Sinorhizobium fredii* HH103 Mutants Affected in Capsular Polysaccharide (KPS) are Impaired for Nodulation with Soybean and *Cajanus cajan

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The *Sinorhizobium fredii* HH103 *rkp-1* region, which is involved in capsular polysaccharides (KPS) production, was isolated and sequenced. The organization of the *S. fredii* genes identified, *rkpUAGHIJ* and *kpsF3*, was identical to that described for *S. meliloti* 1021 but different from that of *S. meliloti* AK631. The long *rkpA* gene (7.5 kb) of *S. fredii* HH103 and *S. meliloti* 1021 appears as a fusion of six clustered AK631 genes, *rkpABCDEF*. *S. fredii* HH103-Rif^r mutants affected in *rkpH* or *rkpG* were constructed. An *exoA* mutant unable to produce exopolysaccharide (EPS) and a double mutant *exoA rkpH* also were obtained. *Glycine max* (soybean) and *Cajanus cajan* (pigeon pea) plants inoculated with the *rkpH*, *rkpG*, and *rkpH exoA* derivatives of *S. fredii* HH103 showed reduced nodulation and severe symptoms of nitrogen starvation. The symbiotic capacity of the *exoA* mutant was not significantly altered. All these results indicate that KPS, but not EPS, is of crucial importance for the symbiotic capacity of *S. fredii* HH103-Rif^r. *S. meliloti* strains that produce only EPS or KPS are still effective with alfalfa. In *S. fredii* HH103, however, EPS and KPS are not equivalent, because mutants in *rkp* genes are symbiotically impaired regardless of whether or not EPS is produced.

Rhizobia are soil α -proteobacteria able to establish symbiotic interactions with leguminous plants leading to the formation of root nodules where these bacteria fix atmospheric nitrogen. This symbiotic process is initiated and maintained by a complex exchange of signal molecules between the host plant and the bacteria (Gage 2004; Geurts and Bisseling 2002; Perret et al. 2000). Plant roots secrete flavonoids that, in conjunction with the bacterial regulator protein NodD, induce the expres-

sion of the bacterial nodulation genes. These events result in the synthesis and secretion of specific signal molecules called Nod factors or lipochitooligosaccharides (LCOs), which in turn induce in the plant root hair curling and nodule meristem initiation. Bacterial penetration into the developing nodule generally takes place via tubular structures called infection threads. Bacteria are released at the tip of the infection thread into the cytoplasm of plant cells, where they are surrounded by a plant membrane and differentiate into the nitrogen-fixing bacteroid form. Nodules induced by rhizobia can be of two general kinds, indeterminate and determinate (Gage 2004; Patriarca et al. 2004). Indeterminate nodules are typically formed by temperate legumes such as *Medicago*, *Pisum*, *Vicia*, and *Trifolium* spp.; this kind of nodule is elongated and has a persistent meristem that continually gives rise to new nodule cells that subsequently are infected by rhizobia residing in the nodule. In the determinate nodule type, which is developed mainly by subtropical and tropical legumes such as *Glycine max* (soybean) and *Lotus japonicum*, the meristematic activity is temporary and the nodule shape is rounded.

In addition to Nod factors, several rhizobial surface polysaccharides are necessary for successful nodulation because they might act as signal molecules or could prevent plant defense responses (Becker and Pühler 1998; Frayse et al. 2003; Kannenberg et al. 1998; Mathis et al. 2005). Up to seven different surface polysaccharides have been reported in *Rhizobium leguminosarum* bv. *viciae*, some of them being of symbiotic relevance (Laus 2004).

Exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS or K-antigens), and cyclic β -glucans are the rhizobial polysaccharides more commonly investigated for their roles in the nodulation process (Frayse et al. 2003; Spaink 2000). The rhizobial EPS is required for the development of nitrogen-fixing nodules on plants forming indeterminate nodules, such as *Medicago sativa* (alfalfa), but apparently it is dispensable on plants that form determinate nodules, such as *Phaseolus* spp. or *G. max* (Gage 2004). Thus, *exo* (EPS) mutants of *Sinorhizobium meliloti* 1021 are unable to infect alfalfa, resulting in nodules that do not contain infected plant cells (Becker and Pühler 1998; Leigh and Coplin 1992). Interestingly, the rhizobial KPS can replace EPS for a successful nodulation of *S. meliloti* with *M. sativa* (Pellock et al. 2000; Petrovics et al. 1993). Due to this symbiotic equivalence between EPS and KPS, *S. meliloti* AK631 (which pro-

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duce 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 biologically inefficient (Frayssé et al. 2005).

At present, three gene clusters required for K-antigen production have been identified in *S. meliloti* Rm41: *rkp-1*, *rkp-2*, and *rkp-3* (Kiss et al. 2001). The *rkp-1* region contains 10 genes (*rkpA* to *rkpJ*) whose encoded products may participate in the synthesis, modification, and transfer of a putative lipophilic molecule that might act as a specific lipid carrier during KPS biosynthesis (Kiss et al. 1997; Petrovics et al. 1993). The *rkp-2* region is constituted by two open reading frames (ORFs), *lpsL* and *rkpK*, which encode for enzymes involved in the metabolism of nucleotide diphospho-sugars (Kereszt et al. 1998), whereas the *rkp-3* region contains genes involved in KPS export but also several strain-specific genes that determine the structure of the K antigen and, therefore, might affect strain-cultivar interactions (Kiss et al. 2001).

The presence of KPS was first reported in *S. fredii* USDA205 and *S. meliloti* AK631 (Reuhs et al. 1993). This polysaccharide also is present in *Rhizobium* sp. NGR234 (Reuhs et al. 1998). Since then, different *S. fredii* KPS structures have been determined (Reuhs et al. 1998; Rodríguez-Carvajal et al. 2001), including that of strain HH103 (Gil-Serrano et al. 1999). Evidence exists that the *S. fredii* KPS could affect bacterial host specificity (Reuhs et al. 1998). However, the isolation of *S. fredii* genes involved in KPS production has not been reported yet.

Here, we report the isolation and characterization of the *rkp-1* region of *S. fredii* HH103, a strain that produces a KPS consisting of a homopolymer of a pseudaminic acid derivative (Gil-Serrano et al. 1999). HH103 *rkp* mutants clearly were impaired in their capacity to nodulate American and Asiatic soybean cultivars as well as *Cajanus cajan* (pigeon pea). Hence, in *S. fredii*, KPS plays an important symbiotic role which cannot be replaced by the presence of EPS. It also is shown that an *S. fredii* *exoA* mutant does not show any significant impairment

in its nodulation capacities with soybean and pigeon pea. These results demonstrate that KPS, but not EPS, is important for the symbioses that *S. fredii* establishes with these two legumes.

RESULTS

Isolation of the *rkp-1* region and the *exoA* gene of *S. fredii* HH103.

Two different strategies were followed in order to isolate the *rkp-1* region of *S. fredii* HH103. First, an *S. meliloti* AK631 *rkpA* mutant was complemented for effective nodulation in alfalfa with a HH103 genomic library constructed in cosmid pLAFR1. Bacterial isolates from effective alfalfa nodules allowed the isolation of an *S. fredii* HH103 cosmid which was termed pMUS766. Second, the nucleotide sequence (X64131) of the *rkp-1* region of *S. meliloti* AK631 was compared with all the nucleotide sequences available on the databases. Primers *rkpC*-f and *rkpD*-r were designed from internal sequences of the *rkpC* and *rkpD* genes of the *S. meliloti* AK631 *rkp-1* region that showed high similarity to the *wcbR* gene of *Burkholderia mallei* (AF285636), which encodes for a putative type-I polyketide synthase involved in the synthesis of the bacterial capsule. Using *S. fredii* HH103 genomic DNA as template, primers *rkpC*-f and *rkpD*-r led to the polymerase chain reaction (PCR) amplification of a 624-bp fragment that showed 86% identity with the 3' end of *rkpC* and the 5' end of *rkpD* of *S. meliloti* AK631. In all, 1,200 clones of a genomic library of *S. fredii* HH103 were screened for positive PCR amplification with primers *rkpC*-f and *rkpD*-r, resulting in the identification of cosmid pMUS759.

A combination of hybridization- and PCR-based analyses showed that pMUS759 and pMUS766 are overlapping cosmids and that they contain the 3' and 5' regions, respectively, of the *rkp-1* cluster of *S. fredii* HH103 (Fig. 1). Following standard genetic procedures, a 16-kb segment covering the complete *rkp-1* region was sequenced (accession number AY882435) and showed to contain seven complete ORFs. The

Fig. 1. Genetic organization of the *rkp-1* region of **A**, *Sinorhizobium fredii* HH103 (accession number AY882435), **B**, *S. meliloti* 1021 (accession number AL591784), and **C**, *S. meliloti* AK631 (accession numbers X64131 and AF247711). The presence, but not the exact position, of the *rkpU* gene in the *rkp-1* region of Rm41, the parental strain of *S. meliloti* AK631, has been reported (accession number AF247710). The triangles indicate the position of the *lacZ*-*Gm^r* cassette in mutants SVQ528 and SVQ535 (into *rkpH*, open triangle), and SVQ537 (into *rkpG*, solid triangle). Cosmids pMUS759 and pMUS766, covering the HH103 *rkp-1* region, are shown. N = *NotI*; E = *EcoRI*.

HH103 *rkp-1* region is constituted by five genes showing the same polarity: *rkpA* (between positions 1,646 and 9,148), *rkpG* (9,145 to 10,560), *rkpH* (10,644 to 11,507), *rkpI* (11,599 to 13,212), and *rkpJ* (13,240 to 14,571). In addition, two other genes involved in polysaccharide production were localized in the extremes of the fragment: the *rkpU* gene (between positions 1,276 to 53) in the 5' terminus and the *kpsF3* gene (15,554 to 14,541) in the 3' terminus of the segment sequenced. Both *rkpU* and *kpsF3* showed polarity opposite to that of *rkpAGHIJ*.

The *rkpI* region of *S. fredii* HH103 presents a genetic organization that is similar to that found in *S. meliloti* 1021 (accession number AL591784) (Fig. 1). The *rkpA* gene of *S. fredii* HH103 encodes a putative protein of 2,500 amino acids. Similarly, in *S. meliloti* 1021, *rkpA* (7.5 kb) also encodes a large protein (2,504 amino acids), which may be involved in the synthesis of a transporting or precursory fatty acid of the KPS. The *rkpU* and *kpsF3* genes also show the same location in the two sinorhizobial strains. Moreover, partial sequencing of *S. fredii* HH103 regions located beyond the 5' and 3' edges of the *rkp-1* region revealed that the genetic organization of these two sinorhizobial strains is likewise maintained. Thus, in both *S. fredii* HH103 and *S. meliloti* 1021, the *pncA* and *pncB* genes (encoding for a probable pyrazinamidase/nicotinamidase and a putative nicotinate phosphoribosyltransferase, respectively) are located downstream of *rkpU*, and the *secD2* gene (encoding for a putative protein-export membrane protein), appears approximately 4 kb upstream of the *kpsF3* gene.

In *S. meliloti* AK631, however, the large *rkpA* of *S. meliloti* 1021 and *S. fredii* HH103 is composed of six individual genes that compose the *rkpABCDEF* operon. Information about the putative functions of the proteins encoded by the *rkp-1* region as well as the percentages of identity between these *S. fredii* and *S. meliloti* proteins is summarized in Table 1.

A 1,056-bp fragment containing the complete *exoA* gene of *S. fredii* HH103 was PCR amplified as described in Material and Methods. This PCR fragment was sequenced and showed to contain the HH103 *exoA* gene (1,016 bp, accession number AY882558), which encodes for a glucosyltransferase protein (EC 2.4.1.-). The *exoA* gene of HH103 was highly similar (95 and 96% identity at the nucleotidic and protein levels, respectively) to that of *S. fredii* USDA208 (accession number AF061245).

In order to investigate the importance of KPS and EPS on the bacterial symbiotic capacity, *S. fredii* HH103 *rkpG*, *rkpH*, and *exoA* mutant derivatives carrying the *lacZ*-Gm^r cassette were constructed and named SVQ537, SVQ528, and SVQ530, respectively. An *rkpH::lacZ*-Gm^r *exoA::Δ* double mutant (called SVQ535) also was constructed, as described below.

Inactivation of *rkpG* or *rkpH* negatively affect KPS production in *S. fredii* HH103.

The production of KPS was investigated in the *S. fredii* *rkpH* (strain SVQ528) and *rkpG* (strain SVQ537) derivatives. For this purpose, bacterial polysaccharides from *S. fredii* HH103 and its mutant derivatives SVQ528 and SVQ537 were isolated by the method of phenol/water and further purified. In all cases, when the crude extracts were chromatographed by size exclusion, using a sephacryl S-500 column, two fractions were obtained: a high (HMW) and a low (LMW) molecular weight fraction. The ¹H NMR (nuclear magnetic resonance) spectra of both fractions from the parental strain HH103 detected the presence of KPS as described by Gil-Serrano and associates (1999). A ¹H NMR spectrum of the HH103 HMW fraction is shown in Figure 2A. In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver-staining experiments indicated that the HMW fraction contained more LPS than the LMW one (data not shown).

Further purification of the HH103 LMW fraction was carried out by a new fractionation step using a Biogel P-6 column. Two new fractions of higher (LMW-H) and lower (LMW-L) molecular weight were obtained. The ¹H NMR spectrum of the LMW-H fraction (Fig. 2B) showed the presence of KPS. The LMW-L fraction mainly contained the glycerophosphorylated cyclic β-1,2-glucan (Fig. 2C) as described by Batley and associates (1987) and Miller and associates (1988).

The ¹H NMR spectra of the HMW (data not shown) and LMW fractions arising from mutants *S. fredii* SVQ528 (*rkpH*) and *S. fredii* SVQ537 (*rkpG*) did not detect the presence of KPS (Fig. 2D and E, respectively). Because of this, fractions from these mutants were not subjected to further purification with Biogel P-6 columns. The presence of a glycerophosphorylated cyclic glucan clearly was detected in the LMW fractions of SVQ528 (Fig. 2D) and SVQ537 (Fig. 2E).

All fractions from *S. fredii* HH103 Rif^r and the LMW fractions from its *rkpH* (SVQ528) and *rkpG* (SVQ537) mutant derivatives also were analyzed by PAGE experiments followed by a treatment with Alcian Blue and silver staining. In the absence of SDS, LPS remained in the upper part of the gel (as a heavily-stained material that appears close to the wells) and did not migrate further (Fig. 3A and B; and C, lane 1). This assumption was confirmed by immunostaining with a monoclonal antibody (NB6-228.22) that recognizes the LPS of HH103 (Fig. 3C, lane 2). In the presence of SDS, the antibody recognized the typical LPS ladder of bands that currently is observed by PAGE and silver staining of crude cell extracts (Fig. 3D and E).

According to the ¹H NMR spectra of the polysaccharides identified in the different HH103 fractions analyzed, the silver-

Table 1. Proteins encoded by the *Sinorhizobium fredii* HH103 *rkp-1* region and identities with the corresponding proteins of *S. meliloti* strains AK631 (accession number X64131) and 1021 (accession number AL591784)^a

Protein	Putative function	Identity with <i>S. meliloti</i> (%)	
		AK631	1021
RkpU (407)	Capsule polysaccharide exporter protein	91 (450) ^b	92 (377)
RkpA (2500)	Fatty acid synthesis transmembrane protein	92 RkpA (372)	85 RkpA (2504)
		88 RkpB (395)	...
		71 RkpC (966)	...
		88 RkpD (188)	...
		79 RkpE (284)	...
		94 RkpF (89)	...
RkpG (471)	2-Amino-3-ketobutirateCoA-ligase (EC 2.3.1.29)	85 RkpG (446)	89 RkpG (471)
RkpH (287)	Ribitol type dehydrogenase (EC 1.1.1.56)	66 RkpH (279)	75 RkpH (267)
RkpI (537)	Phosphoglycerol-transferase (EC 2.7.8.20)	85 RkpI (537)	85 RkpI (538)
RkpJ (443)	Capsular polysaccharide transport protein (KpsS from <i>Escherichia coli</i>)	74 RkpJ (412)	76 RkpJ (441)
KpsF3 (337)	Capsule expression protein	84 KpsF (359) ^b	84 KpsF3 (337)

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

^b The presence of these genes has been reported in Rm41, the parental strain of AK631. Accession numbers: AF247710 (*rkpU*) and AF247711 (*kpsF*).

stained material of the HMW fraction that migrated along the gel (Fig. 3A, lane 1) could correspond to the KPS (Fig. 2A), whereas that of the LMW fraction (Fig. 3A, lane 2) could correspond to a mixture of KPS (detected in the LMW-H subfraction) (Fig. 2B) and the cyclic β -1,2-glucan (detected in the

LMW-L subfraction) (Fig. 2C). Thus, the silver-stained material appearing in Figure 3A, lane 3 (LMW-H) and lane 4 (LMW-L) could correspond mainly to the KPS and the cyclic β -1,2-glucan, respectively. In fact, those fractions (HMW and LMW-H) in which the KPS was detected by ^1H NMR also showed simi-

Fig. 2. Capsular polysaccharides (KPS) analysis of *Sinorhizobium fredii* strains. **A** to **E**, 500-MHz ^1H NMR (nuclear magnetic resonance) spectra of different fractions containing surface polysaccharides isolated from *S. fredii* HH103 and its *rkpH* (SVQ528) and *rkpG* (SVQ537) mutant derivatives. Impurities are marked with asterisks. **A**, High molecular weight fraction of *S. fredii* HH103; ^1H NMR signals were assigned to KPS as in Gil-Serrano and associates (1999). **B**, Low molecular weight (LMW)-H fraction of *S. fredii* HH103; ^1H NMR signals were assigned to KPS. **C**, LMW-L fraction of *S. fredii* HH103; ^1H NMR signals were assigned to glycerophosphorilated cyclic β -1,2-glucan (CG), as assigned in Batley and associates (1987) and Miller and associates (1988), and also to small amounts of KPS. **D**, LMW fraction of *S. fredii* SVQ528; ^1H NMR signals were assigned to CG. **E**, LMW fraction of *S. fredii* SVQ537; ^1H NMR signals were assigned to CG.

lar PAGE and silver-staining patterns (Fig. 3A, lanes 1 and 3). The silver-stained material of the LMW fraction of mutant SVQ537 (*rkpG*) was hardly visible (Fig. 3B, lane 4) and the glycerophosphorylated cyclic β -1,2-glucan was the only polysaccharide positively identified in ^1H NMR experiments.

All the *rkp* or *exoA* HH103 mutants were examined for the production of EPS in solid and liquid yeast extract-mannitol (YM) media. As expected, in solid YM agar, the *exoA* derivatives SVQ530 and SVQ535 formed rough colonies in contrast to the mucous ones of HH103 Rif^r, SVQ528, and SVQ537. In addition, EPS was not recovered from supernatants of SVQ530 and SVQ535 liquid cultures treated with acetone, whereas 60 to 80 mg of dried EPS were collected from cultures of strains HH103 Rif^r, SVQ528, and SVQ537.

Neither *rkpH* and *rkpG* nor *exoA* mutations apparently affected LPS production because the LPS profiles of the different mutants analyzed on SDS-PAGE were undistinguishable from that of their parental strain HH103 (Fig. 3D). Similarly, no alterations between the LPS profile of strain HH103 and that of its KPS- or EPS-mutant derivatives were observed in immunostaining experiments using the monoclonal antibody NB6-228.22 (Fig. 3E).

***rkpH* and *rkpG* play an important role on the *S. fredii* HH103 nodulation capacity with soybean and pigeon pea.**

The symbiotic phenotype of *S. fredii* HH103 *rkp* or *exoA* mutant derivatives was investigated with several host plants: *G. max* cvs. Williams and Peking, and *C. cajan* (pigeon pea). Three different parameters were analyzed: plant-top dry weight, nodule number, and fresh weight of nodules (Table 2).

In the three legumes studied, the symbiotic capacity of the HH103 *exoA* derivative, SVQ530, was statistically indistinguishable from that of its parental strain. In contrast, the three HH103 derivatives carrying mutations in the *rkp* genes, strains SVQ528 (*rkpH*), SVQ537 (*rkpG*), and SVQ535 (*exoA rkpH*), were severely impaired in all the legumes analyzed because significant decreases ($\alpha = 5\%$) in the number and mass of nodules formed were observed with respect to the parental strain HH103 Rif^r. Although, in the three legumes tested, some nodules showing normal morphology and nitrogen-fixation activity (as assessed by acetylene reduction assay [ARA]) were formed, there was a dramatic reduction of the plant-top dry weight of plants inoculated with the *rkp* mutants. Moreover, pseudonodules and very small nodules were observed in soybean cv. Williams and *C. cajan* plants, respectively. SVQ532, a *rkpH* mutant derivative of HH103-Str^r that also was constructed in parallel to SVQ528, also showed the same symbiotic impairment with soybean cv. Williams (data not shown).

Bacterial isolates from soybean nodules formed by mutants SVQ528 (*rkpH*), SVQ535 (*exoA rkpH*), and SVQ537 (*rkpG*) were checked for the presence of the *lacZ*-Gm^r cassette in the corresponding *rkp* gene. In all the nodule isolates tested, PCR amplification using primers *rkpHint*-f and *lacZint*-r (for mutants SVQ528 and SVQ535) or *rkpGint*-f and *lacZint*-r (for SVQ537) amplified DNA bands that showed the same sizes as those observed for their respective mutant strains used as inoculants (data not shown). Hence, soybean nodules formed by the *rkpH* and *rkpG* mutants are not due to reversions caused by loss of the cassette *lacZ*-Gm^r.

All the *exo* or *rkp* derivatives of *S. fredii* HH103 were investigated for their β -galactosidase activity in those nodules of *G. max* cv. Williams that showed normal morphology. All the mutants assayed produced nodules that showed β -galactosidase activity (Fig. 4B through F). Nodules occupied by the parental strain HH103-Rif^r did not show this enzymatic activity (Fig. 4A).

DISCUSSION

S. fredii HH103 nodulates a broad range of legumes, such as *G. max*, *Glycyrrhiza uralensis*, or *C. cajan*, and produces KPS and EPS (Buendía-Clavería et al. 2003; Gil-Serrano et al. 1999; Vinardell et al. 2004). Although *S. fredii* HH103 and *S. meliloti* 1021 produce structurally different KPS, and only the KPS of the former is symbiotically active, the genetic organization of the *rkp*-1 region as well as the genes belonging to this locus are very well conserved between these two strains (Fig. 1; Table 1). This similarity between the *rkp*-1 regions of *S. meliloti* 1021 and *S. fredii* HH103, regardless of whether or

Fig. 3. Polyacrylamide gel electrophoresis (PAGE) and silver- or immunostaining of polysaccharides from *Sinorhizobium fredii* HH103-Rif^r and its *rkp*- or *exoA*-mutant derivatives. **A** and **B**, and **C**, lane 1, PAGE of polysaccharides samples that were run in the absence of detergent (sodium dodecyl sulfate [SDS]), treated with Alcian Blue, and silver stained. **A**, Lane 1, HH103-Rif^r high molecular weight (HMW) fraction; lane 2, HH103-Rif^r low molecular weight (LMW) fraction; lane 3, HH103-Rif^r LMW-H fraction; lane 4, HH103-Rif^r LMW-L fraction. **B**, Lane 1, HH103-Rif^r *rkpH* LMW fraction; lane 2, HH103-Rif^r HMW fraction; lane 3, HH103-Rif^r LMW fraction; lane 4, HH103-Rif^r *rkpG* LMW fraction. **C**, Lane 1, HH103-Rif^r HMW fraction; lane 2, immunostaining of HH103-Rif^r HMW fraction using the monoclonal antibody NB6-228.22. **D**, SDS-PAGE and silver-staining of lipopolysaccharide of crude extracts from lane 1, *S. fredii* HH103-Rif^r; and its lane 2, *rkpH*; lane 3, *exoA*; lane 4, *rkpH exoA*; and lane 5, *rkpG* mutant derivatives. **E**, SDS-PAGE and immunostaining, using the monoclonal antibody NB6-228.22, of crude extracts from lane 1, *S. fredii* HH103-Rif^r; and its lane 2, *rkpH*; lane 3, *exoA*; lane 4, *rkpH exoA*; and lane 5, *rkpG* mutant derivatives.

not a symbiotically active KPS is produced, could be due to the fact that the *rkp-1* region is most probably involved in the transport of KPS rather than in the synthesis of specific moieties of this polysaccharide (Kiss et al. 1997). In *S. meliloti* AK631, however, the *rkpA* gene (as defined in *S. meliloti* 1021) is composed of six individual genes that constitute the *rkpABCDE* operon. The appearance of individual ORFs in AK631 is due to the presence of either +1 or -1 frameshift mutations that cause the appearance of stop codons.

Standard protocols successfully used to isolate and detect, by NMR, the presence of KPS in the wild-type strain HH103 (Gil-Serrano et al. 1999) did not show the presence of this polysaccharide in the SVQ528 (*rkpH*) and SVQ537 (*rkpG*) fractions, suggesting that these mutants might not produce the KPS of the parental strain, a pseudaminic acid-containing homopolysaccharide (Gil-Serrano et al. 1999). However, PAGE analyses of SVQ528 and SVQ537 (Fig. 3B) showed a silver-stained material (a smear in the middle of the lane) that could be due to the presence of a polysaccharide. It is unlikely that

this silver-stained material corresponds to the cyclic β -1,2-glucan, because the HMW fraction of HH103 shows a similar smear and its ^1H NMR only detects KPS. We cannot discard the possibility that this polysaccharide might correspond to a KPS, which might be similar or different from that produced by the parental strain HH103-Rif^r, and that, for unknown reasons, it is not detected by the standard protocols used. In fact, the *rkpH* and *rkpG* mutants of *S. meliloti* AK631 retain the capacity to produce KPS (Kiss et al. 1997).

None of the *S. fredii rkp* mutants investigated showed any detectable alteration of their LPS (Fig. 3D and E). These results are in accordance with those reported in *S. meliloti*, in which it has been shown that mutations in different *rkp* genes, including *rkpH* and *rkpG*, do not affect the LPS of the mutants (Kiss et al. 1997; Petrovics et al. 1993; Reuhs et al. 1995).

The *S. fredii* HH103 *exoA* mutant (SVQ530), which is totally unable to produce EPS, was not impaired to nodulate soybean or *C. cajan* (Table 2). These results are in accordance with previous reports demonstrating that bacterial EPS is not required for the formation of determinate nitrogen-fixing nodules (Becker and Pühler, 1998; Kim et al. 1989).

To our knowledge, this is the first time that the symbiotic importance of KPS in determinate-nodule-forming symbioses has been investigated. Mutants SVQ528 (*rkpH*) and SVQ537 (*rkpG*) clearly were impaired in their symbiotic interaction with soybean cv. Williams, because all the symbiotic parameters analyzed in plants inoculated with the *rkp* mutants were significantly lower ($\alpha = 5\%$) than those of plants inoculated with the parental strain HH103. Previous reports have shown that KPS are strain-specific antigens and that they could be involved in cultivar specificity (Kannenberg et al. 1998; Reuhs et al. 1998, 1999). Hence, we have investigated the symbiotic capacity of mutants SVQ528 and SVQ537 with the soybean cv. Peking. Results show that the HH103 *rkp* mutants also were severely impaired in their nodulation capacity with this Asiatic soybean cultivar (Table 2). Although KPS does not appear to be absolutely required for nodule formation in the *S. fredii*-soybean interaction, the presence of this polysaccharide clearly is necessary for successful nodulation, even if the bac-

Fig. 4. Histochemical staining of 5-week-old soybean nodules occupied by *Sinorhizobium fredii* strains **A**, HH103-Rif^r and its **B**, *rkpH*, **C**, *exoA*, **E**, *exoA rkpH*, and **F**, *rkpG* mutant derivatives. **D**, HH103 Str^r *rkpH*. Magnification = $\times 6.3$. Bar = 500 μm .

Table 2. Plant responses to inoculation of *Glycine max* cvs. Williams and Peking and *Cajanus cajan* with *Sinorhizobium fredii* strains HH103-Rif^r and their *rkp* and/or *exoA* derivatives^a

Legume tested, inoculant ^b	Plant-top dry weight (g)	Number of nodules	Nodule fresh weight (mg)
<i>G. max</i> cv. Williams			
HH103 Rif ^r	5.390 $\pm$ 1.684	215.8 $\pm$ 27.3	2923 $\pm$ 614
SVQ530 (<i>exoA</i>)	6.000 $\pm$ 2.154	243.5 $\pm$ 72.8	2895 $\pm$ 979
SVQ528 (<i>rkpH</i>)	2.018 $\pm$ 0.250 *	52.0 $\pm$ 7.5 *	1138 $\pm$ 40 *
SVQ537 (<i>rkpG</i>)	1.013 $\pm$ 0.305 *	30.8 $\pm$ 17.8 *	230 $\pm$ 120 *
SVQ535 (<i>exoA rkpH</i>)	2.720 $\pm$ 0.774 *	70.5 $\pm$ 13.0 *	1198 $\pm$ 40 *
None	1.054 $\pm$ 0.470 *	...	...
<i>G. max</i> cv. Peking			
HH103 Rif ^r	3.303 $\pm$ 0.557	146.7 $\pm$ 29.3	1571 $\pm$ 318
SVQ530 (<i>exoA</i>)	3.285 $\pm$ 1.113	134.5 $\pm$ 21.8	1472 $\pm$ 360
SVQ528 (<i>rkpH</i>)	1.346 $\pm$ 0.275 **	33.7 $\pm$ 4.6 **	516 $\pm$ 79 **
SVQ537 (<i>rkpG</i>)	0.954 $\pm$ 0.166 **	22.2 $\pm$ 4.5 **	349 $\pm$ 51 **
SVQ535 (<i>exoA rkpH</i>)	0.916 $\pm$ 0.201 **	18.3 $\pm$ 2.9 **	321 $\pm$ 158 **
None	0.640 $\pm$ 0.240 **	...	...
<i>C. cajan</i>			
HH103 Rif ^r	0.35 $\pm$ 0.14	22.8 $\pm$ 14.4	168 $\pm$ 75
SVQ530 (<i>exoA</i>)	0.28 $\pm$ 0.12	17.0 $\pm$ 7.9	116 $\pm$ 53
SVQ528 (<i>rkpH</i>)	0.08 $\pm$ 0.07 **	5.9 $\pm$ 4.8 **	24 $\pm$ 25 **
SVQ537 (<i>rkpG</i>)	0.15 $\pm$ 0.09 **	7.7 $\pm$ 7.0 **	22 $\pm$ 16 **
SVQ535 (<i>exoA rkpH</i>)	0.10 $\pm$ 0.05 **	4.8 $\pm$ 3.5 **	4 $\pm$ 2 **
None	0.11 $\pm$ 0.04 **	...	...

^a Data represent averages of four jars for soybean cv. Williams, six jars for soybean cv. Peking, and eight plants for *C. cajan*. Each jar contained two soybean or pigeon-pea plants. Numbers on the same column followed by a single or a double asterisk are significantly different from those of HH103-Rif^r at the levels $\alpha = 5$ and 1%, respectively.

^b For each legume, each mutant was pairwise compared with the parental strain HH103-Rif^r by using the Mann-Whitney nonparametrical test. Bacteria isolated from 20 nodules formed by each inoculant showed the expected resistance markers.

teria produce EPS. This requirement of KPS is not specific for nodulation with soybeans because mutants SVQ528 and SVQ537 also were impaired to nodulate *C. cajan*.

In the indeterminate-nodule-forming *S. meliloti*–alfalfa symbiosis, EPS and KPS appear, somehow, to play functionally equivalent roles, so that the bacteria can successfully nodulate alfalfa plants if at least one of these two polysaccharides is symbiotically active. These two polysaccharides, however, appear not to play an equivalent role in the determinate-nodule-forming *S. fredii*–soybean and *S. fredii*–*C. cajan* symbioses, because the production of EPS by the HH103 *rkp* mutants does not prevent their symbiotic impairment.

It generally is accepted that EPS production by bacteroids is greatly reduced in comparison with that of free-living bacteria (Frayssé et al. 2003). In the *Bradyrhizobium japonicum*–soybean symbiosis, a new EPS, nodule polysaccharide (NPS), is produced (Streeter et al. 1992). Here, we show that the *exoA* gene of *S. fredii* SVQ530 bacteroids is actively transcribed (Fig. 4). This fact does not necessarily imply that the level of EPS production by *S. fredii* HH103 bacteroids should be similar to that of HH103 cultures. In fact, the presence of the flavonoid genistein in *S. fredii* HH103 cultures inhibits EPS production (Vinardell et al. 2004) but does not clearly repress *exoA* transcription (*unpublished results*). There is also an indication that the *rkpH* and *rkpG* genes are expressed inside soybean nodules (Fig. 4).

Previous reports have shown that bacterial LPS and cyclic β -glucans are necessary for successful nodulation of *B. japonicum* with soybean (Breedveld and Miller 1998; Kannenberg et al. 1998). Here, we show that the symbiosis *S. fredii*–soybean requires the presence of a third polysaccharide, KPS. The fact that this type of polysaccharide has been detected in other bacteria, such as *Erwinia* or *Pseudomonas* spp. (Kannenberg et al. 1998), that also interact with plants makes even more attractive the elucidation of the role of KPS in such bacteria–plant interactions.

MATERIALS AND METHODS

Molecular and microbiological techniques.

The bacterial strains and plasmids used in this work are listed in Table 3. *Sinorhizobium* strains were grown at 28°C on tryptone yeast (TY) medium (Berlinger 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 Lamrabet and associates (1999). Plasmids were transferred from *E. coli* to rhizobia by conjugation as described by Simon (1984).

Recombinant DNA techniques were performed according to the general protocols of Sambrook and associates (1989). For hybridization, DNA was blotted to Amersham HybondTM-N nylon membranes, and the DigDNA method of Roche was employed according to manufacturer's instructions. PCR amplifications were performed as previously described (Vinardell et al. 2004). Primers used were *rkpC*-f (5' TGAATTTCGCGAC GTGATGT); *rkpD*-r (5' CGTAATAGTCGCGCTTGCCGA); *exoA*-f (5' CCCGTCACAACGAAAGTGGGA) and *exoA*-r (5' GGTGCAGACGCCAATGTCGA). The Wisconsin Package for sequence analysis program (Accelrys, Cambridge) was used for basic DNA sequence analysis and assembly. The NCBI ORF-Finder and BLAST programs were used for ORF identification and homology searches, respectively.

In order to generate *rkp* mutants of *S. fredii* HH103, we constructed derivatives of pK18mob, a suicide vector in rhizobia, carrying the *S. fredii* HH103 *rkpH* or *rkpG* coding sequences interrupted by the *lacZ*::Gm^r cassette (subcloned as a 4.5-kb *Sma*I fragment into the unique *Nru*I and *Pml*I sites of *rkpH* and *rkpG*, respectively). These plasmids, named pMUS801 (*rkpH*::*lacZ*-Gm^r) and pMUS837 (*rkpG*::*lacZ*-Gm^r), were individually transferred to HH103-Rif^r, and Rif^r, Gm^r, and Km^r transconjugants were identified in order to obtain putative double recombinants in which the wild-type *rkp* gene had been sub-

Table 3. Bacterial strains and plasmids

Strain or plasmid	Derivation and relevant properties ^a	Source or reference
<i>Sinorhizobium fredii</i>		
HH103-1	HH103 Str ^r	Buendía-Clavería et al. 1989
SVQ269	HH103-Rif ^r	Madinabeitia et al. 2002
SVQ528	SVQ269 <i>rkpH</i> :: <i>lacZ</i> -Gm ^r	This work
SVQ530	SVQ269 <i>exoA</i> :: <i>lacZ</i> -Gm ^r	This work
SVQ532	HH103-1 <i>rkpH</i> :: <i>lacZ</i> -Gm ^r	This work
SVQ535	SVQ269 <i>rkpH</i> :: <i>lacZ</i> -Gm ^r <i>exoA</i> :: Ω	This work
SVQ537	SVQ269 <i>rkpG</i> :: <i>lacZ</i> -Gm ^r	This work
<i>S. meliloti</i>		
AK631	Rm41 <i>exoB</i> derivative.	Putnoky et al. 1990
pp666h	AK631 <i>rkpA</i> ::Tn5 derivative, Km ^r	Petrovics et al. 1993
<i>Escherichia coli</i>		
DH5 α	supE44, Δ lacU169, hsdR17, recA1, endA1, gyrA96, thi-1, relA1, Nx ^r	Stratagene
Plasmids		
pAB2001	Ap ^r vector containing the <i>lacZ</i> Δ p-Gm ^r cassette	Becker et al. 1995
pBluescript II SK+	Cloning and sequencing vector, Ap ^r	Stratagene
pGEM-T-Easy	Polymerase chain reaction products cloning vector, Ap ^r	Promega
pHP45 Ω	Ap ^r vector containing the Ω interposon (Spc ^r Str ^r)	Prentki and Krisch 1984
pK18mob	Cloning vector, Km ^r	Schafer et al. 1994
pRK2013	Helper plasmid, Km ^r	Figurski and Helinski 1979
pMUS759	Cosmid pLAFR1 carrying genes of the <i>rkp</i> -I region of <i>S. fredii</i> HH103	This work
pMUS766	Cosmid pLAFR1 carrying genes of the <i>rkp</i> -I region of <i>S. fredii</i> HH103	This work
pMUS775	pGEM-T-Easy derivative containing <i>S. fredii</i> HH103 <i>exoA</i>	This work
pMUS795	pGEM-T-Easy derivative containing <i>S. fredii</i> HH103 <i>exoA</i> :: <i>lacZ</i> -Gm ^r	This work
pMUS800	pK18mob derivative containing HH103 <i>exoA</i> :: <i>lacZ</i> -Gm ^r	This work
pMUS801	pK18mob derivative containing HH103 <i>rkpH</i> :: <i>lacZ</i> -Gm ^r	This work
pMUS825	pGEM-T-Easy derivative containing <i>S. fredii</i> HH103 <i>exoA</i> :: Ω	This work
pMUS831	pK18mob derivative containing HH103 <i>exoA</i> :: Ω	This work
pMUS837	pK18mob derivative containing HH103 <i>rkpG</i> :: <i>lacZ</i> -Gm ^r	This work

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

stituted by the mutated copy of the gene. Thus, mutants SVQ528 (=HH103-Rif^r *rkpH::lacZ-Gm^r*) and SVQ537 (=HH103-Rif^r *rkpG::lacZ-Gm^r*) were obtained.

The HH103 *exoA* gene was PCR amplified (1,056 bp) by using primers *exoA*-f and *exoA*-r (designed from the available sequence of the *exoA* gene of *S. fredii* USDA208, AF061245) and subcloned into the pGEM-T-Easy vector (Promega, Madison, WI, U.S.A.). The resulting plasmid, pMUS775, was digested with *Cla*I, whose unique site is located in the middle of the *exoA* gene, filled with the Klenow enzyme, and ligated to a 4.5-kb *Sma*I fragment carrying the *lacZ-Gm^r* cassette or to a 2-kb *Sma*I fragment harboring the Ω interposon (Spc^r and Str^r) (Prentki and Krusch 1984), rendering plasmids pMUS795 (*exoA::lacZ-Gm^r*) and pMUS825 (*exoA:: Ω*), respectively. The 5.2-kb *Eco*RI fragment carrying *exoA::lacZ-Gm^r* from pMUS795 and the 3-kb *Eco*RI fragment carrying *exoA:: Ω* from pMUS825 were subcloned into pK18mob, rendering plasmids pMUS800 and pMUS831, respectively. Following the strategy described above, plasmid pMUS800 was transferred to HH103 Rif^r, and strain SVQ530 (= HH103 Rif^r *exoA::lacZ-Gm^r*) was selected. Instead, plasmid pMUS831 was transferred to strain SVQ528 and the identification of Rif^r, Gm^r, Spc^r, and Km^s transconjugants allowed the isolation of the double mutant strain SVQ535 (HH103-Rif^r *rkpH::lacZ-Gm^r exoA:: Ω*). In all the cases, homogenotization of the mutated version of the *rkp* or *exoA* gene was confirmed by hybridization.

Studies of bacterial polysaccharides.

Production of bacterial EPS was carried out in YM medium as described by Vinardell and associates (2004). LPS extraction, separation on SDS-PAGE, and silver staining were performed as described previously (Buendía-Clavería et al. 2003). Immunostaining procedures and the monoclonal antibody NB6-228.22 were described by Buendía-Clavería and associates (2003).

For detection and analysis of KPS, *S. fredii* HH103-Rif^r, SVQ528, and SVQ537 were cultured as follows: 10 liters of TY liquid medium were inoculated with 100 ml of early-stationary phase cultures of bacteria and incubated on an orbital shaker at 160 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, 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 and Jann 1965). The aqueous phase was dialyzed against water, treated with an anion exchange resin IRA400 (AcO⁻), freeze dried, and redissolved in 100 ml of 50 mM Tris-HCl (pH 7.0) containing 10 mM MgSO₄. DNase (1 mg) and RNase (1 mg) were added, and the solution was stirred overnight at 5°C. Proteinase K (2 mg) was added and the solution was shaken for 48 h at 37°C, dialyzed, and then freeze dried. The polysaccharide was chromatographed on Sephacryl S-500 (60 by 2.6 cm) using 0.05 M EDTA-triethylamine (pH 7.0) as eluent, and carbohydrates were detected using a refractive index detector and the orcinol-sulfuric acid method on thin layer chromatography plates. Fractions containing carbohydrates were dialyzed and freeze dried. Chromatography on Biogel P6 (60 by 2.6 cm) was done using 0.02 M HCOONH₄ as eluent, and carbohydrates were detected as above.

For NMR spectroscopy, the samples were deuterium exchanged several times by freeze drying from D₂O and then examined in solution (5 to 10 mg per 750 μ l) in 99.98% D₂O. Spectra were recorded at 303 K on a Bruker AMX500 spectrometer operating at 500.13 MHz (¹H) and 125.75 MHz (¹³C).

Chemical shifts are given in ppm, using the D₂O signal (4.75 ppm) (¹H) and external dimethylsulfoxide (39.5 ppm) (¹³C) as references. The two-dimensional heteronuclear one-bond proton-carbon correlation experiment was registered in the ¹H-detection mode via single-quantum coherence. A data matrix of 256 by 1,000 points was used to digitize a spectral width of 4,845 and 22,522 Hz in F2 and F1; 64 scans were used per increment, with a delay between scans of 1.5 s and a delay corresponding to a *J* value of 150 Hz. ¹³C decoupling was achieved by the GARP scheme. Squared cosine-bell functions were applied in both dimensions and zero filling was used to expand the data to 1,000 by 1,000. This experiment was slightly modified by the implementation of an editing block in the sequence (Parella et al. 1997).

For KPS visualization, PAGE without SDS was carried out. Samples were analyzed by vertical electrophoresis (Bio-Rad, Hercules, CA, U.S.A.), as described by Rodríguez-Carvajal and associates (2001). The acrylamide concentration was 17% (wt/vol) and the acrylamide/*N,N'*-methylenebisacrylamide ratio was 30:0.8. Gels were fixed using Alcian Blue (0.5% in 2% acetic acid) and silver stained.

Histochemical studies (β -galactosidase assay).

The histochemical staining of nodules was carried out following a modification of the method described by Teeri and associates (1989). Frozen sections (30 to 60 μ m thick) obtained on a Kriomat 1703 were fixed with 1.25% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.2) for 15 min under vacuum followed by 1 h at atmospheric pressure, and then rinsed with the buffer for 15 min. The sections were transferred to the staining solution: 800 μ l of 0.2 M sodium cacodylate, pH 7.2; 50 μ l of 100 mM K₃ (Fe(CN)₆); 50 μ l of 100 mM K₄ (Fe(CN)₆); and 40 μ l of 2% (wt/vol) 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-gal) in *N,N*-dimethyl-formamide. After overnight incubation at room temperature, samples were rinsed sequentially with buffer and distilled water and observed by bright-field microscopy in a Leitz (Aristoplan) microscope.

Plant assays.

S. fredii strains were tested on *Glycine max* cvs. Williams and Peking and on *C. cajan* (pigeon pea) as described by Buendía-Clavería and associates (1989). Each Leonard jar contained two plants and each plant was inoculated with approximately 5×10^8 bacteria. Plants were grown for 6 to 7 weeks 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 weighted. Nitrogenase activity of nodules was assessed by ARA as described by Buendía-Clavería and associates (1989). Bacteria were isolated from surface-sterilized nodules as previously reported (Buendía-Clavería et al. 1989). 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. Isolates from soybean nodules formed by mutants SVQ528 (*rkpH*), SVQ535 (*rkpH*, *exoA*), and SVQ537 (*rkpG*) were tested for the maintenance of the cassette *lacZ-Gm^r* insertion into the *rkpH* or *rkpG* genes. For this purpose, one to three nodule isolates per soybean plant inoculated with SVQ528 and SVQ535 were subjected to PCR amplification using primers *rkpHint*-f (5' CATGACCCATGTG ATCATCA) and *lacZint*-r (5' GCCTCTTCGCTATTACGCCA). These primers amplify a DNA segment of approximately 350 bp containing the *rkpH* coding region preceding the cassette insertion and the 5' part of *lacZ*. Primers *rkpGint*-f (5' ATACA AGCAGGTGCAGGCGCA) and *lacZint*-r, which amplify a fragment of about 300 bp, were used for the analyses of bacterial isolates from nodules formed by SVQ537.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The National Center for Biotechnology Information (NCBI) BLAST website: www.ncbi.nlm.nih.gov/BLAST
 NCBI ORF-finder: www.ncbi.nlm.nih.gov/gorf/gorf.html