Microbiology (2003), **149**, 1807–1818 DOI 10.1099/mic.0.26099-0

A *purL* mutant of *Sinorhizobium fredii* HH103 is symbiotically defective and altered in its lipopolysaccharide

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Received 5 November 2002
Revised 13 March 2003
Accepted 18 March 2003

The pleiotropic phenotype of an auxotrophic *purL* mutant (SVQ295) of *Sinorhizobium fredii* HH103 has been investigated. SVQ295 forms colonies that are translucent, produce more slime and absorb less Congo red than those of wild-type strain HH103. SVQ295 did not grow in minimal medium unless the culture was supplemented with thiamin and adenine or with thiamin and AICA-riboside (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside), an intermediate of purine biosynthesis. Bacterial cultures supplemented with AICA-riboside or adenine reached the same culture density, although the doubling time of SVQ295 cultures containing AICA-riboside was clearly longer. *S. fredii* SVQ295 induced pseudonodules on *Glycine max* and failed to nodulate six different legumes. On *Glycyrrhiza uralensis*, however, nodules showing nitrogenase activity and containing infected plant cells were formed. SVQ295 showed auto-agglutination when grown in liquid TY medium and its lipopolysaccharide (LPS) electrophoretic profile differed from that of its parental strain HH103-1. In addition, four monoclonal antibodies that recognize the LPS of *S. fredii* HH103 failed to recognize the LPS produced by SVQ295. In contrast, ^{1}H-NMR spectra of K-antigen capsular polysaccharides (KPS) produced by SVQ295 and the wild-type strain HH103 were similar. Co-inoculation of soybean plants with SVQ295 and SVQ116 (a *nodA* mutant derivative of HH103) produced nitrogen-fixing nodules that were only occupied by SVQ116.

INTRODUCTION

The interaction between rhizobia and legumes to form nitrogen-fixing root nodules involves several communication steps to coordinate gene expression and nodule development. The initial exchange of signals activates rhizobial *nod* gene expression by plant-produced flavonoid compounds. The *nod* gene products are responsible for the production of host-specific lipooligosaccharide signal molecules that cause root hair curling and nodule meristem initiation (Fisher & Long, 1992; Day *et al.*, 2000).

Unlike the bacterial signals specified by *nod* genes, signal molecules that may be required to initiate and maintain infection thread development are poorly understood. Studies with rhizobial mutants defective in exopolysaccharide, lipopolysaccharide (LPS) or K-antigen capsular polysaccharides (KPS) indicate that these polysaccharides are important for infection of a variety of legumes (Becker & Pühler, 1998; Kannenberg *et al.*, 1998; Kannenberg & Carlson, 2001). Interestingly, *Sinorhizobium fredii* and *S. meliloti* produce structurally conserved LPS but strain-specific KPS, indicating that the conserved LPS lack the

Abbreviations: AICA-riboside, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; KPS, K-antigen capsular polysaccharide; LCO, lipo-chitin oligosaccharide.

The GenBank accession number for the sequence determined in this work is AF275718.

structural information necessary to influence host specificity while the variable KPS may affect strain–cultivar interactions (Reuhs *et al.*, 1998). Moreover, studies using a panel of anti-*S. meliloti* monoclonal antibodies that recognizes LPS or KPS have revealed changes of these polysaccharides during symbiosis (Reuhs *et al.*, 1999).

The phenotypes of different *Rhizobium* auxotrophs have also provided interesting clues to the physiology of nodule development. By analysing the symbiotic behaviour of auxotrophic mutants, it has been demonstrated that some metabolic pathways of *Rhizobium* bacteria are important for an efficient symbiotic interaction (Pain 1979; Noel *et al.*, 1988). In some legume–*Rhizobium* associations, the host plant can supply the auxotrophic symbiont with the required nutrient(s) (Djordjevic *et al.*, 1988). However, the success of this physiological complementation will depend on the host plant, the rhizobial strain, and the nature of the auxotrophy (Beringer *et al.*, 1980). One intriguing type of finding is that in some biosynthetic pathways, intermediates, rather than endproducts, may be required for the bacteria to elicit nodule development or infection. For example, studies with *S. meliloti* tryptophan auxotrophs suggest that anthranilate synthesis is important for bacteroid development symbiosis with alfalfa (Barsomian *et al.*, 1992).

One of the most universally deleterious symbiotic defects is purine auxotrophy. Purine auxotrophs of most rhizobial species are unable to nodulate their host plants effectively (Pankhurst & Schwinghamer, 1974; Denarié *et al.*, 1976; Pain, 1979). *S. meliloti* purine auxotrophs have been reported to induce ineffective nodules on alfalfa (Denarié *et al.*, 1976). Several purine auxotrophs of *Rhizobium leguminosarum* bv. *viciae* were described as noninfective or even being unable to induce nodule formation (Pain, 1979). Similarly, a purine auxotroph of the broad-host-range *Rhizobium* sp. NGR234 elicited root hair curling and nodule meristem initiation on *Macroptilium atropurpureum*, but no infection threads were observed (Djordjevic *et al.*, 1988). On soybean, *S. fredii* purine auxotrophs induced pseudonodules that did not contain bacteria (Kim *et al.*, 1988).

Purine auxotrophs (Pur^-) of *R. etli* CFN42 elicited pseudonodules on bean plants (Noel *et al.*, 1988). These mutants caused root hair curling and nodule meristem initiation but did not elicit infection threads. Supplementation of the root medium with 0·1 mM purine, or purine nucleosides, did not enhance nodulation ability of *R. etli pur* mutants. However, the addition of 0·1 mM 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICA-riboside, an intermediate in the biosynthetic purine pathway) to the plant nutritive solution significantly enhanced nodule development (Noel *et al.*, 1988; Newman *et al.*, 1994). Nodules induced by the mutants in the presence of AICA-riboside resembled those formed by the wild-type, but were unpigmented and lacked nitrogenase activity. Examination of these nodules by light microscopy revealed the presence of infection threads filled with bacteria, but infected plant cells were not observed. Time-course experiments showed that the addition of AICA-riboside to *Phaseolus vulgaris* roots within the first 6 days after inoculation with a *R. etli pur* mutant allowed the formation of vascular bundles in the nodule cortex. Beyond this time, AICA-riboside supplementation produced nodules that contained their vascular bundles in a central position (Newman *et al.*, 1992). AICA-riboside also promotes *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium fredii pur* mutants for the formation of root nodules in *Pisum sativum* and *Glycine max*, respectively (Newman *et al.*, 1994). All these results have led to the hypothesis that rhizobia might convert AICA riboside into AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) to initiate and/or sustain infection thread development, possibly by using this substance in the production of a signal molecule.

We report here the isolation and characterization of a *purL* mutant (SVQ295) of *S. fredii* HH103 that requires the addition of adenine and thiamin to grow on minimal medium and forms pseudonodules on *Glycine max* roots. We demonstrate that the symbiotic phenotype of SVQ295 varies depending on the legume assayed. In *Macrotyloma axyllare* no nodules were formed, while in *Glycyrrhiza uralensis*, nodules containing infected cells were observed. Furthermore, the LPS profile of mutant SVQ295 is different from that shown by its wild-type parent HH103, and monoclonal antibodies raised against the HH103 LPS failed to recognize the LPS of SVQ295.

METHODS

Strains, plasmids and media. The bacterial strains and plasmids used in this work are listed in Table 1. *Sinorhizobium* strains were grown in TY medium (Beringer, 1974), yeast mannitol (YM) medium (Vincent, 1970), B^- medium (Spaink *et al.*, 1992) or MMB (Bergensen, 1961) at 28 °C. To screen for auxotrophic strains the minimal medium (MMB) was solidified with agarose at 1·5 % (w/v). *Escherichia coli* was cultured in Luria–Bertani (LB) medium (Maniatis *et al.*, 1982) at 37 °C. When required, the media were supplemented with the appropriate antibiotics as described by Lamrabet *et al.* (1999). When required, MMB medium was supplemented with adenine (12 μg ml^{-1}), AICA-riboside (0·1 or 0·5 mM) and/or thiamin (0·8 μg ml^{-1}).

Genetic manipulations. Plasmids were transferred by conjugation as described by Simon (1984). Random transposon Tn*5*-Mob mutagenesis of *Sinorhizobium fredii* strain HH103-1 was carried out by using the suicide plasmid pSUP5011 as described by Simon (1984). Transconjugants were selected on TY medium containing kanamycin and streptomycin. Single colonies with altered morphology on solid TY or YM media were tested for auxotrophy by using a modification of the Holliday test (Beringer *et al.*, 1984).

Total genomic DNA and plasmid isolations were carried out as described by Maniatis *et al.* (1982). DNA manipulation, including restriction analyses, ligation, bacterial transformation and electrophoresis were performed according to the general protocols of Maniatis *et al.* (1982).

The suicide rhizobial plasmid pSUP202 (Simon *et al.*, 1983) was used to clone the rhizobial DNA that is adjacent to the Tn*5*-Mob insertion in mutant *S. fredii* SVQ295. Briefly, plasmid pSUP202 was transferred from *E. coli* S17-1 to mutant SVQ295, and Tc^R transconjugants were

Table 1. Bacterial strains and plasmids

Strain or plasmid	Derivation and relevant properties	Source or reference
Sinorhizobium fredii		
HH103-1	HH103 Str^R	Buendía-Clavería *et al.* (1989)
SVQ295	HH103-1 *purL*::Tn*5*-Mob	This work
SVQ269	HH103 Rif^R	Madinabeitia *et al.* (2002)
SVQ526	Purine auxotrophic mutant of SVQ269 by Tn*5*-Mob mutagenesis	This work
SVQ116	SVQ269 *nodA*::Tn*5*-B20	Buendía-Clavería *et al.* (1996)
Rhizobium etli		
CE3	Derivative of CFN42, Str^R	Noel *et al.* (1984)
CE382	CE3 *purL*::Tn*5*	Newman *et al.* (1995)
Escherichia coli		
HB101	Restriction-minus, *recA* background, Str^R	Boyer & Roulland-Dussoix (1969)
Plasmids		
pBluescript	Cloning vector, Ap^R	Stratagene
pSUP202	pBR325 containing the Mob region subcloned in *Sau*3A, Ap^R Cm^R Tc^R	Simon *et al.* (1983)
pSUP5011	Suicide plasmid carrying the Tn*5*-Mob transposon	Simon (1984)
pMUS375	pSUP202 containing a 11 kb *Eco*RI fragment of SVQ295 DNA adjacent to the IS*50*R from Tn*5*-Mob	This work
pMUS509	Cosmid pLAFR1 carrying *purL* of *S. fredii* HH103	This work
pCOS110	Cosmid pLAFR1 carrying *purY*, *purQ* and *purL* of *R. etli* CE3	Newman *et al.* (1994)
pJN382A	pCOS110 *purL*::Tn*5*	Newman *et al.* (1994)

selected. Since this plasmid cannot replicate in *S. fredii*, the only possible inheritance of pSUP202 is by homologous recombination between the Mob site of transposon Tn*5*-Mob and the Mob region of plasmid pSUP202. This recombination will lead to the integration of plasmid pSUP202 inside transposon Tn*5*-Mob. Plasmid pSUP202 has a unique *Eco*RI site (inside the Cm^R gene) and this enzyme does not cut the Tn*5*-Mob transposon. Hence, *Eco*RI digestion of total DNA isolated from SVQ295::pSUP202 generates an *Eco*RI fragment that contains the tetracycline-resistance gene, the OriV of pSUP202, the Mob site, part of the transposon including the IS*50*R and a fragment of rhizobial DNA adjacent to the inserted transposon. *Eco*RI-digested SVQ295::pSUP202 total DNA was subjected to self-ligation and *E. coli* HB101 Tc^R transformants were selected.

An oligonucleotide (pdad2: 5′-AGATTTAGCCCAGTCG-3′) was designed from the IS*50*R DNA sequence of transposon Tn*5*-Mob and used as a primer to sequence the rhizobial DNA that is adjacent to the right arm of Tn*5*-Mob in mutant strain SVQ295. DNA sequence was carried out by the dideoxy chain-termination method (Sanger *et al.*, 1977). Computer analysis of the sequences was performed by using the GCG program (University of Wisconsin; Devereux *et al.*, 1984).

Plant assays. The nodulation ability of strain SVQ295 was tested on *Glycine max* (L.) Merr. cv. Williams, *Cajanus cajan* (L.) Millsp., *Macroptilium atropurpureum* (Moc. & Sessé ex DC) Urb., *Vigna radiata* (L.) Wilczek, *Indigofera tinctoria* (L.), *Macrotyloma axillare* (E. Mey.) Verdc., *Albizia lebbek* (L.) Benth., *Kummerowia stipulacea* (Maxim.) Makino, *Neonotonia wightii* (Arn.) Lackey and *Glycyrrhiza uralensis* (L.) as described by Buendía-Clavería *et al.* (1989). Germinated seeds were transferred to Leonard jars containing sterilized vermiculite supplemented with Fåhraeus nutrient solution (Vincent, 1970). When required, the Fåhraeus plant nutritive solution was supplemented with adenine at 12 μg ml^{-1}, thiamin at 0·8 μg ml^{-1} and AICA-riboside at 0·1 or 0·5 mM. Plants were grown for at least 5 weeks with a 16 h photoperiod at 25 °C in the light and 18 °C in the dark. Nitrogenase activity of soybean (*Glycine max*) nodules was determined by acetylene reduction assays as previously reported (Buendía-Clavería *et al.*, 1986). Plant tops were dried at 70 °C for 48 h and weighed. Bacteria were isolated from surface-sterilized nodules as described by Buendía-Clavería *et al.* (1986).

In double-inoculation experiments, soybean plants cv. Williams were inoculated with 3×10^8 bacteria per plant of each co-inoculant, SVQ116 (Rif^r Km^r, prototrophic) and SVQ295 (Str^r Km^r, Ade^- Thi^-). Plants were harvested 82 days after co-inoculation. To determinate nodule occupancy, isolates from soybean nodules were tested for antibiotic resistance and auxotrophic markers.

Radiolabelling of lipo-chitin oligosaccharide (LCO). Bacteria were grown in 5 ml B^- medium in the presence of the inducer naringenin (3·6 μM) and *N*-acetyl[^{14}C]glucosamine. The ^{14}C-labelled LCOs were analysed by TLC as described by Spaink *et al.* (1992).

SDS-PAGE analysis of lipopolysaccharide. Bacterial cultures were grown on solid TY medium or on solid MMB medium supplemented with adenine (or AICA-riboside) and thiamin. Bacterial cells were washed in 0·9 % NaCl and pelleted by centrifugation. The bacterial pellet was resuspended and lysed by heating at 100 °C in 125 μl 60 mM Tris/HCl (pH 6·8) containing 2 % (w/v) SDS and 1 mM EDTA for 5 min and then diluted to 1 ml with the same buffer without SDS. The bacterial crude extract was treated with RNase, DNase and proteinase K as described by Köpling *et al.* (1993). The electrophoresis of crude bacterial extracts was performed on a 16·5 % (w/v) polyacrylamide gel with the Tricine buffer system described by Lesse *et al.* (1990). For the detection of LPS, gels were stained with silver as described by Kittelberger & Hilbink (1993).

Monoclonal antibodies (mAbs) and immunoblotting. Production of mAbs against free-living cultures of HH103 or

nodule-derived bacteroids followed previously published procedures (Lucas *et al.*, 1996). Spleen cells derived from immunized male rats (Line LOU/IAP) were fused to the myeloma line IR983F. For immunoblotting experiments, mAbs that recognize HH103 LPS were derived from four culture supernatants. NB3-4.72 and NB3-4.79 (class IgG 2c) were two clones derived from the same hybridoma line originating from a rat immunized with a preparation of HH103 bacteroids, NB6-228.22 (Class IgG 2b) was also obtained following immunization with a bacteroid preparation, while NB7-242.33 (Class IgG 2c) was obtained following immunization with free-living cultures of HH103.

Polyacrylamide gels were electroblotted onto nitrocellulose sheets, using a Bio-Rad Semi-dry transblot apparatus at 20 V for 1 h at room temperature. Transfer buffer was 48 mM Tris/HCl, 39 mM glycine, containing 20 % (v/v) methanol and 0·0375 % (w/v) SDS (pH 8·0). Western-blotted sheets were immunostained with the appropriate mAb, using as the secondary antibody a goat anti-rat IgG conjugated to alkaline phosphatase.

NMR analyses of KPS. KPS were isolated from *S. fredii* HH103 and its mutant SVQ295 as described by Gil-Serrano *et al.* (1999). The samples containing KPS were deuterium-exchanged several times by freeze-drying from ^2H_2O and then examined in solution (4 mg ml^{-1}) in 99·98 % ^2H_2O. Spectra were recorded at 303 K on a Bruker AMX 500 spectrometer operating at 500·13 MHz. Chemical shifts are given in p.p.m., using the ^2H_2O signal (4·75 p.p.m.) as reference.

Microscopic studies. Small fragments of nodules and pseudonodules were fixed in 4 % (v/v) glutaraldehyde prepared in 0·1 M cacodylate buffer, pH 7·2, for 3 h at 4 °C and post-fixed in 1 % OsO_4 for 2 h at 4 °C. Samples were dehydrated in an acetone series and embedded in Epon (epoxy embedding medium). Toluidine-blue-stained semi-thin sections (0·5 μm thick) were viewed in a Leitz (Aristoplan) light microscope.

RESULTS

Isolation of Tn*5*-Mob mutants showing altered colony morphology

S. fredii HH103-1 was mutagenized with Tn*5*-Mob (Simon, 1984) and 80 000 Nm^R transconjugants were screened for alterations in colony morphology. One mutant, called SVQ295, was further investigated. SVQ295 harbours only one copy of Tn*5*-Mob, as indicated by hybridization experiments using a 0·9 kb *Pst*I internal fragment of the kanamycin-resistance gene of Tn*5*-Mob as a probe (data not shown). It forms colonies that are translucent, produce more slime and absorb less Congo red than those of wild-type strain HH103-1. SVQ295 auto-agglutinates when grown in liquid TY, an indication that this mutant might be altered in its LPS, as reported for *R. leguminosarum* LPS mutants (Priefer, 1989). PAGE showed that the LPS I and LPS II regions of SVQ295 cultures grown on solid TY medium contain bands that migrate faster than those of its parental strain HH103-1 (Fig. 1A, lanes 2 and 1 respectively). On the other hand, the ^{1}H-NMR spectra obtained for the KPS from *S. fredii* HH103 and from its mutant derivative SVQ295 appear to be very similar (Fig. 2). The LCO and plasmid profiles of mutant strain SVQ295 were also identical to those of its parental strain HH103-1 (data not shown).

Mutant SVQ295 does not grow in minimal medium (MMB) unless the bacterial culture is supplemented with thiamin and adenine or with thiamin and AICA-riboside, an intermediate of purine biosynthesis.

Although the doubling time of SVQ295 cultures grown in

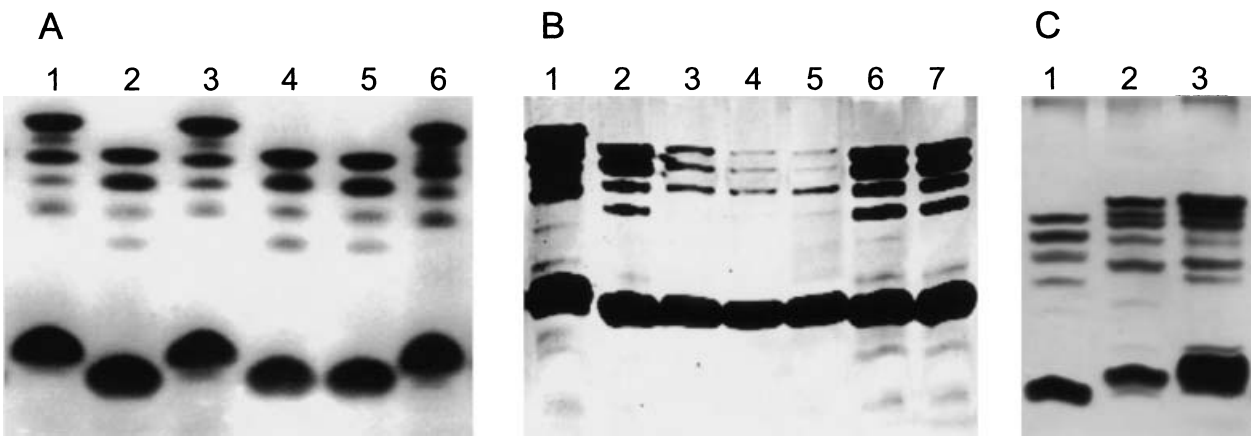

Fig. 1. LPS profiles of strains grown in TY medium, unless otherwise indicated. (A) Lane 1, HH103-1; lane 2, SVQ295; lane 3, SVQ295(pMUS509); lane 4, SVQ295(pJN382A); lane 5, SVQ295; lane 6, SVQ295(pCOS110). (B) Lane 1, HH103-1; lane 2, SVQ295; lane 3, SVQ295 (MMB medium supplemented with 0·5 mM AICA-riboside and thiamin); lane 4, SVQ295 (MMB medium supplemented with 0·1 mM AICA-riboside and thiamin); lane 5, SVQ295 (MMB medium supplemented with adenine and thiamin); lane 6, SVQ295 (TY medium supplemented with 0·5 mM AICA-riboside); lane 7, SVQ295 (TY medium supplemented with 0·1 mM AICA-riboside). (C) Lane 1, SVQ295; lane 2, SVQ526; lane 3, HH103-1.

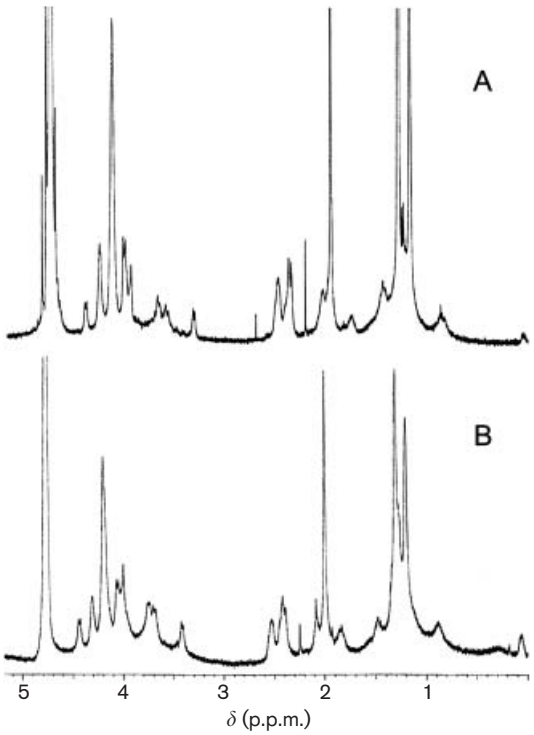

Fig. 2. ^{1}H-NMR spectra (500 MHz) obtained for the capsular polysaccharide from *S. fredii* HH103 (A) and SVQ295 (B).

the presence of AICA-riboside is longer (approx. 9 h) than that in the presence of adenine (approx. 5 h), both bacterial cultures (with AICA-riboside or adenine) reached the same OD_{660} after 6 days of incubation (5×10^8 bacteria ml^{-1}). Inoculation of MMB medium supplemented with adenine or AICA-riboside with SVQ295 previously grown in the presence of AICA-riboside showed again that bacteria grew faster in the presence of adenine than in the presence of AICA-riboside (data not shown). Mutant SVQ295 and its parental strain HH103-1 showed the same doubling time (135 min) in complete TY medium. The presence of adenine or AICA-riboside in SVQ295 cultures did not restore the wild-type LPS electrophoretic profile (Fig. 1B).

Identification of the mutated gene in SVQ295

Plasmid pSUP202 was used to clone the SVQ295 DNA region that is adjacent to the IS*50*R of transposon Tn*5*-Mob. Following the strategy described in Methods, a plasmid (pMUS375) that contained about 16 kb of the SVQ295 DNA that is adjacent to the IS*50*R of transposon Tn*5*-Mob was isolated. A *Xho*I fragment of pMUS375 that contains 485 bp of the IS*50*R and about 4 kb of SVQ295 DNA was subcloned into plasmid pBluescript. By using primer pdad2, a DNA fragment containing the last 120 bp of the IS*50*R and 500 bp of the adjacent rhizobial DNA was sequenced. Computer analysis of this sequence indicates homology with the *purL* gene of *S. meliloti*, which encodes a 5′-phosphoribosylformylglycinamidine synthase (FGAR amidotransferase, EC 6.3.5.3). This enzyme catalyses the step from FGAR (5′-phosphoribosyl-*N*-formylglycinamide) to FGAM (5′-phosphoribosyl-*N*-formylglycinamidine) in the biosynthetic purine pathway and it is essential for biosynthesis of both adenine and thiamin. These results are in accordance with the auxotrophic phenotype exhibited by mutant SVQ295.

Symbiotic properties of mutant SVQ295

SVQ295 induces Fix^- pseudonodules on soybean cv. Williams. Microscopic analysis showed that plant cells of these pseudonodules were devoid of bacteria. Nodulation assays were carried out to investigate whether the addition of thiamin and/or adenine or AICA-riboside to the plant nutrient solution restored soybean nodulation ability of mutant SVQ295. Adenine, thiamin, or both together, did not enhance the symbiotic properties of mutant strain SVQ295 whereas 0·1 or 0·5 mM AICA-riboside enabled SVQ295 to form infected nodules that did not fix nitrogen and were smaller than those formed by HH103. Bacteria isolated from these nodules had the appropriate antibiotic resistance (Str^R Nm^R) and were auxotrophic for adenine and thiamin.

The symbiotic properties of SVQ295 were also studied in other leguminous plants that establish nitrogen-fixing symbioses with the wild-type strain HH103-1. Mutant SVQ295 failed to nodulate *Cajanus cajan*, *Indigofera tinctoria*, *Macrotyloma axillare*, *Albizia lebbek*, *Kummerowia stipulacea* and *Neonotonia wightii*. It formed small ineffective nodules on *Macroptilium atropurpureum*. Although infected nodule cells were observed (Fig. 3D, E), bacteria were not recovered from these nodules. In contrast, SVQ295 was able to form nitrogen-fixing nodules on *Glycyrrhiza uralensis* (Fig. 3A) as demonstrated by acetylene reduction assays. *G. uralensis* nodules contained infected plant cells with a large centrally positioned vacuole (Fig. 3B, C) and bacteria isolated from these nodules showed the markers of SVQ295.

Isolation and characterization of the *purL* gene of *S. fredii* HH103-1

For unknown reasons, it is often difficult to isolate cosmid clones that complement HH103 mutants by conjugational transfer of the HH103 genomic library to the recipient mutant. Because of this difficulty, an HH103 gene library, constructed in cosmid pLAFR1, was transferred to *R. etli* CE382, a derivative of CE3 containing Tn*5* in *purL* (Newman *et al.*, 1995). CE382 Tc^R Km^R transconjugants were selected on MMB agar and a cosmid named pMUS509 was isolated from one of them. Cosmid pMUS509 was transferred (by transformation) to *E. coli* S17-1 and from this donor strain it was reintroduced (by conjugation) into *R. etli* CE382. Transconjugants carrying pMUS509 did not require the presence of adenine and thiamin to grow on MMB medium and were able to form nitrogen-fixing nodules on *Phaseolus vulgaris*.

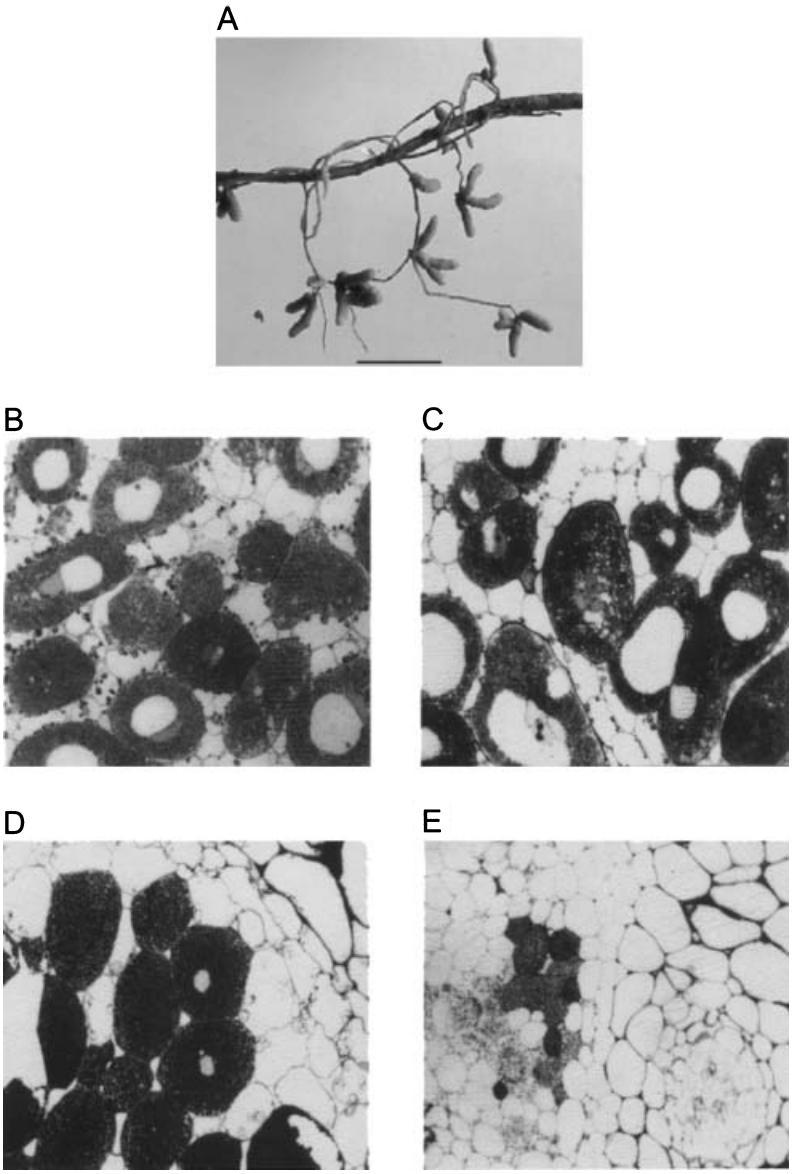

Fig. 3. Nodulation phenotype of SVQ295. (A) Morphology of nodules induced by SVQ295 on *Glycyrrhiza uralensis*. Bar, 1 cm. (B–E) Light microscopy of sections of nodules induced on *G. uralensis* by HH103-1 (B) and SVQ295 (C), and on *Macroptilium atropurpureum* by HH103-1 (D) and SVQ295 (E). Magnification: 63× in (B–D); 16× in (E).

Cosmids pMUS509, pCOS110 and pJN382A were transferred to *S. fredii* SVQ295. Cosmid pCOS110 carries the *purYQL* region of *R. etli* and pJN382A is a derivative of pCOS110 carrying Tn*5* in *purL*. SVQ295 transconjugants carrying pMUS509 or pCOS110 were able to grow on MMB, demonstrating that the *R. etli purL* gene can complement the *purL* mutation of *S. fredii* HH103-1. SVQ295 carrying pMUS509, pCOS110 or pJN382A was tested for nodulation ability on soybean cv. Williams. Plants were grown in the presence or absence of thiamin. Soybean plants inoculated with SVQ295(pCOS110) or SVQ295(pMUS509) induced nitrogen-fixing nodules, while those inoculated with SVQ295(pJN382A) only formed Fix^- pseudonodules. These results demonstrated that the mutation on the *purL* gene is responsible for the symbiotic-defective phenotype of mutant SVQ295. The presence of thiamin in the plant nutrient solution did not have any significant effect on bacterial nodulation.

A 3·2 kb *Bam*HI fragment of cosmid pMUS509, which positively hybridized to a probe containing 0·5 kb of the SVQ295 DNA that is adjacent to the transposon IS*50*R in plasmid pMUS375, was subcloned into pBluescript. From the resultant plasmid a *Kpn*I–*Bam*HI 2870 bp fragment was sequenced (accession number AF275718). By matching this sequence with that obtained from the partial sequencing of pMUS375 using the IS*50*R primer pdad2, it was possible to position the transposon at nucleotide 832 of the final 2870 bp sequence.

The sequenced fragment contains one ORF with a high probability of encoding protein, as indicated by the Testcode algorithm. This ORF encodes a protein that is homologous to several PurL proteins of different organisms such as *S. meliloti*, *Mesorhizobium loti* and *Agrobacterium tumefaciens* (Table 2). *purL* begins at position 461 and extends for 2129 bp, encoding a deduced polypeptide of

Table 2. Comparison of HH103 PurL protein with PurL proteins from different organisms.

Organism	Identity (%)	Similarity (%)	Amino acids overlapping	Length of PurL protein	Accession no.
Sinorhizobium meliloti	92	96	743	743	Q92PH7
Mesorhizobium loti	81	90	739	743	Q98NN7
Agrobacterium tumefaciens	86	91	769	775	AAL42846
Zymomonas mobilis	61	73	745	734	AAF23789*
Bacillus subtilis	48	61	725	742	AAA22679*
Synechocystis sp.	48	61	741	777	BAA16646
Lactobacillus lactis	46	61	731	739	AAD12626
Streptomyces coelicolor	42	59	729	752	CAB56359
Mycobacterium tuberculosis	42	56	748	754	P54876
Campylobacter jejuni	44	62	701	728	CAB73212
Lactobacillus casei	43	58	744	741	P35852
Methanococcus jannaschii	41	56	737	733	Q58660
Escherichia coli	25	41	771	1295	P15254
Salmonella typhimurium	24	40	770	1295	AAB08888
Vibrio cholerae	25	41	667	1297	AAF94031
Pseudomonas aeruginosa	24	40	685	1298	AAG07150

*Defined in the protein library as proteins encoded by the *purQ* gene.

743 amino acids with a predicted size of 79·1 kDa. The ORF is preceded by a putative ribosome-binding site, GAAGGA, at positions −19/−14. The sequence AGCGGCCGAACCGGCT at positions −229/−214 of the putative ATG start codon of the *S. fredii purL* gene shows similarities to the PurBox reported in *Lactococcus lactis* (Kilstrup *et al.*, 1998). In addition, the sequence CGCAAAGCGCCCCCTGTTTGTGTTGCGGTTC at positions −90/−59 from the ATG codon resembles the *E. coli* PurBox (GGCAAACGGTTTCGTC) as defined by Sampei & Mizobuchi (1989). DNA sequences upstream and downstream of the identified ORF do not show clear homology to any known gene. The abundance of stop codons in these two regions indicates that they should not contain any coding region.

Serological studies on the LPS produced by *S. fredii* SVQ295

PAGE showed that SVQ295 carrying cosmid pMUS509 has an LPS profile that is indistinguishable from that of HH103-1 (Fig. 4A, lanes 3 and 1 respectively).

Four mAbs raised against free-living cultures of HH103 (NB7-242.33) or nodule-derived bacteroids (NB3-4.72; NB3-4.79 and NB6-228.22) and that recognize the same pattern of bands that are visible in silver-stained LPS gels of *S. fredii* HH103 were tested for their reactivity against the LPS of mutant SVQ295. Fig. 4 shows that the four mAbs reacted with the HH103-1 LPS bands corresponding to the LPS I and LPS II regions (lane 1 of panels B–E) but failed to recognize any LPS band in mutant strain SVQ295 (lane 2 of panels B–E). SVQ295 carrying pMUS509 or pCOS110 produced LPS that were fully recognized by all the assayed mAbs (lanes 3 and 4 of panels B–E).

Co-inoculation experiments

We investigated the effect of co-inoculating soybean plants with SVQ295 and the *S. fredii* HH103 *nodA* mutant SVQ116, which cannot produce nodulation factors (LCOs) (Buendía-Clavería *et al.*, 1996). Hence, the inoculum mixture was composed of a mutant (SVQ295) that produces LCOs but is unable to infect soybean roots and another one (SVQ116) that should be infective but is totally unable to trigger nodule development. Soybean plants growing in a plant nutritive solution containing adenine were also inoculated with SVQ295 or with SVQ295 and SVQ116.

As expected, single inoculation with SVQ295 only induced pseudonodules on soybean. In this experiment, however, SVQ295 was able to induce non-nitrogen-fixing nodules on soybean when adenine was added to the plant nutritive solution. Soybean plants inoculated with both mutants (in the presence or absence of adenine) formed a mixture of pseudonodules and true nitrogen-fixing nodules (Table 3). No colonies were formed when nodules were directly crushed on TY medium containing streptomycin (chromosomal marker of SVQ295). All bacterial isolates (900 colonies tested) from nodules crushed on TY medium showed the markers of SVQ116 (Rif^r, Km^r, Str^s and prototrophy). These results demonstrate that, regardless of the presence or absence of adenine, only the prototrophic LCO^- mutant SVQ116 was able to penetrate into the plant cells.

DISCUSSION

Previous reports have clearly shown that purine auxotrophic mutants of (*Sino*)*Rhizobium* are symbiotically defective,

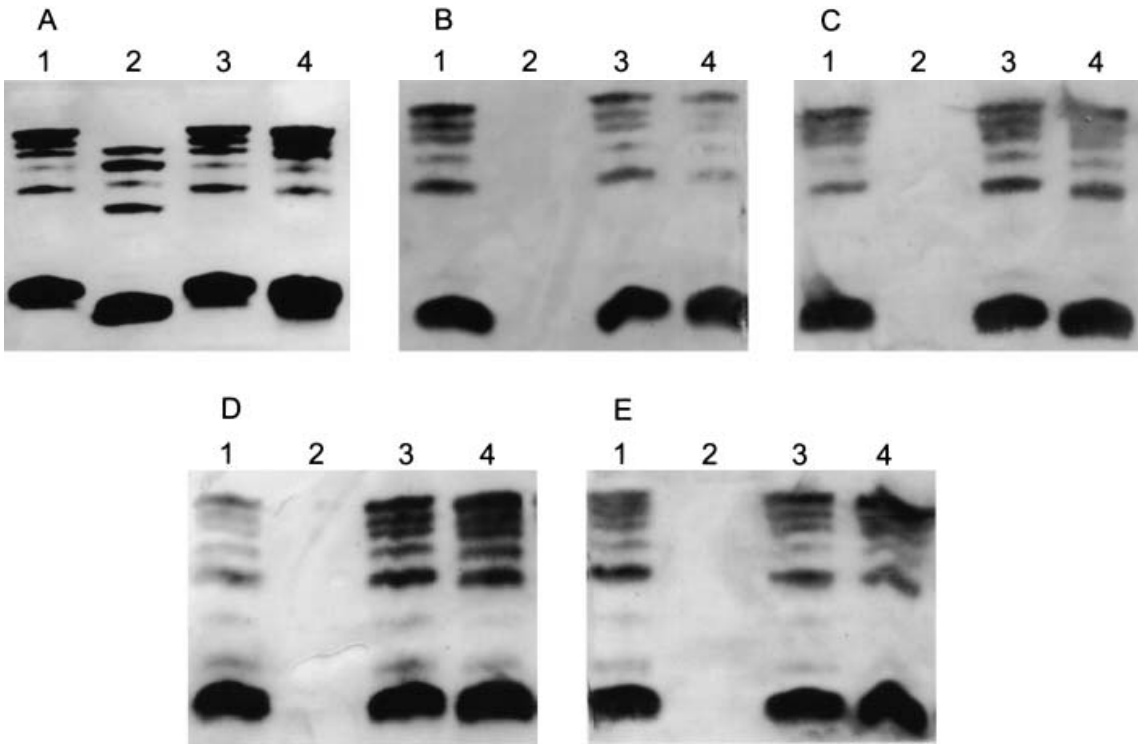

Fig. 4. Silver staining (A) and immunostaining (B–E) of LPS profiles from *S. fredii* strains using the mAbs NB3-4.72 (B), NB3-4.79 (C), NB-6-228.22 (D) and NB7-242.33 (E). Lanes: 1, HH103-1; 2, SVQ295; 3, SVQ295(pMUS509); 4, SVQ295(pCOS110).

forming Fix^- pseudonodules that show centrally located vascular bundles (Pankhurst & Schwinghamer 1974; Denarié *et al.*, 1976; Pain, 1979; Noel *et al.*, 1984, 1988; Newman *et al.*, 1992, 1995; Djordjevic *et al.*, 1996).

A possible explanation for the linking of purine auxotrophy and symbiotic deficiency is that, during nodule formation, adenine might act as a precursor for cytokinins, which are N-6 substituted derivatives of adenine (Pankhurst & Schwinghamer, 1974; Pain, 1979). This possibility does not exclude that adenine auxotrophs might suffer other alterations that, for any reason, could prevent normal nodule development. In fact, purine auxotrophs usually exhibit pleiotropic phenotypes that indicate the occurrence of multiple alterations of the bacterial physiology. For instance, *R. etli* purine auxotrophs expressed, in free-living

Table 3. Co-inoculation of soybean plants cv. Williams with *S. fredii* mutants SVQ116 and SVQ295 in the presence or absence of adenine

Inoculant(s)	Nodulation*	Appearance of plants†	Markers of the nodule isolates‡
SVQ116	Nod^-	Yellow	–
SVQ295	Pseudonodules	Yellow	–
SVQ295 + ade§	Pseudonodules and small nodules (8)	Yellow	Str^r Km^r, Ade^- Thi^- (28)
SVQ116 + SVQ295	Pseudonodules and nodules (41)	Green	Rif^r Km^r Str^s, Ade^+ Thi^+ (50)
SVQ116 + SVQ295 + ade§	Pseudonodules and nodules (43)	Green	Rif^r Km^r Str^s, Ade^+ Thi^+ (40)
HH103-1	Fix^+ (160)	Green	Str^r Km^s, Ade^+ Thi^+ (4)

*Six plants were used for each treatment. The numbers in parentheses indicate the mean number of nodules per soybean plant. Plants were analysed 82 days after inoculation.

†Nitrogenase activity of green soybean plants was confirmed by acetylene reduction assay.

‡The numbers in parentheses indicate the number of nodules analysed. From each nodule 10 colonies were checked for antibiotic resistance and auxotrophy markers.

§Fåhraeus nutritive solution was supplemented with 0·8 μg adenine ml^{-1}.

conditions, higher levels of the *fixNOQP* operon, which codes for the symbiotic cytochrome terminal oxidase ccb_3 (Soberon *et al.*, 1997). Moreover, *Rhizobium* sp. NGR234 *purY* and *purF* mutants exhibited multiple differences in gene expression when compared to the parental wild-type strain (Guerreiro *et al.*, 1998).

In this investigation we have studied an auxotrophic mutant of *S. fredii* HH103 that was isolated in a screen for the appearance of alterations in colony morphology. Mutant SVQ295 requires adenine and thiamin for growth in minimal media, which is in agreement with the fact that the reaction catalysed by the PurL enzyme occurs before AICAR in the biosynthetic pathway for purines (Neuhard & Nygaard, 1987). In addition, SVQ295 can also grow in minimal media if the supplementation with adenine is replaced by the purine precursor AICA-riboside. Although SVQ295 cultures grown in the presence of 0·5 mM AICA-riboside showed a longer doubling time than that observed in MMB medium with adenine, the final optical density was the same in both bacterial cultures (with AICA-riboside or adenine). Fully grown SVQ295 cultures in the presence of AICA-riboside did not contain ade^+ revertants, since bacterial subcultures failed to grow in MMB medium devoid of adenine or AICA-riboside. Hence, we conclude that AICA-riboside can indeed act as a purine source for *pur* mutants. Previous reports showed that *pur* mutants of *S. fredii* HH303 and *R. leguminosarum* bv. *viciae* 128C53 grew poorly 18 h after the addition of AICA-riboside (Newman *et al.*, 1994). Our results also support this observation, since an initial population of approximately 4×10^5 bacteria ml^{-1} in MMB supplemented with AICA-riboside did not show any increase after 5 h incubation and only increased to 7×10^5 bacteria ml^{-1} after 18 h. Hence, the latency period exhibited by SVQ295 cultures in the presence of AICA-riboside and their long doubling time may explain the poor growth of these *pur* mutants during their first hours of incubation in the presence of AICA-riboside. However, after 18 h incubation, a *purF* mutant of *R. etli* did not show poor growth in minimal medium supplemented with AICA-riboside (Newman *et al.*, 1994).

Mutant SVQ295 failed to nodulate six different legumes (see Results), and formed pseudonodules on *G. max*. On *Macroptilium atropurpureum*, small ineffective nodules were formed. Bacterial release in *M. atropurpureum* nodules was only observed in a few clustered plant cells (Fig. 3E). In *Glycyrrhiza uralensis*, however, bacterial release was much more extended through the nodule tissue and nitrogenase activity was detected (Fig. 3C). This variable symbiotic phenotype of SVQ295 might be due to the differential ability of each legume host in supplying purine, or purine precursors, to the bacteria during the nodulation process. The fact that *G. uralensis* forms indeterminate nodules (Fig. 3A) is not relevant to the ability of SVQ295 to infect nodule cells, since *Albizia lebbek* also forms indeterminate nodules (with the parental strain HH103) but fails to nodulate with the *purL* mutant.

Previous reports have clearly shown that the addition of AICA-riboside to the plant nutritive solution promotes nodulation ability of different rhizobial *pur* mutants (Newman *et al.*, 1994, 1995; Djordjevic *et al.*, 1996). In addition to supporting these observations our results also show that, in long-term experiments (82 days), the addition of adenine can also promote the formation of small soybean nodules by mutant SVQ295. This late nodulation appears to be the consequence of the exogenous addition of adenine to the plant root medium because, in the absence of adenine, soybean plants inoculated with SVQ295 only formed pseudonodules (Table 3).

Mutant SVQ295 carries a transposon Tn*5*-Mob insertion between nucleotides 831 and 832 of the sequenced DNA fragment, which corresponds to the 5′ end of a 2690 bp ORF showing homology to the *purL* gene of *S. meliloti*. This ORF would encode a putative polypeptide of 743 amino acids, clearly shorter than the *E. coli* PurL protein (1295 amino acids). The assumption that SVQ295 is mutated in its *purL* gene is supported by the fact that the mutant was complemented by cosmid pCOS110 (it carries *R. etli purYQL* genes), but not by cosmid pJN382A (a derivative of pCOS110 carrying a Tn*5* insertion in the *purL* gene).

In *E. coli*, the *purL* gene encodes a 5′-phosphoribosyl-formylglycinamidine synthase (FGAR amidotransferase) that catalyses the conversion of 5′-phosphoribosyl-*N*-formylglycinamide into 5′-phosphoribosyl-*N*-formyl-glycinamidine (Sampei & Mizobuchi, 1989). In *Bacillus subtilis* and *Lactobacillus casei*, however, this enzyme is formed by the products of two different genes, *purQ* and *purL* (Ebbole & Zalkin, 1989; Peltonen & Mäntsälä, 1999). The PurL enzyme in *E. coli* has three different domains, I, II and III, which have been assigned as potential ATPase, triosephosphate-isomerase-like isomerase, and glutaminase, respectively (Sampei & Mizobuchi, 1989). The *E. coli* PurL domain III aligns with the *B. subtilis* and *L. casei* PurQ protein, while domains I and II align with the PurL protein of *B. subtilis*, *L. casei* and *S. fredii* HH103. This indicates that *S. fredii* HH103 might also need at least two different genes for the formation of a functional FGAR amidotransferase. Other bacteria, such as *Methanococcus jannaschii*, *Mycobacterium tuberculosis* and *Synechocystis* sp. also appear to have a multi-subunit form of the FGAR amidotransferase enzyme (Bult *et al.*, 1996; Jackson *et al.*, 1996; Kaneko *et al.*, 1996). In all these cases, in which the enzyme appears to be composed of different subunits, the ORF defined as *purL* encodes a polypeptide of 705–777 amino acids, which is clearly shorter than the FGAR amidotransferase (1295 amino acids) encoded by the *purL* gene of *E. coli* (Table 2).

The *L. casei* and *B. subtilis purQ* and *purL* genes belong to an operon composed of a cluster of other purine genes, while the *E. coli purL* gene constitutes a single transcriptional unit which is independent of other purine genes. The *purL* of *S. fredii* HH103 also appears to constitute a single transcriptional unit, since putative ORFs were not found

upstream (460 bp) or downstream (178 bp) of the *purL* gene.

The fact that colonies produced by mutant SVQ295 in complete TY medium differ in morphology from those formed by its prototrophic parental strain HH103-1 prompted us to investigate the existence of possible alterations in surface polysaccharides. *S. fredii* HH103 produces a capsular homopolysaccharide (also called K-antigen polysaccharide, or KPS) in which the repeating unit is a derivative of the pseudaminic acid (Gil-Serrano *et al.*, 1999). NMR analyses of the KPS produced by mutant SVQ295 showed a spectrum that was similar to that previously reported for its parental strain HH103 (Fig. 2). Hence, we conclude that a major change in the structure has not occurred, although other changes (such as subtle alterations or differences in the level of polymerization) cannot be discarded.

In contrast, the LPS electrophoretic profile of mutant SVQ295 is distinct from that shown by HH103-1 (Fig. 1), a clear indication that the *purL* mutation has affected the bacterial LPS structure. This possible alteration in the LPS produced by SVQ295 was further confirmed by the finding that four mAbs that react against the HH103 LPS failed to recognize the LPS produced by the *pur* mutant. The presence of cosmids pMUS509 and pCOS110 in SVQ295 restored the wild-type LPS electrophoretic profile and also the presence of epitopes recognized by the four mAbs (Figs 1 and 4). Since these two cosmids also corrected the purine auxotrophy and restored effective nodulation on soybean, we conclude that the *purL* mutation is actually responsible for the pleiotropic phenotype exhibited by mutant SVQ295. The fact that cosmid pJN382A (a derivative of cosmid pCOS110 carrying a Tn*5* insertion in the *purL* gene) failed to correct the LPS profile of mutant SVQ295 supports this hypothesis. The PurL protein acts in the purine biosynthetic pathway before the formation of AIR (5′-phosphoribosyl-5-aminoimidazole), an intermediate compound from which a pathway for thiamin biosynthesis emerges. This fact explains why *purL* mutants require the presence of adenine and thiamin to grow in minimal medium. We have isolated another HH103 derivative (SVQ526) that forms Fix$^-$ pseudonodules on *Glycine max* and requires the presence of adenine, but not thiamin, to grow in minimal medium. The gene mutated in SVQ526 has not been identified. The LPS profile of mutant SVQ526 is clearly different from that shown by mutant SVQ295 (Fig. 1C, lane 2). Although the LPS profile of SVQ526 appears similar to that of its wild-type parent HH103 (Fig. 1C, lanes 2 and 3), some differences can be observed, suggesting that the LPS of mutant SVQ526 has also suffered some alterations. Alterations of the SVQ295 and SVQ526 LPS were detected in bacteria cultures grown on complete TY medium; therefore a functional purine biosynthetic pathway appears to be necessary for the production of LPS showing the wild-type electrophoretic profile and for the expression of epitopes recognized by the mAbs assayed.

Previous reports have shown that a *purY* mutant (ANU2866) of *Rhizobium* sp NGR234 does not show any apparent alteration in the electrophoretic profile of its LPS. However, a *purM* mutant of NGR234 (ANU2861) produces a LPS that differs from that of the wild-type strain (Djordjevic *et al.*, 1996). The presence of AICA-riboside in ANU2861 cultures eliminated these differences, in contrast to our observation that AICA-riboside does not restore the wild-type LPS electrophoretic profile in *S. fredii* SVQ295. All these results show that although the mentioned *pur* genes (*purM* and *purY* of NGR234 and *purL* of *S. fredii*) code for enzymes acting before the formation of AICAR, their respective mutants exhibit differences in their pleiotropic phenotypes. It is also possible that the effect of *pur* mutations on rhizobial surface polysaccharides varies among different strains even if they are closely related, such as *S. fredii* and *Rhizobium* sp. NGR234.

LPS-defective mutants of *R. leguminosarum* triggered a host defence response in pea and induced ineffective nodules (Perotto *et al.*, 1994). Similarly, the LPS defective *purM* mutant of NGR234 also induced on *Macroptilium atropurpureum* a reaction analogous to a hypersensitive response at the site of infection (Djordjevic *et al.*, 1988). These facts suggest that it is possible that the defective symbiotic response of SVQ295 on soybean and other legumes might be due to a combinative effect of the purine auxotrophy and bacterial LPS alterations.

In general, co-inoculation of legume plants with Exo$^-$ and Nod$^-$ mutants results in the formation of nodules that are always occupied by both bacterial strains (Klein *et al.*, 1988; Müller *et al.*, 1988; Kapp *et al.*, 1990). These results led to the notion that the successful formation of nodules required the cooperation of both co-inoculants during the whole nodulation process. However, Djordjevic *et al.* (1996) described that *M. atropurpureum* plants co-inoculated with *purM* and *exoY* mutants of NGR234 (ANU2861 and ANU2811, repectively) formed Fix$^-$ nodules that only contained the *exoY* co-inoculant. In addition, no nodules were formed when ANU2861 and ANU265 (a pSym$^-$ derivative of NGR234) were used as co-inoculants.

In this report we show that co-inoculation of soybean plants with mutants SVQ295 and SVQ116 (a *nodA* derivative of *S. fredii* HH103) produced nodules that were only occupied by the non-nodulating *nodA* mutant. Although the results presented in Table 3 correspond to soybean plants analysed 82 days after inoculation, it is not a requisite to grow soybean plants for such a long time to find nodules formed by co-inoculation with SVQ295 and SVQ116. In additional experiments we have found that a few nodules can be formed 24 days after inoculation (data not shown). Although *pur* mutants can enable *exo* or *nod* mutants to penetrate inside the root and to invade plant cells, the former appears to be outcompeted by the prototrophic co-inoculant. This circumstance makes *pur* mutants a very suitable 'launcher strain' for introducing into legume nodules certain rhizobial mutants that, by themselves, are

not able to infect plant roots. In general, rhizobial mutants affected at very early events of the nodulation process cannot be investigated for their behaviour at late nodulation stages. Since the '*pur*-mutant launchers' appear to be absent inside the nodules, the behaviour of the non-infective co-inoculant can be studied inside the nodules.

ACKNOWLEDGEMENTS

This work was supported by the Comisión Interministerial de Ciencia y Tecnología grant BIO99-0614-C03 and the PAI-II scientific programme of the Andalusian government. We are grateful to Teresa Cubo for providing strain SVQ116 and to Rocío Gutiérrez for technical assistance with the LPS work. We also thank Josep Casadesús for critical reading of the manuscript.

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