPS production has been studied in rhizobia. Both *rkpJ* and *rkpU* mutants were unable to produce KPS. The *rkpU* derivative also showed alterations in its lipopolysaccharide (LPS). Neither KPS production nor *rkpJ* and *rkpU* expression was affected by the presence of the flavonoid genistein. Soybean (*Glycine max*) plants inoculated with the *S. fredii* HH103 *rkpU* and *rkpJ* mutants showed reduced nodulation and clear symptoms of nitrogen starvation. However, neither the *rkpJ* nor the *rkpU* mutants were significantly impaired in their symbiotic interaction with cowpea (*Vigna unguiculata*). Thus, we demonstrate for the first time to our knowledge the involvement of the *rkpU* gene in rhizobial KPS production and also show that the symbiotic relevance of the *S. fredii* HH103 KPS depends on the specific bacterium–legume interaction.

## INTRODUCTION

Rhizobia are soil alphaproteobacteria able to establish nitrogen-fixing symbioses with plants belonging to the family *Leguminoseae*. This symbiotic interaction eventually results in the development of a new plant organ, the root nodule, in which rhizobia differentiate into a special bacterial form able to fix nitrogen, the bacteroid. The establishment of this symbiosis requires a molecular dialogue in which several signals are interchanged by the

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Abbreviations: EPS, exopolysaccharides; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; Kdx, Kdo-derivative; KPS, K-antigen polysaccharides; LPS, lipopolysaccharides.

two partners (Jones *et al.*, 2007; Gibson *et al.*, 2008; Masson-Boivin *et al.*, 2009). Among the different bacterial molecules involved, rhizobial surface polysaccharides appear to play a crucial role acting as signals required for the progression of the interaction and/or preventing host defence mechanisms. At present, exopolysaccharides (EPS), lipopolysaccharides (LPS), K-antigen polysaccharides (KPS) and cyclic $\beta$-glucans are the rhizobial polysaccharides most commonly investigated for their roles in the nodulation process (Fraysse *et al.*, 2003; Becker *et al.*, 2005; Crespo-Rivas *et al.*, 2009; Downie, 2010).

Rhizobial KPSs are acidic polysaccharides which show structural analogy with the group II K-antigens found in *Escherichia coli* (Kannenberg *et al.*, 1998). Rhizobial KPSs were first described by Reuhs *et al.* (1993). To our knowledge, this polysaccharide has been found only in sinorhizobial strains belonging to *Sinorhizobium meliloti* and *Sinorhizobium fredii* species (Becker *et al.*, 2005; Parada *et al.*, 2006). KPS has also been found in *Rhizobium* sp. NGR234 (Reuhs *et al.*, 1998). Most probably, this broad host range strain that does not nodulate soybeans belongs to the *S. fredii* species (Saldaña *et al.*, 2003). Although the KPS structure is strain-specific, the presence of a dimeric repeating unit composed of one hexose and a 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) or a Kdo-derivative (Kdx) is commonly found in *S. meliloti* (Reuhs *et al.*, 1993; Kannenberg *et al.*, 1998). The presence of this sugar–Kdx repeating unit has also been found in *S. fredii* strains that form nitrogen-fixing nodules with Asiatic soybeans but fail to nodulate effectively with American soybean cultivars (Reuhs *et al.*, 1993; Kannenberg *et al.*, 1998). Other *S. fredii* strains, including HH103, that effectively nodulate Asiatic and American soybean cultivars produce a KPS in which the sugar–Kdx structural motif is not present (Reuhs *et al.*, 1998; Gil-Serrano *et al.*, 1999; Rodríguez-Carvajal *et al.*, 2001, 2005). The chemical structure of the *S. fredii* HH103 KPS appears to be unique among the rhizobial strains analysed as it is a homopolysaccharide in which the repeating unit is a derivative of the pseudoaminic acid (Gil-Serrano *et al.*, 1999).

In *S. meliloti*, the KPS can replace EPS for a successful nodulation with *Medicago sativa* (alfafa) (Pellock *et al.*, 2000). Due to this symbiotic equivalence between EPS and KPS, *S. meliloti* AK631 (which produce KPS but not EPS) is able to form nitrogen-fixing nodules with alfalfa. EPS mutants of *S. meliloti* 1021 are symbiotically impaired because the KPS produced by this strain is symbiotically inefficient (Fraysse *et al.*, 2005). In *Rhizobium* sp. NGR234 and *S. fredii* HH103, the absence of KPS leads to a decrease in the bacterial symbiotic capacity with all the host plants tested: *Vigna unguiculata* (cowpea), *Flemingia congesta*, *Leucaena leucocephala* and *Tephrosia vogelii* for the former (Le Quéré *et al.*, 2006) and *Glycine max* (soybean) and *Cajanus cajan* (pigeon-pea) for the latter (Parada *et al.*, 2006).

At present, three gene clusters required for K-antigen production have been identified in *S. meliloti* strains Rm41 and 1021: *rkp-1*, *rkp-2* and *rkp-3* (Kiss *et al.*, 2001; Becker *et al.*, 2005). The *rkp-1* and *rkp-3* regions are involved in KPS transport, whereas the *rkp-2* and *rkp-3* regions participate in the synthesis of the repeating unit of this polysaccharide. Recently, the *rkp-3* region of *Rhizobium* sp. NGR234 has been characterized (Le Quéré *et al.*, 2006). In a previous study, we sequenced the *rkp-1* region from *S. fredii* HH103 (Parada *et al.*, 2006). As in *S. meliloti* 1021, this region is composed of the *rkpAGHIJ* genes whose encoded products are predicted to participate in the synthesis, modification and transfer of a lipophilic molecule that might act as a specific lipid carrier during KPS biosynthesis and also as an anchor to the bacterial external membrane (Kiss *et al.*, 1997; Fraysse *et al.*, 2005). We have also demonstrated that the inactivation of *rkpG* or *rkpH* leads to the inability to produce the wild-type KPS and to a severe decrease in the symbiotic capacity of this strain with soybean and pigeon-pea (Parada *et al.*, 2006). The *rkp-1* region of *S. meliloti* 1021 and *S. fredii* HH103 is preceded by the *rkpU* gene, which codes for a protein related to export of capsular polysaccharides but whose putative involvement in KPS export has not been established. Recently, Pobigaylo *et al.* (2008) reported that an *rkpU* mutant of *S. meliloti* 1021 shows reduced competitiveness for nodulation of alfalfa.

In this study, we have mutated the *rkpU* gene of *S. fredii* HH103 and provided evidence of its involvement in KPS production. The inactivation of *rkpU* not only abolished KPS production but also altered the electrophoretic profile of the bacterial LPS as well as its recognition by the monoclonal antibody NB6-228.22. We have also constructed a *S. fredii* HH103 *rkpJ* mutant and investigated its symbiotic capacity with soybean and cowpea. This gene is also present in the *S. meliloti* Rm41 *rkp-1* region and its inactivation provokes the highest symbiotic impairment among the different *S. meliloti rkp-1* mutants investigated (Kiss *et al.*, 1997). Although *S. fredii rkpU* and *rkpJ* mutants were severely impaired for nodulation with soybean, they were fully effective with *Vigna unguiculata* (cowpea). We also showed that the presence of the *nod* gene inducer genistein has no effect on *rkp* gene expression and KPS production.

## METHODS

**Molecular and microbiological techniques.** The bacterial strains and plasmids used in this work are listed in Table 1. *Sinorhizobium* strains were grown at 28 °C on TY medium (Beringer, 1974) or yeast extract/mannitol (YM) medium (Vincent, 1970). *E. 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 *et al.* (1999). 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 described previously (Crespo-Rivas *et al.*, 2009). To investigate EPS production on solid YM medium, rhizobial strains

**Table 1.** Bacterial strains and plasmids

| Strain or plasmid | Derivation and relevant properties | Source or reference |
|---|---|---|
| ***S. fredii*** | | |
| SVQ269 | HH103-Rif$^R$ | Madinabeitia *et al.* (2002) |
| SVQ528 | SVQ269 *rkpH*::*lacZ*-Gm$^R$ | Parada *et al.* (2006) |
| SVQ530 | SVQ269 *exoA*::*lacZ*-Gm$^R$ | Parada *et al.* (2006) |
| SVQ535 | SVQ269 *rkpH*::*lacZ*-Gm$^R$ *exoA*::Ω | Parada *et al.* (2006) |
| SVQ566 | SVQ269 *rkpJ*::Ω | This work |
| SVQ566C | SVQ566 derivative harbouring a wild-type copy of *rkpJ* (by integration of plasmid pMUS1056) | This work |
| SVQ567 | SVQ269 *rkpJ*::*lacZ*-Gm$^R$ | This work |
| SVQ575 | SVQ269 *rkpU*::Ω | This work |
| SVQ575C | SVQ575 derivative harbouring a wild-type copy of *rkpU* (by integration of plasmid pMUS1048) | This work |
| ***E. coli*** | | |
| DH5α | *supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, Nx$^R$ | Stratagene |
| **Plasmids** | | |
| pAB2001 | Ap$^R$ vector containing the *lacZ*Δp-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 & Krisch (1984) |
| pK18mob | Cloning vector, Km$^R$ | Schäfer *et al.* (1994) |
| pRK2013 | Helper plasmid, Km$^R$ | Figurski & Helinski (1979) |
| pMUS759 | Cosmid pLAFR1 carrying the *rkpGHIJ* genes of the *rkp-1* region of *S. fredii* HH103 | Parada *et al.* (2006) |
| pMUS766 | Cosmid pLAFR1 carrying *rkpU* and the *rkpAGH* genes of the *rkp-1* region of *S. fredii* HH103 | Parada *et al.* (2006) |
| pMUS899 | pK18mob derivative containing HH103 *rkpJ*::Ω | This work |
| pMUS901 | pK18mob derivative containing HH103 *rkpJ*::*lacZ*-Gm$^R$ | This work |
| pMUS936 | pK18mob derivative containing HH103 *rkpU*::Ω | This work |
| pMUS1048 | pK18mob derivative containing HH103 *rkpU* | This work |
| pMUS1056 | pK18mob derivative containing HH103 *rkpJ* | This work |

were grown for 120 h at 28 °C followed by 48 h growth at room temperature.

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

Recombinant DNA techniques were performed according to general protocols (Sambrook *et al.*, 1989). For hybridization, DNA was blotted to Amersham Hybond-N nylon membranes, and the DigDNA method (Roche) was carried out according to the manufacturer's instructions. PCR amplifications were performed as described previously (Vinardell *et al.*, 2004b). All primers pairs used in this work are listed in Table 2.

**Construction of mutants.** In order to generate *rkp* mutants of *S. fredii* HH103, we constructed derivatives of pK18mob, a Km$^R$ suicide vector in rhizobia, carrying the *S. fredii* HH103 *rkpJ* or *rkpU* coding sequences interrupted by the Ω (Spc$^R$) interposon (subcloned as a 2.0 kb *Bam*HI fragment into the unique *Bgl*II site of *rkpJ* or as a 2.0 kb *Sma*I fragment into the unique *Sal*I site, refilled with Klenow, of *rkpU*). The different plasmids thus generated were individually transferred to HH103-Rif$^R$, and Rif$^R$ Spc$^R$ Km$^S$ transconjugants were identified in order to obtain putative double recombinants in which the wild-type *rkp* gene had been substituted by the mutated copy of the gene. Thus, mutants SVQ566 (=HH103-Rif$^R$ *rkpJ*::Ω) and SVQ575 (=HH103-Rif$^R$ *rkpU*::Ω) were obtained. In both cases, homogenotization of the mutated version of the *rkp* gene was confirmed by PCR and DNA–DNA hybridization. Primers used were: for *rkpJ*, *rkpJ*ext-F and *rkpJ*int-R, leading to the amplification of a 519 bp fragment (2.5 kb with the Ω interposon); for *rkpU*, n*rkpU*-F and n*rkpU*-R, leading to the amplification of a 133 bp fragment (2.1 kb with the Ω interposon). Following the same strategy, strain SVQ567, a *rkpJ*::*lacZ*-Gm$^R$ mutant derivative of *S. fredii* HH103, was also obtained. For this purpose, the *lacZ*Δp-Gm$^R$ cassette (as a 4.3 kb *Sma*I fragment) was subcloned into the unique *Bgl*II site (refilled with Klenow) of *rkpJ*. The *rkpJ*::*lacZ*-Gm$^R$ fusion was then cloned into pK18mob, the resulting plasmid was transferred to HH103-Rif$^R$, and Rif$^R$ Gm$^R$ Km$^S$ transconjugants were selected for further characterization.

**Complementation of mutants.** In order to complement the *rkpJ* and *rkpU* mutations *in cis*, DNA fragments containing the complete *rkpU* gene (a 1863 bp *Sma*I–*Xba*I fragment from cosmid pMUS766) or the complete *rkpJ* gene (a 2418 bp *Eco*RI–*Sma*I fragment from cosmid pMUS759) were subcloned in pK18mob, rendering plasmids pMUS1048 and pMUS1056, respectively. These plasmids were transferred to strains SVQ575 and SVQ566, respectively, and Rif$^R$ Spc$^R$ Km$^R$ transconjugants were identified in order to obtain putative single recombinants in which the wild-type *rkp* gene had been reconstituted in the genome next to the mutated copy of the gene. In these candidates, the presence of both the mutated and the wild-type copies of the *rkp* gene was checked by PCR using primers n*rkpU*-F/n*rkpU*-R for *rkpU* and *rkpJ*ext-F/*rkpJ*int-R for *rkpJ*. For these PCRs, the Expand Long Template PCR System (Roche) was employed. Among several candidates, strains SVQ575C and SVQ566C were selected for further study. The relative positions of the wild-type and the mutated copies of *rkpJ* were investigated by using primers n*cisrkpJ*-F/*cisrkpJ*-R.

**Table 2.** Primers used in PCR experiments

| Primer | Sequence (5′–3′) | Position of priming site | Predicted length of PCR product (bp) |
|---|---|---|---|
| *rkpJ*ext-F | agaccttcgcaggacgagga | *rkpI* internal | 519 |
| *rkpJ*int-R | ggcaggaaaatgcgagttgga | *rkpJ* internal | |
| n*cisrkpJ*-F | gccgccgtccatgatctgatc | *rkpI* internal | 1247 |
| *cisrkpJ*-R | gtcttcgtcgtcgagatggg | *rkpJ* internal | |
| n*rkpU*-F | ctgaagcagaaggccgtcga | *rkpU* internal | 133 |
| n*rkpU*-R | cgttgatcgtttcgctgacga | *rkpU* internal | |
| *rkpU*int-F | ccgctcaatctcgtcagcgaa | *rkpU* internal | 362 |
| *rkpU*int-R | acgatgtccggctcctcgta | *rkpU* internal | |
| inter*UpncA*-F | ccgacaatctcgacgatctc | 3′-end of *rkpU* | 301 |
| inter*UpncA*-R | tcgttctgcatgtcgatgac | 5′-end of *pncA* | |
| *qrkpJ*-F | cagctcgagaccgacttc | *rkpJ* internal | 129 |
| *qrkpJ*-R | gtgcaccttgatggcaag | *rkpJ* internal | |
| *qrkpU*-F | agcgtttcaacaccatgc | *rkpU* internal | 139 |
| *qrkpU*-R | atcacgtcgcgattcttg | *rkpU* internal | |
| *q*HH16S-F | gataccctggtagtccac | rRNA 16S internal | 167 |
| *q*HH16S-R | taaaccacatgctccacc | rRNA 16S internal | |

**RT-PCR analysis.** For RT-PCR experiments, *S. fredii* HH103 Rif$^R$ was incubated in YMB in an orbital shaker (180 r.p.m.) 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 Qiagen) following the manufacturer's instructions. Retrotranscription of the RNA was carried out using the Quantitect kit (Qiagen).

Quantitative (real-time) RT-PCRs (qRT-PCRs) were performed in a 20 µl final volume containing 1 µl cDNA, 0.6 pmol each primer and 10 µl FastStart SYBR Green Master Mix (Roche). The PCR was conducted on an iCycler IQ (Bio-Rad). For analysis of *rkpU* and *rkpJ* expression, the primers pairs used were *qrkpU*-F and *qrkpU*-R, and *qrkpJ*-F and *qrkpJ*-R, respectively. To normalize the data, a 167 bp internal fragment of the *S. fredii* HH103 16S rRNA (GenBank accession no. AY260145) was employed as an internal control in each sample by using primers *q*HH16S-F and *q*HH16S-R. Three independent experiments performed in triplicate were carried out.

**PAGE analysis of LPS and KPS.** At least two independent cultures of each strain were used for extraction of LPS and/or KPS, giving reproducible results. LPS extraction from bacterial cultures grown in either liquid or 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 *et al.* (2003). When LPS was extracted from liquid cultures, in order to avoid differences due to the different optical density of the cultures, the volume of crude extracts employed in PAGE experiments were adjusted as follows: 5-fold (for samples extracted from cultures with $OD_{600}$ 0.3), 2.5-fold ($OD_{600}$ 0.6), 1.2-fold ($OD_{600}$ 1.2), 1-fold ($OD_{600}$ 1.3-1.4).

For visualization by PAGE, KPS was extracted from bacterial cultures grown on liquid TY medium for 48 h. Bacterial cells were washed three times in 0.9 % NaCl and pelleted by centrifugation. The bacterial pellet was resuspended and lysed by heating at 100 °C in 500 µl KPS extraction buffer [50 mM Tris/HCl (pH 8.5); 13 mM EDTA; 15 mM $H_3BO_3$] for 6 min. The bacterial crude extract was then treated with RNase (60 µg ml$^{-1}$) and DNase (20 µg ml$^{-1}$) for 5 h at 37 °C, followed by two 24 h treatments with proteinase K (30 µg ml$^{-1}$) at 37 °C. Finally, the resultant mixture was centrifuged at 10 000 ***g*** for 5 min and 400 µl of the supernatant was mixed with 680 µl of 1 M sucrose and 120 µl of absolute ethanol and stored at –20 °C. Samples were analysed by PAGE as described by Parada *et al.* (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 % (v/v) in all cases. The acrylamide concentration of the running gel was 18 % (w/v), and the acrylamide/*N*,*N*′-methylenebisacrylamide ratio was 29 : 1. Gels were fixed using Alcian Blue (0.5 % in 2 % acetic acid) and silver-stained.

**NMR analysis of KPS and cyclic glucans (CGs).** For the detection of KPS and CGs by NMR, *S. fredii* HH103 Rif$^R$, SVQ566, SVQ575, SVQ566C and SVQ575C were cultured as follows: 8 l of TY liquid medium were inoculated with 100 ml early stationary phase cultures of bacteria and incubated on an orbital shaker at 160 r.p.m. 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 % (w/v) NaCl, freeze-dried and stored in sealed bottles at room temperature. Bacterial polysaccharides were extracted from the freeze-dried bacterial cells (5 g) with 1 : 1 hot phenol–water mixture (100 ml) and the two phases were separated (Westphal & Jann, 1965). The aqueous phase was dialysed against water, treated with an ion exchange resin IRA400 ($AcO^-$), freeze-dried and redissolved in 100 ml 50 mM Tris/HCl (pH 7.0) containing 10 mM $MgSO_4$. DNase (1 mg) and RNase (1 mg) were added, and the solution was stirred overnight at 5 °C, dialysed and then freeze-dried.

Cell extract samples were deuterium-exchanged several times by freeze-drying from $^2H_2O$ and then examined in solution (5 mg per 750 µl of 99.98 % $^2H_2O$) by NMR. Spectra were recorded at 303 K on a Bruker AV500 spectrometer operating at 500.13 MHz ($^1H$). Chemical shifts are given in p.p.m., using the $H^2H_2O$ signal (4.71 p.p.m.) ($^1H$) as reference.

**Plant assays.** *S. fredii* strains were tested on *G. max* cv. Williams and on *V. unguiculata* (cowpea) as described by Crespo-Rivas *et al.* (2007). Each Leonard jar contained two plants, and each plant was inoculated with about $5 \times 10^8$ bacteria. Plants were grown for 5–6 (for cowpea) or 7 (for soybean) weeks in a plant growth chamber with a 16 h photoperiod at 25 °C in the light and 18 °C in the dark. Plant tops and nodules were dried at 70 °C for 48 h and weighed. Bacteria were isolated from surface-sterilized nodules as reported previously (Crespo-Rivas *et al.*, 2007). Nodule isolates were tested on appropriately supplemented medium to determine whether they retained the antibiotic-resistance markers of the bacteria used to inoculate the

plants. All plant tests were made at least twice independently. Table 3 shows representative experiments.

Optical microscopy and histochemical staining ($\beta$-galactosidase activity) of nodules were done as described previously (Buendía-Clavería *et al.* 2003; Parada *et al.*, 2006).

## RESULTS

### Computer analyses predict that the *S. fredii* HH103 *rkpJ* and *rkpU* genes could be involved in polysaccharide transport

The *S. fredii* HH103 *rkp-1* region (GenBank accession no. AY882435) is composed of five genes showing the same polarity: *rkpA*, *rkpG*, *rkpH*, *rkpI* and *rkpJ*. These *rkp* genes are flanked by two other genes, *rkpU* (upstream *rkpA*) and *kpsF3* (downstream *rkpJ*), that show opposite polarity to *rkpA–rkpJ* and could also be involved in polysaccharide export (Parada *et al.*, 2006). The same genetic organization is present in *S. meliloti* 1021 (GenBank accession no. AL591688; Müller *et al.*, 2009). The *rkpU* sequence of *S. meliloti* 41 was also determined (GenBank accession no. AF247710) but its exact position remains unknown (Pálvölgyi *et al.*, 2009).

In this work, we studied the *rkpJ* and *rkpU* genes of *S. fredii* HH103. Although both genes have been suggested to participate in capsular polysaccharide transport in *S. meliloti* (Kiss *et al.*, 1997; GenBank accession no. AF247710), this putative involvement in KPS production has only been demonstrated for *rkpJ*.

The NCBI Conserved Domains Database (CDD; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) predicts that *S. fredii* HH103 RkpJ (GenBank accession no. AAW78654, 443 residues) can be assigned to the KpsS superfamily composed of cytoplasmic proteins participating in capsule polysaccharide export (the KpsS domain is located between residues 11 and 401, score $1 \times 10^{-119}$). In the *S. fredii* HH103 RkpU polypeptide (GenBank accession no. AAW78649, 407 residues), the CDD utility determines the presence of a Wza domain between residues 49 and 280 (score $5 \times 10^{-33}$). This domain is characteristic of periplasmic proteins involved in polysaccharide export (reviewed by Collins & Derrick, 2007).

As described in Methods, we constructed *S. fredii* HH103 *rkpJ* and *rkpU* mutant derivatives (see Table 1). Following the same methodology and several approaches, we have tried, unsuccessfully, to obtain *kpsF3* mutant derivatives of HH103. These results suggest a possible lack of viability of *kpsF3* derivatives in this strain. In fact, preliminary analysis of a draft of the *S. fredii* HH103 genome suggests the presence of a single copy of the *kpsF* gene in this strain (our unpublished results), which is in contrast with the presence of three copies in the *S. meliloti* 1021 genome (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi). The KpsF3 protein, an

**Table 3.** Plant responses to inoculation of *G. max* cv. Williams and *V. unguiculata* with *S. fredii* strains HH103-Rif$^R$ and their *rkp* and/or *exoA* derivatives

Data represent averages of five jars. Each jar contained two soybean or cowpea plants. Determinations were made 7, 6 and 5 weeks after inoculation for *G. max* cv. Williams, *V. unguiculata* assay 1 and *V. unguiculata* assay 2, respectively. For each legume, each mutant was compared pair-wise with the parental strain HH103-Rif$^R$ by using the Mann–Whitney non-parametric test.

| Legume tested | Inoculant* | Plant-top dry weight (g) | No. of nodules | Nodule fresh weight (mg) |
|---|---|---|---|---|
| *G. max* cv. Williams | HH103 Rif$^R$ | 5.183 ± 0.809 | 173.3 ± 33.0 | 1907 ± 269 |
| | SVQ566 (*rkpJ*) | 1.729 ± 0.528† | 23.3 ± 14.9† | 484 ± 331† |
| | SVQ575 (*rkpU*) | 1.363 ± 0.623† | 15.3 ± 8.0† | 309 ± 247† |
| | SVQ566C | 5.232 ± 1.409 | 195.3 ± 35.0 | 2349 ± 372 |
| | SVQ566 (pMUS759) | 1.400 ± 0.338† | 8.8 ± 4.9† | 192 ± 113† |
| | SVQ575C | 2.882 ± 0.102†‡ | 86.8 ± 25.5†‡ | 1050 ± 355†‡ |
| | SVQ575 (pMUS766) | 3.580 ± 0.407†‡ | 120.3 ± 23.7†‡ | 1781 ± 544†‡ |
| | None | 0.973 ± 0.196† | – | – |
| *V. unguiculata* (assay 1) | HH103 Rif$^R$ | 4.032 ± 1.558 | 146.0 ± 21.6 | 2070 ± 668 |
| | SVQ566 (*rkpJ*) | 5.722 ± 1.961 | 121.8 ± 24.7 | 2752 ± 662 |
| | SVQ575 (*rkpU*) | 5.166 ± 0.951 | 105.6 ± 20.4 | 2859 ± 335 |
| | None | 0.387 ± 0.103† | – | – |
| *V. unguiculata* (assay 2) | HH103 Rif$^R$ | 4.182 ± 1.593 | 95.4 ± 34.1 | 1975 ± 818 |
| | SVQ528 (*rkpH*) | 3.156 ± 0.290 | 60.0 ± 10.1 | 1359 ± 198 |
| | SVQ530 (*exoA*) | 3.350 ± 0.549 | 76.0 ± 17.4 | 1713 ± 233 |
| | SVQ535 (*rkpH exoA*) | 3.394 ± 0.713 | 60.0 ± 5.5 | 1364 ± 298 |
| | None | 0.490 ± 0.175† | – | – |

*Bacteria isolated from 20 nodules formed by each inoculant showed the expected resistance markers.
†Numbers on the same column are significantly different from those of HH103-Rif$^R$ at the level $\alpha = 5\%$.
‡For strains SVQ575C and SVQ575 (pMUS766), the value is significantly different from that of SVQ575 at the level $\alpha = 5\%$.

arabinose-5-phosphate isomerase, has been also suggested to participate in capsular polysaccharide synthesis due to its identity level (39 %) with the KpsF protein from *E. coli*.

## The *rkpJ* and *rkpU* genes of *S. fredii* HH103 are required for KPS production

Mutant strains SVQ566 and SVQ575, which are affected in the *rkpJ* and *rkpU* genes, respectively, were investigated for the production of KPS by PAGE experiments performed in the absence of SDS (for the visualization of KPS without interferences of LPS). Alcian Blue silver-stained polyacrylamide gels of crude cell extracts showed the presence of two silver-stained materials in samples of HH103 Rif$^R$ (Fig. 1, lanes 1, 4 and 8), presumably corresponding to the high and low molecular weight forms of KPS. None of these silver-stained materials were present in the samples of the HH103 *rkpJ* (strain SVQ566, lane 6) or *rkpU* (strain SVQ575, lane 2) mutant derivatives. In fact, the distribution of the silver-stained material of SVQ566 and SVQ575 is very similar to that of SVQ528 (lane 9), an *S. fredii* HH103 *rkpH* mutant that does not produce KPS (Parada *et al.*, 2006).

The absence of KPS in mutants SVQ566 (*rkpJ*) and SVQ575 (*rkpU*) was confirmed by $^1$H-NMR analyses. Bacterial polysaccharides of HH103 Rif$^R$, SVQ566 and SVQ575 were isolated by the hot phenol–water method and further purified as described in Methods. The KPS produced by the parental strain (Gil-Serrano *et al.*, 1999) (Fig. 2a) was not detected by $^1$H-NMR analyses in any of the mutants analysed (Fig. 2b and c), revealing a role for both *rkpJ* and *rkpU* in the production of KPS in *S. fredii*. Fig. 2 also shows that CGs produced by mutants SVQ566 and SVQ575 appear to be identical to those produced by the wild-type strain, since the $^1$H-NMR spectra corresponding to the CGs produced by the mutants (Fig. 2b, c) were identical to those of *S. fredii* HH103 Rif$^R$ (Fig. 2a) (Crespo-Rivas *et al.*, 2009).

In order to confirm the role of *rkpU* and *rkpJ* in KPS production by *S. fredii* HH103, we carried out complementation studies. For this purpose, two strategies were followed: (i) the introduction of a cosmid carrying a part of the *rkp-1* region which includes either *rkpU* (cosmid pMUS766) or *rkpJ* (cosmid pMUS759); thus, strains SVQ575 (pMUS766) and SVQ566 (pMUS759) were obtained; (ii) the integration of a wild-type copy of *rkpU* or *rkpJ* in the genome of SVQ575 and SVQ566, respectively, leading to the construction of the complemented strains SVQ575C and SVQ566C.

PAGE experiments showed that both strains SVQ575 (pMUS766) (Fig. 1, lane 5) and SVQ575C (lane 3) regained the capacity to produce KPS, although the average degree of polymerization of the KPS of the latter seems to be higher than that observed in both SVQ575 (pMUS766) and the wild-type strain. NMR experiments confirmed the production of the wild-type KPS by SVQ575C (Fig. 2e). In the case of mutant SVQ566, the presence of cosmid pMUS759 (which contains the *rkpGHIJ* genes) did not restore the ability to produce KPS (Fig. 1, lane 7). In contrast, SVQ566C regained the capacity to produce the wild-type KPS, as demonstrated by both PAGE (Fig. 1, lane 10) and NMR analyses (Fig. 2d).

## The *S. fredii* HH103 *rkpU* mutant showed an altered electrophoretic LPS pattern

The LPSs of mutants SVQ566 (*rkpJ*) and SVQ575 (*rkpU*) were also analysed (Fig. 3) by PAGE experiments performed in the presence of SDS as well as by immunostaining experiments using a monoclonal antibody (NB6-228.22) that recognizes the HH103 Rif$^R$ LPS (Buendía-Clavería *et al.*, 2003).

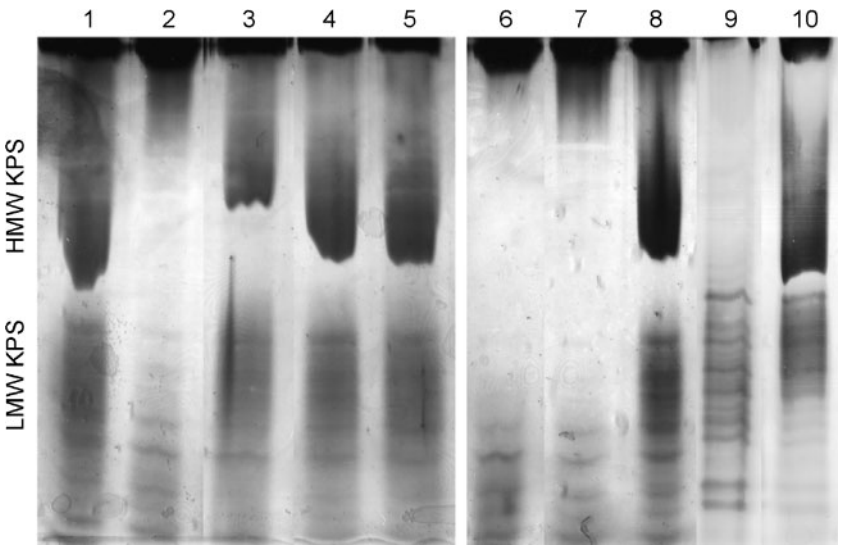


**Fig. 1.** PAGE analysis of KPS production by *S. fredii* HH103 Rif$^R$ and different *rkp* derivatives. Samples were run in the absence of detergent (SDS), treated with Alcian Blue and silver-stained. Lanes 1, 4 and 8, HH103 Rif$^R$; lane 2, SVQ575 (*rkpU*::Ω); lane 3, SVQ575C; lane 5, SVQ575 (pMUS766); lane 6, SVQ566 (*rkpJ*::Ω); lane 7, SVQ566 (pMUS759); lane 9, SVQ528 (*rkpH*::*lacZ*-Gm$^R$); lane 10, SVQ566C.

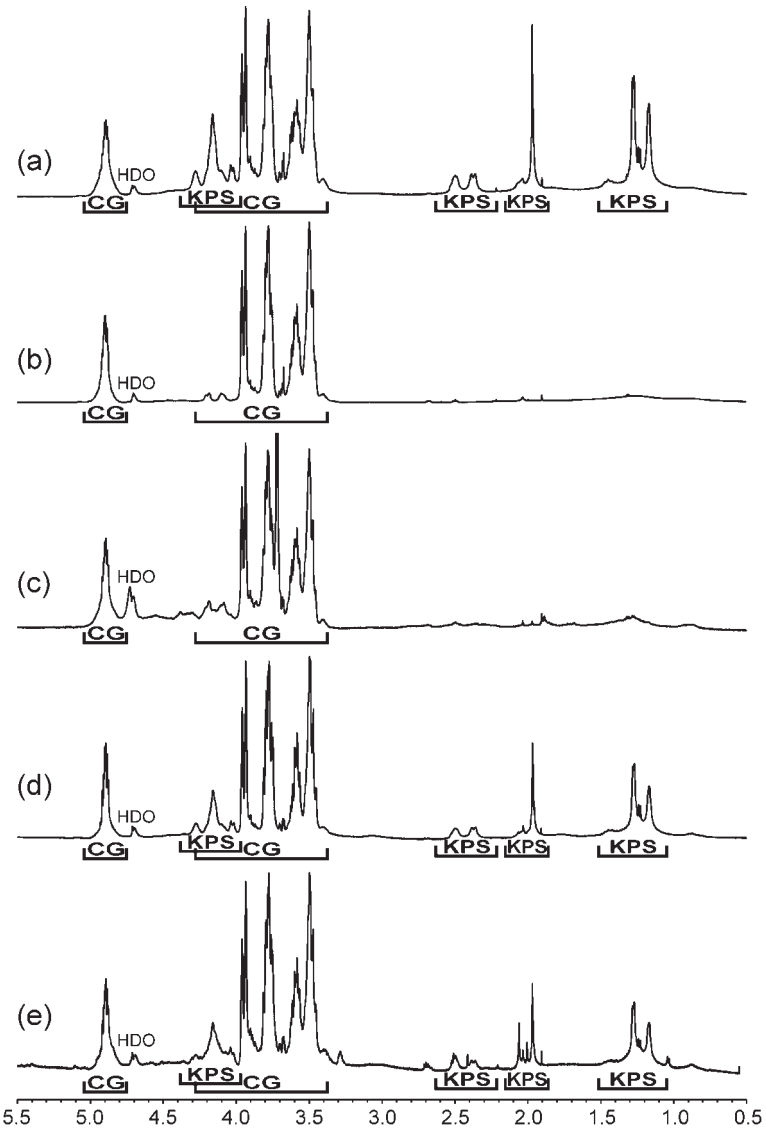


**Fig. 2.** NMR analysis of KPS production by *S. fredii* strains. $^{1}$H NMR spectra (500 MHz) of crude cell extracts containing surface polysaccharides isolated from (a) *S. fredii* HH103 Rif$^{R}$, (b) SVQ566 (*rkpJ*::Ω), (c) SVQ575 (*rkpU*::Ω), (d) SVQ566C and (e) SVQ575C. Signals corresponding to KPS or CG are indicated.

The LPS electrophoretic (Fig. 3a, lane 2 vs lane 1) and immuno-staining (Fig. 3b, lane 2 vs lane 1) profiles of SVQ566 (affected in *rkpJ*) did not show any differences with respect to that of strain HH103. The same situation was found for mutant SVQ575 (*rkpU*) when LPS was extracted from 3-day-old solid cultures (Fig. 3c and d, lanes 3 and 4). However, when LPS was extracted from 5-day-old solid cultures, the HH103 *rkpU* derivative SVQ575 produced an LPS showing an altered electrophoretic profile with regard to that of HH103 (Fig. 3c and e, lane 2 vs lane 1). These alterations, which were not detected in SVQ566 samples obtained from 5-day-old solid cultures (data not shown), affected both the smooth (slow-migrating bands) and the rough (fast-migrating bands) LPS. The introduction of a wild-type copy of the *rkpU* gene, either *in cis* (strain SVQ575C, Fig. 3e, lane 3) or *in trans* (strain SVQ575pMUS766, Fig. 3e, lane 4), restored the wild-type LPS electrophoretic pattern. Curiously, no differences were detected between the immuno-staining profiles exhibited by the LPS of SVQ575 and HH103 (Fig. 3f), indicating that the new LPS bands produced by SVQ575, but not by HH103, are not recognized by the monoclonal antibody NB6-228.22. These results indicate that the inactivation of the *S. fredii* HH103 *rkpU* gene has an effect on not only KPS but also LPS production.

LPS production by mutant SVQ575 was also investigated at different stages of growth in liquid medium. Thus, we extracted LPS from SVQ575 and HH103 Rif$^{R}$ cultures with $OD_{600}$ values of 0.3, 0.6 and 1.2 (early, medium and late exponential phase, respectively), as well as of cultures that reached the transition between log and stationary phases ($OD_{600}$ of 1.3–1.4). Although differences were found between the LPS electrophoretic patterns from SVQ575 and HH103 in all the growth curve points analysed, the highest differences were found when the incubation time was extended to the transition between log and stationary phases (Fig. 4a). In agreement with our previous observa-

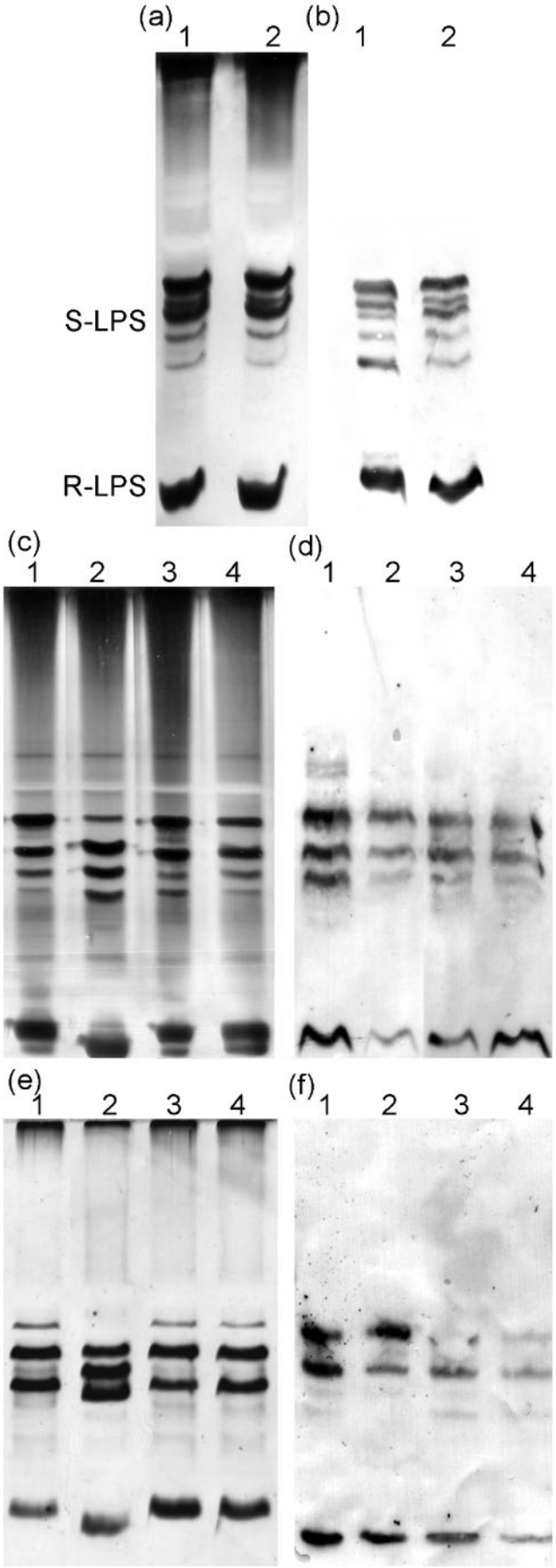


**Fig. 3.** SDS-PAGE and silver-staining (a, c, e) or immuno-staining using the monoclonal antibody NB6-228.22 (b, d, f) of LPS crude extracts from *S. fredii* HH103-Rif$^R$ and its *rkpJ*::Ω (SVQ566) and *rkpU*::Ω (SVQ575) mutant derivatives grown in solid TY medium. The rough and smooth forms of LPS are indicated as R-LPS and L-LPS, respectively. (a, b) Lane 1, HH103 Rif$^R$; lane 2, SVQ566. (c, d) Lanes 1 and 3, HH103 Rif$^R$; lanes 2 and 4, SVQ575. LPS was extracted from 5- (lanes 1 and 2) or 3- (lanes 3 and 4) day-old cultures. (e, f) Lane 1, HH103 Rif$^R$; lane 2, SVQ575; lane 3, SVQ575C; lane 4, SVQ575 (pMUS766). LPS was extracted from 5-day-old cultures.

tions, those LPS bands that were present in SVQ575 but not in HH103 Rif$^R$ were not detected by immuno-staining experiments (Fig. 4b).

Sequence analysis of cosmid pMUS766 revealed that the *S. fredii* HH103 *rkpU* gene is followed (231 bp downstream) by *pncA* and *pncB* (GenBank accession no. AY882435). These genes, which show the same polarity as *rkpU*, encode, respectively, a nicotinamidase and a nicotinate phosphoribosyltransferase, two enzymes involved in the NAD salvage synthesis pathway, which serves to recycle NAD degradation products. These two genes are also present in cosmid pMUS766. In order to analyse the possibility that the *rkpU* mutation could have a polar effect on the transcription of the *pncAB* genes, we carried out RT-PCR experiments. Two different sets of primers were used: *rkpU*int-F and *rkpU*int-R, which allow the amplification of a 362 bp internal fragment of the *rkpU* gene, and inter*UpncA*-F and inter*UpncA*-R, which lead to the amplification of a 301 bp DNA fragment that includes the 3′ end of *rkpU* and the first 41 nt of *pncA*. Using both primer pairs, a DNA fragment of the expected size could be amplified when HH103 gDNA was used as template. However, when HH103 cDNA was employed as template, only the internal fragment of the *rkpU* gene was amplified (data not shown). This result, which was confirmed by qRT-PCR experiments (data not shown), indicated that *rkpU* and *pncA* are not cotranscribed. Therefore, the KPS and LPS defects observed in SVQ575 can be assigned to the inactivation of the *rkpU* gene.

Mutants SVQ566 and SVQ575 were also examined for the production of EPS. On solid YMA, no obvious differences between the mucus of these mutants and that of HH103 Rif$^R$ were detected (data not shown). We have also investigated one characteristic that can be influenced by alterations in bacterial surface polysaccharides: motility in semi-solid media (Crespo-Rivas *et al.*, 2009). Motility of mutants SVQ566 and SVQ575 was analysed in either YMA or TY media. No differences were found with respect to that of the parental strain HH103 Rif$^R$.

## Expression of *rkpU* and *rkpJ* was not affected by the presence of the flavonoid genistein

Previous studies performed on *S. fredii* USDA205 and *Rhizobium* sp. NGR234 have shown that the presence of

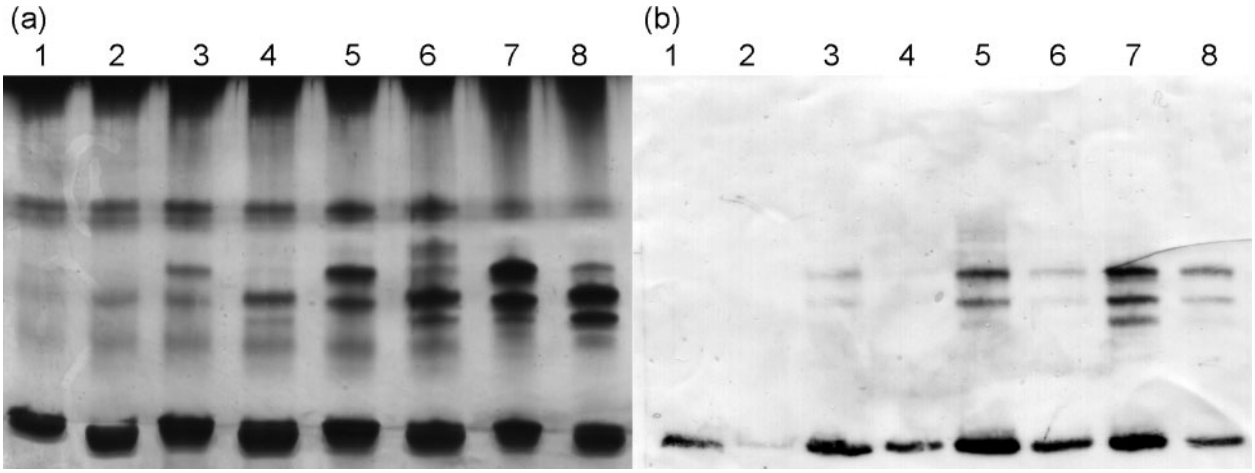


**Fig. 4.** SDS-PAGE and silver-staining (a) or immuno-staining using the monoclonal antibody NB6-228.22 (b) of LPS crude extracts from liquid cultures of *S. fredii* HH103-Rif$^R$ and its *rkpU* (SVQ575) mutant derivative. Lanes 1, 3, 5, 7, HH103 Rif$^R$; lanes 2, 4, 6, 8, SVQ575. The $OD_{600}$ of the cultures was approximately 0.3 (lanes 1 and 2), 0.6 (lanes 3 and 4), 1.2 (lanes 5 and 6) and 1.3–1.4 (lanes 7 and 8). Similar amounts of cell material were loaded in all lanes.

flavonoids influences KPS production (Reuhs *et al.*, 1994; Simsek *et al.*, 2009). Therefore, we investigated whether a similar situation could exist in *S. fredii* HH103. For this purpose, we carried out two experiments. First, we studied the expression of the *rkpJ* and *rkpU* genes on HH103 Rif$^R$ grown in either the absence or the presence of genistein, an effective *nod* gene inducer of this strain (Vinardell *et al.*, 2004a). qRT-PCR experiments showed that in the presence of genistein, the expression of both *rkpJ* ($1.20 \pm 0.58$) and *rkpU* ($0.73 \pm 0.30$) was similar to that observed in the absence of flavonoids. The expression of *rkpJ* was also analysed by performing $\beta$-galactosidase assays with strain SVQ567, an *rkpJ*::*lacZ*-Gm$^R$ derivative of *S. fredii* HH103 Rif$^R$. The levels of $\beta$-galactosidase activity shown by SVQ567 grown in the absence and presence of genistein were $76.6 \pm 4.2$ and $73.8 \pm 2.0$, respectively.

In addition, the KPS produced by *S. fredii* HH103 Rif$^R$ grown in the absence and presence of genistein was analysed by PAGE experiments. As controls, the KPS of HH103 grown in the presence of the ineffective *nod* inducer flavone (Vinardell *et al.*, 2004a) or in the presence of 0.1% (v/v) ethanol (flavonoids are dissolved in ethanol before being added to the cultures) were also analysed. No relevant differences were observed between the different treatments (data not shown), suggesting that the presence of genistein did not have a significant impact on HH103 KPS production.

## Inactivation of *rkp* genes severely impaired the symbiotic relationship of *S. fredii* HH103 with soybean but not with cowpea

The symbiotic phenotype of *S. fredii* HH103 *rkpU* and *rkpJ* mutant derivatives was investigated with two host plants, *G. max* cv. Williams (soybean) and *V. unguiculata* cv. Bisbee Red (cowpea), which form determinate nodules. Three different parameters were analysed: plant-top dry-weight, nodule number and fresh weight of nodules (Table 3).

Mutants SVQ566 (*rkpJ*) and SVQ575 (*rkpU*) presented a clear reduction in their capacity to nodulate soybean Williams effectively, as they showed significant statistical differences in the three parameters analysed when compared with the parental strain HH103 Rif$^R$ (Table 3, Fig. 5a). Although they were still able to induce the formation of a few nitrogen-fixing nodules, soybean roots inoculated with either SVQ566 or SVQ575 showed numerous pseudonodules (Fig. 5c and d, marked with white arrows) that were absent in roots inoculated with HH103 (Fig. 5b). The few nitrogen-fixing nodules induced by both SVQ566 and SVQ575 strains were similar in size and aspect to those produced by soybean plants inoculated with HH103 Rif$^R$, contained symbiotic cells fully invaded by bacteroids (as revealed by optical microscopy analysis; Fig. 5i–k) and fixed nitrogen as demonstrated by an acetylene reduction assay (data not shown). In the case of SVQ575, the symbiotic defect could be partially complemented by the introduction of a wild-type copy of the *rkpU* gene either *in cis* (strain SVQ575C; Fig. 5a and g) or *in trans* (presence of cosmid pMUS766; Fig. 5a and h): in both cases, the three parameters analysed were significantly higher than those of mutant SVQ575 but also significantly lower than those of HH103 Rif$^R$. Soybean plants inoculated with strain SVQ575C, but not those inoculated with SVQ575 (pMUS766), still formed some pseudonodules. The symbiotic defect of mutant SVQ566 could be fully complemented by the introduction of a wild-type copy of *rkpJ in cis* (Fig. 5a and e), but the presence of cosmid pMUS759 (Fig. 5a and f) did not restore the ability of this mutant to efficiently nodulate soybean.

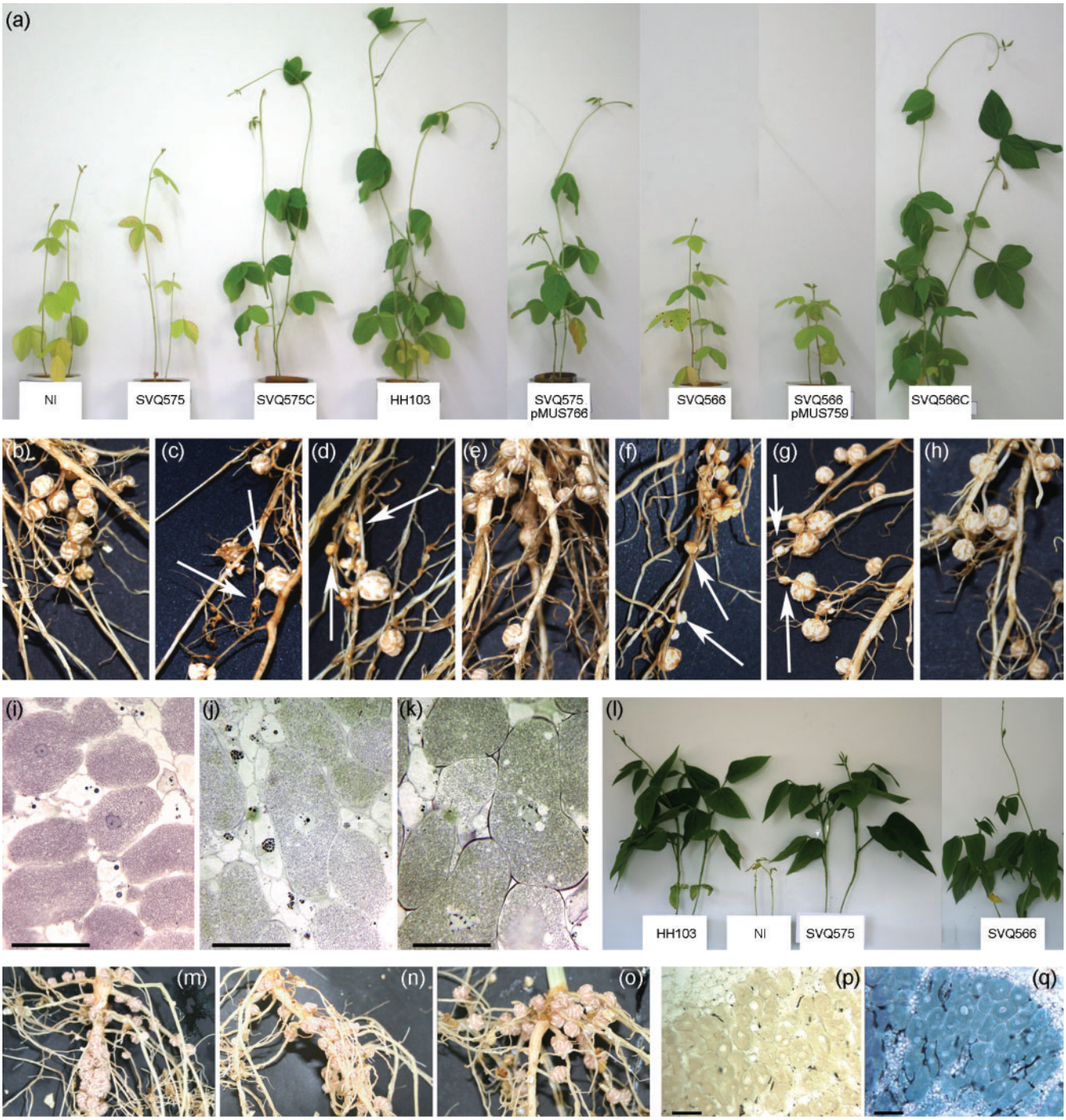


**Fig. 5.** Plant responses to inoculation of *G. max* cv. Williams (a–k) and *V. unguiculata* (l–q) with *S. fredii* strain HH103-Rif$^R$ and the *rkp* mutant derivatives. (a) Aerial parts of soybean plants inoculated with (from left to right): non-inoculated (NI), SVQ575, SVQ575C, HH103Rif$^R$, SVQ575 (pMUS766), SVQ566, SVQ566 (pMUS759) and SVQ566C. (b–k) Macroscopic (b–h) and microscopic (i–k) aspect of soybean Williams nodules induced by *S. fredii* HH103 Rif$^R$ and different *rkp* derivatives. (b) and (i) HH103-Rif$^R$; (c and j) SVQ566 (*rkpJ*::Ω); (d and k) SVQ575 (*rkpU*::Ω); (e) SVQ566C; (f) SVQ566 (pMUS759); (g) SVQ575C; and (h) SVQ575 (pMUS766). Some pseudonodules are marked with white arrows. (l) Aerial parts of cowpea plants inoculated with (from left to right): HH103Rif$^R$, non-inoculated (NI), SVQ575, SVQ566. (m–o) Macroscopic aspect of cowpea nodules induced by HH103 Rif$^R$ (m), SVQ566 (n) and SVQ575 (o). Histochemical staining of cowpea nodules infected with HH103 Rif$^R$ (p) and SVQ567 (q). Bar, 50 μm, (i–k); 100 μm (p, q).

In contrast with soybean Williams, the number and dry-weight of nodules as well as plant-top dry-weight of *V. unguiculata* plants inoculated with the *S. fredii* HH103 *rkpU* or *rkpJ* mutant derivatives were not significantly different ($\alpha$=5 %) from those following inoculation with the parental strain (Table 3, Fig. 5l–o). Strain SVQ567, a *rkpJ*::*lacZ*-Gm$^R$ derivative of HH103 Rif$^R$, was also investigated for its nodulation capacity with cowpea, showing the same phenotype as the *rkpJ*::Ω mutant SVQ566 (data not shown). Histochemical staining revealed that cowpea nodules induced by SVQ567 showed $\beta$-galactosidase activity (Fig. 5q). Nodules occupied by the parental strain HH103-Rif$^R$ did not show this enzymic activity (Fig. 5p).

The fact that mutations in the *rkpJ* and *rkpU* genes did not decrease the symbiotic capacity of *S. fredii* HH103 Rif$^R$ with cowpea plants prompted us to investigate whether other *S. fredii* HH103 Rif$^R$ mutants affected in the *rkp-1* region also showed differences in their symbiotic capacity with soybean and cowpea. For this purpose, cowpea plants were inoculated with mutant SVQ528, an *S. fredii* HH103 Rif$^R$ *rkpH* derivative whose symbiotic capacity with soybean has been reported to be impaired (Parada *et al.*, 2006). In these studies, we also included an *S. fredii* HH103 Rif$^R$ *exoA* mutant (SVQ530, unable to produce EPS) as well as an *rkpH*::*lacZ*-Gm$^R$ *exoA*::Ω double mutant, called SVQ535. Results presented in Table 3 (*V. unguiculata*, assay 2) show that the symbiotic capacity of mutants SVQ528 (*rkpH*), SVQ530 (*exoA*) and SVQ535 (*rkpH exoA*) with *V. unguiculata* plants was not significantly different from that of their parental strain HH103 Rif$^R$.

## DISCUSSION

In this work, we have demonstrated the participation of the *rkpJ* and *rkpU* genes in the production of KPS of *S. fredii* HH103. The *rkpJ* gene had been previously characterized in *S. meliloti* Rm41 (Kiss *et al.*, 1997); however, this is the first time that the involvement of the *rkpU* gene in KPS production has been investigated in rhizobia.

Both RkpJ and RkpU are absolutely required for KPS production in *S. fredii* HH103 as determined by both PAGE experiments and $^1$H-NMR analyses. These results are in agreement with those previously obtained with *rkpG* and *rkpH* mutant derivatives of *S. fredii* HH103 (Parada *et al.*, 2006) but different from those reported for different *rkp-1* mutants of *S. meliloti*, as the latter retain the capacity to produce KPS (Kiss *et al.*, 1997). The rhizobial RkpJ protein is orthologous to the *E. coli* KpsS protein, which is involved in the ABC transport of the capsule polysaccharide across the bacterial inner membrane. The *E. coli* KpsF protein is also involved in this process, which, in sinorhizobia, could be encoded by the *kpsF3* gene (located downstream of the *rkp-1* region).

Computer analysis predicted that RkpU belongs to the Wza protein family, which is involved in polysaccharide export (Collins & Derrick, 2007). For instance, a Wza protein carries out the translocation across the periplasm and the outer membrane in the export of the *E. coli* type 1 capsule (Whitfield, 2006). The fact that RkpU was absolutely required for *S. fredii* HH103 KPS production and that in *S. fredii* and *S. meliloti* the *rkpU* gene was linked to *rkp* genes involved in KPS export suggests that RkpU may participate in the translocation of rhizobial KPS from the periplasm to the outer membrane.

The inactivation of the HH103 *rkpJ* gene (mutant SVQ566) apparently did not affect the production of other surface polysaccharides such as LPS, EPS and CG, in accordance with previous reports for HH103 *rkp-1* mutants in the *rkpG* and *rkpH* genes (Parada *et al.*, 2006). However, mutation of *rkpU* (mutant SVQ575) affected the production of LPS by HH103 when bacterial growth in solid medium reached the stationary phase. Curiously, when bacteria grew in liquid medium, differences between the LPS patterns exhibited by HH103 and its *rkpU* derivative could be observed in the different growth curve points analysed (Fig. 4). Nevertheless, the alterations exhibited by the LPS of SVQ575 increased as the age of the culture did. Because the chemical structure of the *S. fredii* HH103 LPS is not known, it is difficult to predict which kind of changes have occurred in the LPS structure of mutant SVQ575 and why these changes are enhanced in 'aged cultures'. However, the alterations observed in the LPS fast-migrating bands of SVQ575 (the rough LPS) strongly suggest that the LPS core might be altered.

The presence of genistein, in contrast with reports for *S. fredii* USDA205 and *Rhizobium* sp. NGR234 (Reuhs *et al.*, 1994; Simsek *et al.*, 2009), did not affect KPS production by *S. fredii* HH103. In accordance with this observation, the expression of the *rkpJ* and *rkpU* genes was not altered by the presence of this flavonoid.

The symbiotic phenotype of rhizobial KPS mutants was first analysed in *S. meliloti*, in which a functional equivalence between KPS and EPS was found (Pellock *et al.*, 2000). This equivalence was not observed in *S. fredii* HH103 since mutants in *rkpG* or *rkpH* are symbiotically impaired in the two host plants analysed (soybean and pigeon-pea) regardless of whether EPS is produced or not (Parada *et al.*, 2006). In *Rhizobium* sp. NGR234, the deletion of several genes belonging to the *rkp-3* region led to the absence of KPS and to a general decrease in the symbiotic capacity with all the host legumes tested (Le Quéré *et al.*, 2006). In the present work, we have found that two new HH103 mutants lacking KPS, the *rkpJ* and *rkpU* derivatives, are also severely impaired for nodulation of soybean. However, they were still able to induce the formation of a small number of nitrogen-fixing nodules that seem to be properly infected. Thus, KPS seems to play an important (but not essential) role in the infection of soybean roots, as the absence of this surface polysaccharide diminished the number of successful infections.

Surprisingly, the HH103 *rkpH*, *rkpJ* and *rkpU* derivatives were not affected in their symbiotic interaction with

cowpea, indicating that KPS does not play an important role in the HH103 interaction with this legume. In this assay, we also determined that the absence of EPS and/or KPS did not significantly affect the nodulation ability of *S. fredii* HH103 with cowpea. It is remarkable that, in contrast with our observations, the *Rhizobium* sp. NGR234 KPS mutant was more severely impaired for cowpea nodulation than other legumes tested (Le Quéré *et al.*, 2006). Histochemical staining of cowpea nodules induced by SVQ567 (a *rkpJ*::*lacZ*-Gm$^R$ derivative of HH103 Rif$^R$) revealed that the *rkpJ* gene is transcribed in bacteroids. A similar result was reported for the *S. fredii* HH103 *rkpG* and *rkpH* genes in soybean Williams nodules (Parada *et al.*, 2006).

The different effects (on symbiosis and on surface polysaccharide production) of both *rkpJ* and *rkpU* mutations could be (at least partially) complemented by the introduction *in cis* of a wild-type copy of the corresponding gene (strains SVQ566C and SVQ575C, respectively). In the case of the *rkpU* derivative, the introduction of cosmid pMUS766 (which harbours the HH103 *rkpU* gene), in addition to restoring wild-type KPS and LPS production, provoked a higher recovery of the symbiotic capacity with soybean than the introduction *in cis* of a single copy of the *rkpU* gene. One possible explanation of this fact is the higher degree of polymerization exhibited by the KPS produced by SVQ575C with respect to those of strains HH103 Rif$^R$ and SVQ575 (pMUS766). On the other hand, the introduction of cosmid pMUS759 (which contains the 3′ part of *rkpA* and the *rkpGHIJ* genes) into SVQ566 did not restore KPS production and nodulation ability with soybean. Although we have not investigated the reasons for this complementation failure, one possible explanation might be the absence of the promoter of the entire operon in cosmid pMUS759. In strain SVQ566C, the mutated copy of *rkpJ* was located between *rkpI* and the wild-type copy of *rkpJ* (data not shown). In another candidate, in which the mutated copy of *rkpJ* was placed downstream of the wild-type copy of *rkpJ*, KPS production was also restored (as assessed by PAGE experiments, data not shown). The fact that the restoration of KPS production was independent of the site of integration of the wild-type copy of *rkpJ* suggests that in strain SVQ566C, *rkpJ* could be transcribed from a promoter located in the vector employed for the integration (pK18mob).

It has been generally accepted that the relative importance of each surface polysaccharide in nodule invasion and development is influenced by the nodule type formed by the host plant (Fraysse *et al.*, 2003). Both *G. max* (soybean) and *V. unguiculata* (cowpea) are determinate-nodule-forming legumes. Most of soybean-nodulating rhizobia are also able to nodulate cowpea. However, at least in the case of *S. fredii* HH103, these two legumes seem to have different requirements in order to allow bacterial infection. Thus, the *S. fredii* HH103 *rkp* mutants analysed so far are impaired for nodulation of soybean but not of cowpea. This was also the case for an HH103 mutant affected in the symbiotic regulator *nolR* (Vinardell *et al.*, 2004b). On the other hand, an HH103 *ndvB* mutant, unable to produce CGs, was still able to induce pseudonodules in soybean but did not provoke any macroscopic response in *V. unguiculata* roots (Crespo-Rivas *et al.*, 2009). The *S. fredii* HH103 *rkp* mutants are also negatively affected in their ability to nodulate *Glycyrrhiza uralensis*, an indeterminate-nodule-forming legume (our unpublished data). Thus, the symbiotic relevance of the KPS of *S. fredii* HH103 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.

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