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A *pyrF* auxotrophic mutant of *Sinorhizobium fredii* HH103 impaired in its symbiotic interactions with soybean and other legumes

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Summary. Transposon Tn*5*-Mob mutagenesis allowed the selection of a *Sinorhizobium fredii* HH103 mutant derivative (SVQ 292) that requires the presence of uracil to grow in minimal media. The mutated gene, *pyrF*, codes for an orotidine-5´-monophosphate decarboxylase (EC 4.1.1.23). Mutant SVQ 292 and its parental prototrophic mutant HH103 showed similar Nod-factor and lipopolysaccharide profiles. The symbiotic properties of mutant SVQ 292 were severely impaired with all legumes tested. Mutant SVQ 292 formed small ineffective nodules on *Cajanus cajan* and abnormal nodules (pseudonodules) unable to fix nitrogen on *Glycine max* (soybean), *Macroptitlium atropurpureum, Indigofera tinctoria*, and *Desmodium canadense*. It also did not induce any macroscopic response in *Macrotyloma axillare* roots. The symbiotic capacity of SVQ 292 with soybean was not enhanced by the addition of uracil to the plant nutritive solution. [**Int Microbiol** 2007; 10(3):169-176]

Key words: *Sinorhizobium fredii* HH103 · *Macrotyloma axillare* · *pyrF* · symbiotic defects · soybean · legumes

Introduction

In the symbiotic relationship that is established between rhizobia and their host legumes, a complex set of molecular interactions takes place during the development and maintenance of nitrogen-fixing nodules on the plant roots. Inside the nodules, rhizobia provide the plant with an assimilable source of nitrogen. In return, the legume supplies compounds that can be used by the bacteria as sources of carbon and energy [14]. Analyses of the symbiotic properties of auxotrophic rhizobial mutants are one way to identify some of the metabolic functions that are necessary to establish a successful symbiotic relationship.

Symbiotically-defective auxotrophic mutants have been isolated from several fast-growing strains of rhizobia, and their effects in the nodulation process are highly variable [reviewed in 14]. Auxotrophic mutants for leucine, histidine, ornithine, aspartic acid, or cysteine induce ineffective nitrogen-fixing nodules. However, the nodules induced by *Sinorhizobium meliloti* mutants affected in glutamine biosynthesis are nitrogen-fixing, which indicates that this amino acid is not essential for symbiotic nitrogen fixation in alfalfa plants. Furthermore, *S. meliloti* auxotrophic mutants for glycine are more effective in nitrogen fixation than their parental prototrophic strain.

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Purine auxotrophs (*pur* mutants) of most rhizobia species are unable to effectively nodulate their host plants [7,12,14]. While adenine auxotrophs of *Rhizobium leguminosarum* bv. *viciae* are unable to nodulate pea plants, those of *S. meliloti* form nodules on alfalfa that fail to fix nitrogen [14]. A purine auxotrophic mutant of *Rhizobium* sp. NGR234 elicited root-hair curling and nodule-meristem initiation on *Macroptilium atropurpureum*, but infection threads (inward-facing tubular structures through which rhizobia penetrate the root) were not observed [14]. A *purL* mutant of *S. fredii* HH103 induces pseudonodules on soybean roots but it is still able to induce a few nitrogen-fixing nodules on *Glycyrrhiza uralensis* [7]. In contrast, a *purF* mutant of *R. leguminosarum* bv. *trifolii* L1 has been described to retain the capacity to induce fully developed nitrogen-fixing nodules on its legume host [24].

A pyrimidine auxotrophic *R. etli* mutant, unable to synthesize uracil, induces small ineffective pseudonodules on *Phaseolus vulgaris* (bean). Similarly, uracil auxotrophic derivatives of *S. meliloti* 2001 and *S. fredii* HH303 only induce pseudonodules on alfalfa and soybean, respectively [14]. *S. meliloti* Rmd201 pyrimidine auxotrophic mutants affected in carbamoyl phospate synthetase (*car)* and in pyrimidine biosynthesis (*pyr*) induce white ineffective nodules on alfalfa plants. The nodules induced by *pyr* mutants are more advanced than those induced by *car* mutants [22].

In some cases, rhizobia require a particular intermediate of a biosynthetic pathway (but not the end products) to establish successful symbiotic interactions. Studies with tryptophan auxotrophic mutants of *S. meliloti* suggested that anthranilate synthesis, rather than tryptophan, is required for bacteroid development in alfalfa nodules [14]. Information about the importance of the different metabolic pathways for the *Sinorhizobium fredii* symbiotic capacity is scarce. Interestingly, *S. fredii* strains show a broad host-range of nodulation, a characteristic that facilitates studies of the impact of any particular mutation on the bacterial symbiotic capacity with legumes in which determined or undetermined nodules are formed [8]. In this work, we characterized genetically and symbiotically a uracil auxotrophic mutant of *S. fredii* HH103 affected in the *pyrF* gene.

Material and methods

Strains, media, and genetic manipulations. Bacterial strains and plasmids used in this work are listed in Table 1. *Sinorhizobium* strains were grown in TY [2], yeast mannitol (YM) [23], B^- [20], or MMB [1] minimal medium at 28ºC. Auxotrophic strains were plated on MMB solidified with 1.5% (w/v) agarose. *Escherichia coli* was cultured in Luria-Bertani (LB) medium [16] at 37ºC. When required, the media were supplemented with the appropriate antibiotics, as described by Lamrabet et al. [10], or with uracil at 12 µg/ml.

Plasmids were transferred by conjugation as described by Simon [17]. Tn*5*-Mob mutagenesis of *S. fredii* strain SVQ269 was carried out using the suicide plasmid pSUP5011 [17]. *Sinorhizobium* plasmid profiles were obtained using the Ekhardt method, as described by Ollero et al. [13]. Those substances required by the auxotrophic mutant SVQ292 to grow in minimal media were identified as described by Beringer et al. [3]. DNA was manipulated according to the general protocols of Sambrook et al. [16]. The primer pdad2 (5′ AGATTTAGCCCAGTCG 3′), located inside the IS50R of transposon Tn*5*-Mob, was used as a primer to sequence the rhizobial DNA adjacent to the right site of the inserted transposon in mutant strain SVQ292.

The UWGCG program was used for basic DNA sequence analysis and assembly. The NCBI ORF-Finder [http://www.ncbi.nlm.nih.gov/gorf/gorf.html] and BLAST [http://www.ncbi.nlm.nih.gov/BLAST/] programs

Table 1. Bacterial strains and plasmids used in this work

Strains	Derivation and relevant properties	Source or reference
Sinorhizobium fredii		
SVQ269	HH103 Rif^r	[6]
SVQ292	SVQ269 *pyrF*::Tn5-Mob	This work
Escherichia coli		
HB101	Restriction-minus, *recA* background, Str^r	[4]
Plasmids		
pBluescript	Cloning vector, Ap^r	Stratagene®
pSUP202	pBR325 containing the Mob region subcloned in Sau3A, Ap^r, Cm^r, Tc^r	[18]
pSUP5011	Suicide plasmid carrying the Tn*5*-Mob transposon	[19]
pMUS390	pSUP202 derivative plasmid harboring about 0.5 kb of SVQ292 DNA adjacent to right of Tn*5*-Mob transposon	This work
pMUS344	Cosmid pLAFR1 carrying the *pyrF* gene of S. fredii HH103	This work

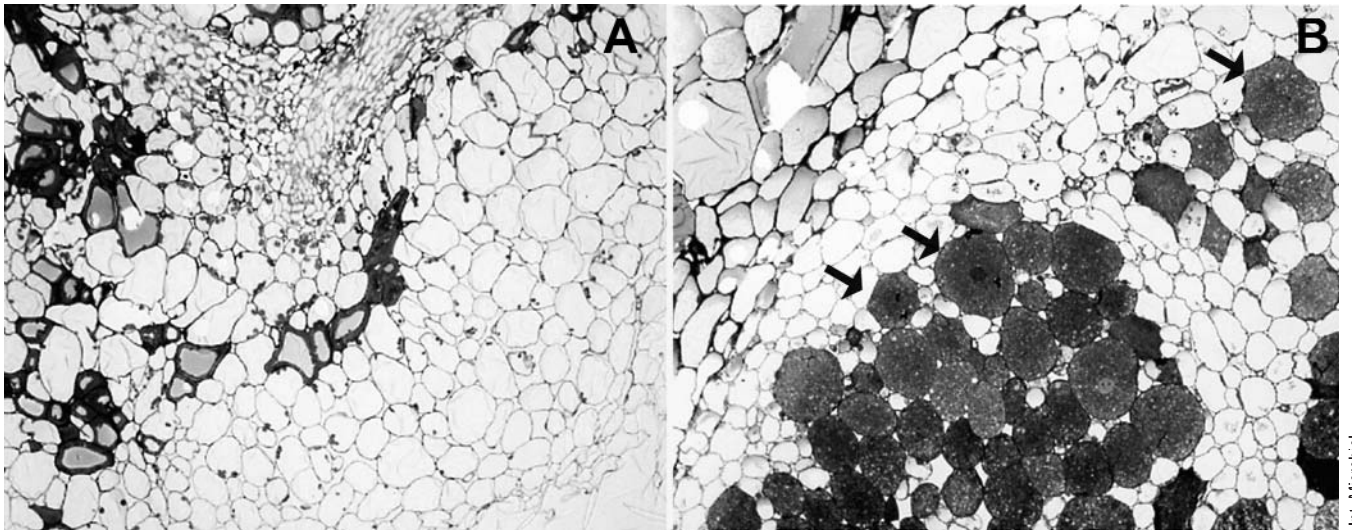

Fig. 1. Toluidine blue-stained semithin sections of pseudonodule induced by the pyrF mutant SVQ292 (**A**) and by the parental strain SVQ269 (**B**) on soybean *Glycine max* cv. Williams. The arrows mark nodule cells full of bacteroids. (Magnification, ca. 16×)

were used for open reading frame (ORF) identification and homology searches, respectively. The ClustalW and Box-shade programs were used to align peptide sequences. Nucleotide and/or amino acid sequence data are to be found at the EMBL database as accession no. AF311321.

In vivo isolation of SVQ292 mutation. The mutated gene in SVQ292 was cloned following the method described by Madinabeitia et al. [11] and was used to construct plasmid pMUS390. This plasmid harbors about 0.5 kb of the SVQ292 DNA adjacent to the IS*50*R of the inserted transposon.

Plant assays. Nodulation assays on *Glycine max* (L.) Merr. cv. Williams, *Cajanus cajan* (L.) Millsp., *Desmodium canadense* (L.) DC, *Macroptilium atropurpureum* (Moc.& Sessé ex DC) Urb, *Indigofera tinctoria* (L.), and *Macrotyloma axillare* (E. Mey) Verdc, were carried out as described by Buendía-Clavería et al. [6]. Germinated seeds were transferred to Leonard jars containing sterilized vermiculite supplemented with Fåhraeus nutrient solution [23]. Each Leonard jar contained two plants. Each plant was inoculated with about 10^8 bacteria and then grown for 40 days with a 16 h-photoperiod at 25ºC in the light and 18ºC in the dark [8]. Nitrogen fixation was determined by acetylene reduction assay [5]. Plant tops were dried at 80ºC for 48 h and weighted. Bacteria were isolated from surface-sterilized nodules as previously reported [10]. When required, the Fåhraeus plant nutritive solution was supplemented with uracil at 12 µg/ml.

Lipo-chitin oligosaccharide (LCO) detection. Bacteria were grown in 5 ml of B^- medium in the presence of the inducer naringenin (3.6 µM) and *N*-acetyl[^{14}C] glucosamine. The ^{14}C-labeled LCO were analyzed by reverse thin-layer chromatography, as described by Spaink et al. [20].

Lipopolysaccharide SDS/PAGE analysis. To investigate lipopolysaccharide (LPS) profiles, bacterial cultures were grown on solid TY medium. Bacterial cells were washed in 0.9% NaCl and pelleted by centrifugation. Treatments applied to bacterial pellet, electrophoresis of crude bacteria extracts, and the silver-staining procedures were done as described by Buendía-Clavería et al. [7].

Microscopic studies. 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 (v/v) 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 and characterization of a *S. fredii* HH103 Tn*5*-Mob auxotrophic mutant. S. *fredii* SVQ269 (= HH103 Rif^r) was subjected to Tn*5*-Mob mutagenesis using plasmid pSUP5011, after which transconjugant colonies were selected on TY supplemented with rifampicin and kanamycin. In order to select auxotrophic mutants, transconjugants were replica-plated on MMB solidified with agarose. One of the colonies that failed to grow in MMB was selected for further investigation (auxotrophic mutant SVQ292). SVQ292 was able to grow in MMB if the minimal medium was supplemented with uracil.

In order to identify and subclone the gene mutated in SVQ292, plasmid pMUS390 was obtained as described in Material and Methods. This plasmid harbors about 0.5 kb of the rhizobial DNA adjacent to the IS*50*R of the Tn*5*-Mob inserted in mutant SVQ292. An *Eco*RI*/Xho*I 0.9-kb fragment of pMUS390 was cloned into pBluescript and about 450 bp were sequenced by using primer pdad2. Computer analysis of this sequence indicated homology with the *pyrF* gene of *S. meliloti*, which encodes an orotidine-5´-monophosphate decarboxylase (EC 4.1.1.23). This enzyme catalyzes the final step in pyrimidine biosynthesis, the conversion of orotidine-5´-monophosphate (OMP) to uridine-5´-monophosphate.

SVQ292 only induced pseudonodules on soybean cv. Williams that did not fix nitrogen. Microscopic analysis showed that plant cells of these pseudonodules were devoid of bacteria, while many cells of nodules induced by the parental strain SVQ269 were filled of rhizobia (Fig. 1A,B). This symbiotic defect was used to construct a genomic library of the wild-type strain HH103 (in cosmid pLAFR1) and then to isolate the wild-type *pyrF* gene. The genomic library was transferred "en masse" to mutant SVQ292, and the tetracycline-resistant transconjugants were used to inoculate soybean cv. Williams plants. Soybean roots developed many pseudonodules and a few nitrogen-fixing nodules. All bacteria re-isolated from the effective nodules were Tc^r (presence of cosmid pLAFR1), Nm^r, and Km^r (presence of Tn*5*-Mob) and contained the same cosmid. This cosmid, named pMUS344, was isolated, introduced into *E. coli* HB101, and then re-transferred to the *pyrF* mutant SVQ292. Strain SVQ292 (pMUS344) was able to grow in MMB without uracil and formed normal nitrogen-fixing nodules on soybean.

A 1.5-kb *Pst*I fragment of cosmid pMUS344, which positively hybridized to the 0.9-kb *Eco*RI/*Xho*I fragment of plasmid pMUS390, was subcloned into pBluescript. The 1511 bp sequence appears in the EMBL nucleotide data library as accession number AF311321. By matching this sequence with that obtained by partial sequencing of pMUS390 using the IS50R primer pdad2, it was possible to locate the insertion of the transposon Tn*5*-Mob at nucleotide 955 of the 1511-bp sequence.

The sequenced fragment comprised one complete and two partial ORFs, each with the same polarity and with a high probability of encoding proteins, as indicated by the Testcode algorithm. The left side of the sequenced fragment contained the 3´ fragment of a putative ORF that is 87.5% identical to *smc00413*, a putative *S. meliloti* gene of unknown function (GenBank accession no. AL591783). The putative protein encoded by this HH103 ORF was 66% identical to protein AGR_C_57 of *Agrobacterium tumefaciens* C58 (NP_353329) and contained the conserved domain GpmB, which is characteristic of fructose 2,6 biphosphatases (EC 3.1.3.46).

The second ORF was 85% identical to the *pyrF* gene of *S. meliloti*. This ORF began at position 397 and extended for 702 bp, encoding a deduced polypeptide of 234 amino acids with a predicted molecular mass of 24.5 kDa. In mutant SVQ292, the Tn*5*-Mob transposon was inserted 558 nucleotides after the start codon of the *pyrF* gene. At position –79/–66 there is a putative $GGN_{10}GC$ motif (GGcaatctc tttGC), described as a promoter [21]. The percentages of identity between the *S. fredii* HH103 PyrF protein and those of different rhizobia and other gram-negative bacteria are shown in Table 2. In addition, alignment of the PyrF proteins of *S. fredii* HH103 and different rhizobial strains is shown in Fig. 2.

The final 175 nucleotides (positions 1323–1511) corresponded to the 5´ region of a putative ORF that is 82% identical to the *S. meliloti* gene *smc00411*, which encodes a hypothetical protein of 95 amino acids. In the *S. fredii* sequence, the intergenic region between *pyrF* and *sm00411* spans 221

Table 2. Comparison of HH103 PyrF protein with PyrF proteins from different bacteria

Bacterial strains	Identity (%)	Similarity (%)	Length of the PyrF protein (amino acids)	Amino-acid overlap	Accession number
Sinorhizobium meliloti	86	94	234	233	CAC41769
Sinorhizobium medicae	83	91	232	231	ABR62357
Mesorhizobium sp. BNC1	79	89	249	223	ZP_00194064
Rhizobium leguminosarum bv. *viciae*	79	89	235	228	CAK05821
Rhizobium etli	78	89	235	228	ABC89136
Agrobacterium tumefaciens	77	88	255	225	AAK86113
Mesorhizobium loti	74	87	237	225	BAB51336
Brucella suis	71	85	238	227	AAN31018
Brucella melitensis	70	85	259	227	AAL53180
Bartonella bacilliformis	68	84	235	225	Q44843
Bradyrhizobium japonicum	54	72	237	229	CAB91880
Vibrio cholerae	40	60	231	215	AAF95059
Pseudomonas aeruginosa	38	57	232	231	CAA46564
Escherichia coli	36	55	245	227	NP_415797

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Sf HH103        1 ------------------MSTSARDRLIVGLDLPTIAEAERIVSTLGDDVAFYKIGYQLA
Sm 1021         1 ------------------MSTSARDRLIVGLDLPTVTEAEKIVSTLGEEVLFYKIGYQLA
Sme WSM419       1 ------------------   MSARDRLIVGLDLPTLAEAEKIVSALGEEVLFYKIGYQLA
Msp BNC1        1 ---MRLGADADISGELTMPADEMRGRLIVGLDVPTLAEAEKVVSALGDTVSFYKIGYQLA
Rlv 3841        1 --------------------MGARERLIVGLDVPTIGEAERLVSTLGDDILFYKIGYQLV
Re CFN42        1 --------------------MDARERLIVGLDVPTIGEAERLVSTLGDDILFYKIGYQLV
At C58          1 MQRPPPHATGRQWKHGRDKTMTAREKLIVGLDVPTVQQAEDIVSKIGDEVLFYKIGYQLV
Ml MAFF303099   1 ------------MQAKSIQAQSIQERLIVGLDLPTVREAEQAVRELDGVVSFYKIGYQLA

Sf HH103       43 FAGGLEFARDLAASGKKVFLDMKLLDIDNTVAKGVENIVRMGMSMLTLHAYPKAMKAAVD
Sm 1021        43 FAGGLDFARDLAASGKQVFLDMKLLDIDNTVAKGVENIVKMGVSMLTLHAYPKAMKSAVE
Sme WSM419      41 FAGGLDFARDLAASGKQVFLDMKLLDIDNTVAKGVENIVKMGVSMLTLHAYPKAMKSAVE
Msp BNC1       58 FAGGLDFAGDLIRSGRQVFLDMKLLDIDNTVAKGVENIARMGVAMLTLHAYPKAMRAAVA
Rlv 3841       41 FAGGLEFARDLAASGKKIFLDMKLLDIDNTVASGVENIAKMGMSMLTLHAYPKAMKAAVE
Re CFN42       41 FAGGLEFARDLAASGKKIFLDMKLLDIDNTVASGVENIAKMGMSMLTLHAYPKAMRAAVE
At C58         61 FAGGLEFARDLVQSGKKVFLDMKLLDIDNTVASGVENIARMGMSMLTLHAYPKAMRAAVK
Ml MAFF303099  49 FAGGLDFARELASGGTKIFLDMKLLDIDHTVAKGVENIVKMGMTMLTIHAYPKAMRAAVE

Sf HH103      103 AAKGSDLCLLGVTVLTSMDAQDVIDAGYGSDPHTLVLRRAEQARAAGMGGIVCSAEEATA
Sm 1021       103 AARGSNLCLLGVTVLTSMDEQDVIDAGYEYDPHSLVLRRAEQARAAGMGGIVCSAEEAAA
Sme WSM419     101 AARGSNLCLLAVTVLTSMDEQDVMDAGYNYDPQSLVLRRAEQAHAAGMGGIVCSAAEAAA
Msp BNC1      118 AAEGSGLCLLGVTVLTSMDAQDLIDAGYEHDPQTLVLRRAEQARAAGMGGVVCSAVEAAL
Rlv 3841      101 AAAGSGLCLLGVTVLTSMDADDLAEAGYSQDPHSLVLRRAGQARAAGMGGIVCSAEEAAA
Re CFN42      101 AAAGSGLCLLGVTVLTSMDAEDLAEAGYNQDPHSLVLSRAGQARAAGMGGIVCSAAEAAE
At C58        121 AAEGSGLCLLGVTVLTSMDDSDLVEAGYASDARSLVLRRAEQAREAGMGGIVCSAEESTA
Ml MAFF303099 109 AARGSDLCLLAVSVLTSMDEQDMIDVGYEYDPHTLVLRRSEQALHAGMGGIVCSAAEAEA

Sf HH103      163 VRRIIGAEMAVVTPGIRPTGAEKGDQKRVMTPAEAIRAGSSHLVVARPIVKAADPLSASR
Sm 1021       163 VRKIIGGDMALVTPGIRPAGAEKGDQKRVMTPADALRAGSSHLVVGRPIVAAPDPLAASR
Sme WSM419     161 VRKIIGGDMALVTPGIRPAGAEKGDQKRVMTPADALAAGSSHLVVGRPIVAAPDPLAASR
Msp BNC1      178 VRSVVGPDMAVVTPGIRPAGADHGDQKRVVTPADAIRAGASHLVVARPIVAADDPKSAAE
Rlv 3841      161 VREIVGPDMAIVTPGIRPDGSDKGDQKRVMTPFDALKAGATHLVVGRPIVKAPDPRDAAR
Re CFN42      161 VREVVGPDMAIVTPGIRPTGSDKGDQKRVMTPFDALKAGSTHLVVGRPIVKAPDPKQAAR
At C58        181 VREILGPDLAVVTPGIRPAGADLGDQKRVMTPYDAIKAGSSHLVVARPIVRAEDPKAAAR
Ml MAFF303099 169 VRRIVGPDMAVVTPGIRPAGSDHGDQKRVVTPAQAIRNGASHLVVARPIVAASDRRAAAQ

Sf HH103      223 AILAEMEGVLSA---
Sm 1021       223 AILAEMESALSR---
Sme WSM419     221 AILAEMESALSG---
Msp BNC1      238 AILREMHRAVGN---
Rlv 3841      221 AILSEMVSALWPANR
Re CFN42      221 AVLNEMVGALWPANR
At C58        241 AILDDMLRASFPANQ
Ml MAFF303099 229 AILDEMRSA------
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Fig. 2. Alignment of the PyrF proteins of different rhizobia. Sf, *Sinorhizobium fredii*; Sm, *S. meliloti*; Sme, *S. medicae*; Msp, *Mesorhizobium* sp.; Rlv, *Rhizobium leguminosarum* bv. *viciae*; Re, *Rhizobium etli*; At, *Agrobacterium tumefaciens*; Ml, *Mesorhizobium loti*.

nucleotides, instead of the 106 nucleotides present in the corresponding region of *S. meliloti*.

Symbiotic properties of mutant SVQ292 on several legumes. *S. fredii* HH103 is considered a broad host-range nodulation strain. Thus, we studied whether the mutation in *pyrF* could affect the symbiotic capacity of HH103 with different legumes, as it did for soybean. Mutant SVQ292 formed pseudonodules with *Glycine max* cv. Williams, *M. atropurpureum*, *I. tinctoria*, and *D. canadense*, and ineffective nodules with *C. cajan*. Inoculation of *M. axillare* with mutant SVQ292 did not induce any macroscopic root response.

Plant tests were carried out to determine whether the presence of uracil in the plant nutritive solution would comple-

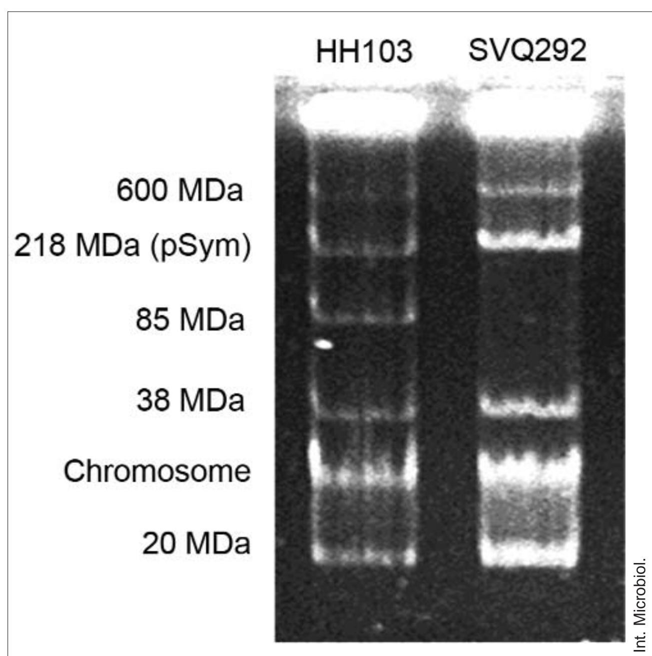

Fig. 3. Plasmid profiles of *S. fredii* SVQ269 and its *pyrF* derivative SVQ292. The indicated molecular masses of the plasmids are approximate.

ment the symbiotic defects of mutant SVQ292. The presence of uracil did not enhance the symbiotic capacity of SVQ292 with any of the legumes tested. Since SVQ292 was symbiotically defective, we decided to investigate whether this mutant was altered either in symbiotic signals (such as LCO and LPS) or in its plasmid profile. The LCO and LPS profiles produced by SVQ292 were similar to those observed for the parental strain SVQ269, as demonstrated by thin-layer chromatography and SDS-PAGE, respectively (data not shown). However, plasmid agarose electrophoresis showed that, apparently, SVQ292 lacked a cryptic plasmid of about 85 MDa (Fig. 3).

Discussion

Purine and pyrimidine auxotrophic rhizobial mutants are usually unable to nodulate their host plants or to induce nitrogen-fixing nodules [7,14]. Vineetha et al. [22] obtained different pyrimidine auxotrophs of *S. meliloti* that induce white ineffective nodules on alfalfa plants. These reports suggest that the expression of some purine and pyrimidine biosynthetic genes is essential for an effective symbiosis of rhizobia with host plants.

We cloned and sequenced a 1.5-kb *Pst*I DNA fragment that contained the *pyrF* gene of *S. fredii* HH103. This DNA fragment harbored three ORFs (5´ *sm00413-pyrF-smc00411* 3´) with the same organization as the corresponding ORFs of *S. meliloti*. The only difference detected was in the intergenic region between *pyrF* and *smc00411*. In *S. meliloti*, this region spans 106 nucleotides. Of these, only the six immediately after *pyrF* and the 54 preceding *smc00411* are conserved in HH103. Moreover, between these two conserved fragments, HH103 harbors 161 additional nucleotides without homology to any known DNA sequence. In *M. loti*, a gene homologous to *smc00411* is located 17 nucleotides after the end of *pyrF*. However, the *mrl7505* gene (NC_002678), which is homologous to *smc00413*, is at a distance of 3000 kb from the other two genes.

In this work, we found that strain SVQ292, a *pyrF* mutant of *S. fredii* HH103 that requires uracil to grow, induced Fix$^-$ pseudonodules on plants—including *Glycine max* cv. Williams and even *Macrotyloma axillare*—that were effectively nodulated by the parental strain. These results suggested that in *S. fredii* HH103 the *pyrF* gene, which encodes orotidine-5´-monophosphate decarboxylase, is essential for the establishment of an effective symbiosis between the bacterium and its hosts. The symbiotic impairment with *M. axillare* should occur at the very early stages of nodulation, since neither pseodunodules nor any other macroscopic root responses to the presence of SVQ292 were observed. Pseudonodules induced by SVQ292 on Williams soybean were devoid of bacteria (Fig. 1A). Similarly, pyrimidine auxotrophs of *S. meliloti* 2011 induced white ineffective nodules in alfalfa plants. The alfalfa nodules induced by *S. meliloti pyrE*/*pyrF* mutants are more developed than those induced by a *pyrC* mutant, and the latter are more advanced than those induced by *car* mutants [22]. In the pyrimidine biosynthetic pathway, enzymes encoded by *car* genes act earlier than Pyr enzymes. All these findings indicated that the pyrimidine biosynthetic pathway, or a particular intermediate, is essential for nodule morphogenesis. Also, the impaired nodule development caused by the auxotrophy varies according to the position at which the biosynthetic pathway is blocked.

The symbiotic defect of SVQ292 was not reverted by the addition of uracil to the nutritive plant solution. Similar results were described for pyrimidine auxotrophic mutants of *R. etli* CFN42, *S. fredii* HH303, and *S. meliloti* Rmd2, in which the addition to the plant nutrient medium of uracil or any other intermediate in the biosynthetic pyrimidine pathway does not restore the symbiotic defects [14,22]. However, in different rhizobial purine auxotrophs, the addition of AICA-riboside (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, an intermediate in the biosynthetic purine pathway) to the plant nutritive solution significantly enhances nodule development [7,12,14]. This is also the case

of other auxotrophic rhizobial mutants that induce ineffective nodules but are able to effectively nodulate their host plants if the auxotrophic requirement is added to the plant nutritive solution. For example, histidine auxotrophs of *Bradyrhizobium japonicum* and *S. meliloti* and a riboflavin-requiring mutant of *R. leguminosarum* induce nitrogen-fixing nodules on their host plants only when the nutritive plant solution is supplemented with the required growth factor [14].

SVQ292 lacked a cryptic plasmid of about 85 MDa (Fig. 3) but this was not the cause of the mutant's symbiotic defect because SVQ292 harboring cosmid pMUS344, which contained the wild-type *pyrF* gene, induced nitrogen-fixing nodules on soybean. These results suggested that the cryptic plasmid cured in mutant SVQ292 is not essential for the establishment of an effective symbiosis between *S. fredii* HH103 and soybean.

Nodulation factors (LCO) and rhizobial surface polysaccharides act as signal molecules in the nodulation process. Rhizobial mutants that do not produce LCO are totally unable to initiate nodule formation [10,20]. Many rhizobial mutants affected in the production of bacterial surface polysaccharides form ineffective pseudonodules that are devoid of bacteria [14]. Since a leucine auxotrophic mutant of *S. meliloti* is unable to produce LCO [15] and a purine auxotrophic mutant of *S. fredii* has an altered LPS profile [7], we investigated whether mutant SVQ292 produced normal LCO and LPS profiles. Indeed, the the LCO and LPS profiles produced by SVQ292 were similar to those produced by its wild-type parental strain HH103. Thus, the symbiotic impairment of SVQ292 was not apparently due to alterations in these two symbiotic signals.

Although the possible symbiotic role of amino acids, purines, and pyrimidines is not clear, a recent report provided new insights indicating that purines and pyrimidines, or any intermediary of their biosynthetic pathways, play an important role in triggering nodule formation [9]. Photosynthetic *Bradyrhizobium* sp. strain ORS278 does not require LCO production for its symbiotic interaction with the legume *Aeschynomene sensitiva*. However, different mutants affected in the biosynthesis of purines and a strain ORS278 *pyrF* mutant only formed a few pseudonodules on this legume. Thus, purines and pyrimidines are essential for the formation of nodules, regardless of whether the mechanism of nodule organogenesis requires the presence of bacterial nodulation factors.

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References

1. Bergensen FJ (1961) The growth of *Rhizobium* in synthetic media. Aust J Biol Sci 14:349-360
2. Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84:188-198
3. Beringer JE, Ruiz-Sainz JE, Johnston AWB (1984) Methods for the genetic manipulation of *Rhizobium.* In: Grainger JM, Lynch JM (eds) Microbiological methods for environmental biotechnology. Academic Press, New York, pp 79-94
4. Boyer HW, Roulland-Dossoix D (1969) Complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol 41:459-472
5. Buendía-Clavería AM, Ruiz-Sainz JE, Cubo-Sánchez T, Pérez Silva J (1986) Studies of symbiotic plasmids in *Rhizobium trifolii* and fast-growing bacteria that nodulate soybean. J Appl Bacteriol 17:155-160
6. Buendía-Clavería AM, Chamber M, Ruiz-Sainz JE (1989) A comparative study of the physiological characteristics, plasmid content and symbiotic properties of different *Rhizobium fredii* in European soils. Syst Appl Bacteriol 17:55-160
7. Buendía-Clavería AM, Moussaid A, Ollero FJ, et al. (2003) A *purL* mutant of *Sinorhizobium fredii* HH103 is symbiotically defective and altered in its lipopolysaccharide. Microbiology 149:1807-1818
8. de Lyra MCCP, López-Baena FJ, Madinabeitia N, et al. (2006) Inactivation of the *Sinorhizobium fredii* HH103 *rhcJ* gene abolishes nodulation outer proteins (Nops) secretion and decreases symbiotic capacity with soybean. Int Microbiol 9:125-133
9. Giraud E, Moulin L, Vallenet D, et al. (2007) Legumes symbioses: absence of *nod* genes in photosynthetic bradyrhizobial. Science 316:1307-1312
10. Lamrabet Y, Bellogín RA, Cubo T, et al. (1999) Mutation in GDP-fucose synthesis genes of *Sinorhizobium fredii* alters Nod factors and significantly decreases competitiveness to nodulate soybeans. Mol Plant-Microbe Interact 12:207-217
11. Madinabeitia N, Bellogín RA, Buendía-Clavería A, et al. (2002) *Sinorhizobium fredii* HH103 has a truncated *nolO* gene due to a –1 frame-shift mutation that is conserved among other geographically distant *S. fredii* strains. Mol Plant-Microbe Interact 15:150-159
12. Newman JD, Rosovitz MJ, Noel KD (1995) Requirement for rhizobial production of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) for infection of bean. Mol Plant-Microbe Interact 8:407-414
13. Ollero FJ, Espuny MR, Bellogín RA (1989) Mobilization of the symbiotic plasmid from a strain of *Rhizobium* sp. (*Hedysarum coronarium*). System Appl Microbiol 11:217-222
14. Randhawa GS, Hassani R (2002) Role of rhizobial biosynthetic pathways of amino acids, nucleotide bases and vitamins in symbiosis. Indian J Exp Biol 40:755-764
15. Sanjuán-Pinilla JM, Muñoz S, Nogales J, Olivares J, Sanjuán J (2002) Involvement of the *Sinorhizobium meliloti leuA* gene in activation of nodulation genes by NodD1 and luteolin. Arch Microbiol 178:36-44.
16. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Lab Press, Cold Spring Harbor, NY
17. Simon R (1984) High frequency mobilization of gram-negative bacterial replicons by the in vivo constructed Tn*5*-Mob transposon. Mol Gen Genet 196:413-420
18. Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in gram negative bacteria. Bio/Technology 1:784-791

19. Simon R, Quandt J, Klipp W (1989) New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions, and induction of genes in gram-negative bacteria. Gene 80:161-169
20. Spaink HP, Aarts A, Stacey G, Bloemberg GV, Lugtenberg BJJ, Kennedy EP (1992) Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. Mol Plant-Microbe Interact 5:72-80
21. Strych U, Wohlfarth S, Winkler UK (1994) Orotidine-5´-monophosphate decarboxylase from *Pseudomonas aeruginosa* PAO1: cloning, overexpression, and enzyme characterization. Curr Microbiol 29: 353-359
22. Vineetha KE, Vij N, Prasad CK, Hassani R, Randhawa GS (2001) Ultrastructural studies on nodules induced by pyrimidine auxotrophs of *Sinorhizobium meliloti.* Indian J Exp Biol 39:371-377
23. Vincent JM (1970) The modified Fåhraeus slide technique. In: Vincent JM (ed) A manual for the practical study of root nodule bacteria. Appendix III. Blackwell Scientific Pub, Oxford, pp 144-145
24. Worland S, Guerreiro N, Yip L, Djordjevic MA, Djordjevic SP, Weinman J, Rolfe BG (1999) *Rhizobium* purine auxotrophs, perturbed in nodulation, have multiple changes in protein synthesis. Aust J Plant Physiol 26:511-519