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Symbiotic properties and first analyses of the genomic sequence of the fast growing model strain *Sinorhizobium fredii* HH103 nodulating soybean

Isabel Margaret[a], Anke Becker[b], Jochen Blom[c], Ildefonso Bonilla[d], Alexander Goesmann[c], Michael Göttfert[e], Javier Lloret[d], Virginie Mittard-Runte[c], Christian Rückert[c], José E. Ruiz-Sainz[a,*], José María Vinardell[a], Stefan Weidner[c]

[a] *Departamento de Microbiología, Universidad de Sevilla, Sevilla, Spain*
[b] *Fakultät für Biologie, Institut für Biologie III, Universität Freiburg, Freiburg, Germany*
[c] *Centrum für Biotechnologie, Universität Bielefeld, Bielefeld, Germany*
[d] *Departamento de Biología, Universidad Autónoma de Madrid, Madrid, Spain*
[e] *Institut für Genetik, Technische Universität Dresden, Dresden, Germany*

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ABSTRACT

Glycine max (soybean) plants can be nodulated by fast-growing rhizobial strains of the genus *Sinorhizobium* as well as by slow-growing strains clustered in the genus *Bradyrhizobium*. Fast-growing rhizobia strains with different soybean cultivar specificities have been isolated from Chinese soils and from other geographical regions. Most of these strains have been clustered into the species *Sinorhizobium fredii*. The *S. fredii* strain HH103 was isolated from soils of Hubei province, Central China and was first described in 1985. This strain is capable to nodulate American and Asiatic soybean cultivars and many other different legumes and is so far the best studied fast-growing soybean-nodulating strain. Additionally to the chromosome *S. fredii* HH103 carries five indigenous plasmids. The largest plasmid (pSfrHH103e) harbours genes for the production of diverse surface polysaccharides, such as exopolysaccharides (EPS), lipopolysaccharides (LPS), and capsular polysaccharides (KPS). The second largest plasmid (pSfrHH103d) is a typical symbiotic plasmid (pSym), carrying nodulation and nitrogen fixation genes. The present mini review focuses on symbiotic properties of *S. fredii* HH103, in particular on nodulation and surface polysaccharides aspects. The model strain *S. fredii* HH103 was chosen for genomic sequencing, which is currently in progress. First analyses of the draft genome sequence revealed an extensive synteny between the chromosomes of *S. fredii* HH103 and *Rhizobium* sp. NGR234.

1. Introduction to rhizobia fixing nitrogen in symbiosis with legumes

An essential element of agricultural sustainability is the effective management of N in the environment. This usually implies at least some use of nitrogen derived from a process called Biological Nitrogen Fixation (BNF). BNF is an activity only performed by some prokaryotic microorganisms, which are collectively called *nitrogen fixers*. Nitrogen fixers are able to catalyse the reduction of atmospheric nitrogen to ammonia, which then becomes available to a broad range of living forms such as microbes and plants and, through further transformations, to animals.

It has been known since ancient times that crop-rotation practices, alternating between a non-legume (such as a cereal) and a legume, have advantages over continuous cultivation of any particular non-legume crop. It has also been known that legume roots contain a kind of special out-growths, nowadays called *nodules*, showing well defined structures. These two facts, however, were not recognized as related until the 19th century, when it was demonstrated that legume plants are able to reduce (fix) atmospheric nitrogen to ammonium ions and that only those plants having root nodules were able to perform this process. Soon thereafter, it was demonstrated that the formation of root nodules is the result of a bacterial infection. Bacterial strains that carry out the infection and occupy the nodules are collectively called rhizobia. Bacteria inside nodules undergo a series of structural and physiological changes resulting in the formation of the so-called *bacteroids*, the bacterial symbiotic form responsible for the BNF process. Reduction of atmospheric nitrogen is performed by the bacteria and not by the plant.

* Corresponding author at: Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012 Sevilla, Spain.
Tel.: +34 954 557121; fax: +34 954 557830.
E-mail address: rsainz@us.es (J.E. Ruiz-Sainz).

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The rhizobial group comprises soil α- and β-proteobacteria (Downie, 2010; Sawada et al., 2003) that belong to different genera, all of which are Gram-negative rods (0.5–1.0 × 1.2–3.0 μm), motile, and aerobic, although growth under conditions of low oxygen concentrations can also take place. Their optimal growth conditions are at pH 7 and 28–30 °C. Traditionally, two main groups of rhizobia have been distinguished:

Fast-growers: Rhizobial strains with a generation time of about 1.5–4 h in YM (Yeast-extract Mannitol) medium. Many of these strains belong to the genera *Rhizobium*, *Sinorhizobium* or *Mesorhizobium*. These strains usually acidify media containing mannitol, such as YM, or other hexoses as the carbon source.

Slow-growers: Rhizobial strains with a generation time of at least 6 h, many of them belonging to the genus *Bradyrhizobium*. These strains usually alkalinize YM medium.

The formation of nitrogen fixing nodules is the result of a very specific bacteria–plant interaction because there is a marked specificity between species of legumes and rhizobia. As a result of this specificity, a single rhizobial strain will be able to infect certain species of legumes but not others. Rhizobial strains able to nodulate many different legumes are called "broad-host-range rhizobia". In contrast, strains that only nodulate with a few legumes are defined as "narrow-host-range rhizobia".

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 sativa* (alfalfa), *Pisum sativum* (pea), and *Trifolium* spp. (clovers). This kind of nodule is elongated and has a persistent meristem that continuously 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), *Phaseolus vulgaris* (bean) and *Lotus japonicus*, the meristematic activity is temporary and the nodule shape is rounded.

2. The soybean rhizobia group consisting of fast and slow growing rhizobial strains

Taxonomic studies carried out in the last three decades have shown that *G. max* (soybean) is nodulated by fast- and slow-growing rhizobia, which we collectively refer to as "soybean rhizobia". Until 1982, soybeans were believed to be nodulated only by slow-growing bacteria belonging to the species *Bradyrhizobium japonicum*. In this year, however, Keyser and co-workers isolated fast-growing rhizobial strains from soybean nodules grown in Chinese soils (Keyser et al., 1982).

Since then, new groups of soybean rhizobia have been isolated and classified. Soybean nodulating bacteria are distributed over different species belonging to three different genera: *Bradyrhizobium* (*B. japonicum*, *B. elkanii* and *B. liaoningense*, slow-growing bacteria with generation times over 6 h); *Mesorhizobium* [*M. tianshanense*, generation times are variable (Chen et al., 1995)]; and *Sinorhizobium* [*S. fredii* and *S. xinjiangense*, fast-growers, generation time between 1.5 and 4 h (Chen et al., 1988)].

The fast-growing soybean rhizobia strains isolated by Keyser et al. (1982) are able to form nitrogen-fixing nodules on Asiatic soybean (*G. max*) cultivars, such as cultivars Peking or Jing Dou 19, but fail to nodulate or are very poorly effective, with modern cultivars from North America, such as cultivar Williams (Buendía-Clavería and Ruiz-Sainz, 1985; Buendía-Clavería et al., 1994; Keyser et al., 1982). Thus, these rhizobial strains exhibit marked soybean cultivar specificity. Years later, the isolation of new fast-growing soybean rhizobia strains that were able to form nitrogen-fixing nodules with both Asiatic and American soybean cultivars was reported (Camacho et al., 2002; Dowdle and Bohlool, 1985; Rodríguez-Navarro et al., 1996; Thomas-Oates et al., 2003; Yang et al., 2001). Although most of the fast-growing soybean rhizobia strains have been isolated from Chinese soils, isolations from other geographical regions have also been reported (Hernandez and Focht, 1984; Young et al., 1988).

Fast-growing soybean rhizobia strains were finally clustered into the species *S. fredii* (Chen et al., 1988). It has recently been proposed that the genus *Sinorhizobium* should be renamed as *Ensifer* (Young, 2003; Young, 2010). Taxonomic studies have shown that *S. fredii* is closely related to the alfalfa microsymbiont *Sinorhizobium meliloti* (de Lajudie et al., 1994), although their host-ranges and nodulation factor structures (Nod factors or LCOS) are different (Denarie et al., 1996; Gil-Serrano et al., 1997). *S. fredii* is also related to other *Sinorhizobium* species that nodulate a variety of legumes, such as different species of *Acacia*, *Sesbania*, *Medicago* or *Leucaena leucocephala*. Table 1 lists the different *Sinorhizobium* species and some of their host legumes.

The host range of nodulation of *S. fredii* USDA257 and *Rhizobium* sp. NGR234 has been examined in great detail by inoculating 452 species of legumes that represent all the three subfamilies of the Leguminosae family (Pueppke and Broughton, 1999). Both strains, which are very closely related, share very wide overlapping host ranges. *S. fredii* USDA257 and *Rhizobium* sp. NGR234 nodulated with 79 and 112 genera, respectively (Pueppke and Broughton, 1999).

At present, only slow-growing soybean rhizobia (*Bradyrhizobium*) strains are being used in the production of commercial inoculants for soybeans. In general, *S. fredii* strains are not as effective as *B. japonicum* USDA110, a strain that is highly effective with American soybean cultivars.

However, field experiments carried out in Andalusia (Spain) showed that seed yields of soybean plants inoculated with some *S. fredii* strains are as high as those obtained with *B. japonicum* strain USDA110 or with high levels of nitrogen fertilization, indicating that some *S. fredii* strains could be valid soybean inoculants (Buendía-Clavería et al., 1994; Rodríguez-Navarro et al., 2003). These studies, however, were carried out in alkaline soils (pH 8.0) that, as with all European soils, are devoid of indigenous soybean microsymbionts. Since the only reports in which *S. fredii* strains appeared as valid inoculants for American soybean cultivars are those based on field experiments in Andalusian soils, it remains to be determined whether any particular characteristic(s) of these soils (such as alkaline pH), climatic conditions and/or any other unknown factor are favouring this symbiotic interaction.

3. Basic knowledge on the fast growing *S. fredii* strain HH103 nodulating soybean

S. fredii strain HH103 was isolated from a soil sample of Honghu county (Hubei province, Central China) and was first described in 1985 (Dowdle and Bohlool, 1985). This Honghu soil sample was taken from a traditional soybean cropping area. The Asiatic soybean cultivar Ou Huang no. 3 was used as the trap host to isolate soybean rhizobia from the Honghu soil sample. *S. fredii* strain HH103 is capable to nodulate American and Asiatic soybean cultivars and is so far the best studied fast-growing soybean-nodulating strain. It also nodulates many other legumes from different legume tribes (Table 2). Legumes nodulated by *S. fredii* HH103 can form determinate or indeterminate nodules (Fig. 1). Up to now, all the legumes tested that form nitrogen-fixing nodules with strain HH103 are also nodulated by *S. fredii* USDA257 (Pueppke and Broughton, 1999). *S. fredii* USDA257, however, fails to nodulate most of the American soybean cultivars while strain HH103 is able to form nitrogen fixing nodules on these agronomically advanced cultivars (Buendía-Clavería et al., 1989; Dowdle and Bohlool, 1985; Keyser et al., 1982).

Table 1
List of species belonging to the *Sinorhizobium* genus and some of their host legumes.

Species of *Sinorhizobium*	Host plants	Reference
S. abri	*Abrus precatorius*	Ogasawara et al. (2003)
S. americanus	*Acacia* spp.	Toledo et al. (2003)
S. arboris	*Acacia senegal*, *Prosopis chilensis*	Nick et al. (1999)
S. chiapanecum	*Acaciella angustissima*	Rincón-Rosales et al. (2009)
S. fredii Former name: *Rhizobium fredii*	*Cajanus cajan*, *C. scarabaeoides*, *Glycine max*, *Glycine soja*, *Vigna unguiculata*, *V. angularis*, *Sesbania grandiflora*, *Medicago sativa*, *Amorpha fruticosa*, *Tephrosia vogelii*, *Robinia pseudoacacia*, *Macrotyloma axillare*, *Lotus japonicus*, *Psophocarpus tetragonolobus* and many others	Chen et al. (1988)
S. indiaense	*Sesbania rostrata*	Ogasawara et al. (2003)
S. kostiense	*Acacia senegal*, *Prosopis chilensis*	Nick et al. (1999)
S. kummerowiae	*Kummerowia stipulacea*	Wei et al. (2002)
S. medicae Former name: *Sinorhizobium meliloti*	*Medicago orbicularis*, *M. polymorpha*, *M. rugosa*, *M. truncatula*	Rome et al. (1996)
S. meliloti Former names: *Rhizobium meliloti*	*Melilotus* spp., *Medicago*, spp., *Trigonella* spp.	Jordan (1984)
S. morelense	*Leucaena leucocephala*	Wang et al. (2002)
S. saheli	*Sesbania cannabina*, *S. grandiflora*, *S. rostata*, *S. pachycarpa*, *Acacia seyal*, *Leucaena leucocephala*, *Neptunia natans*	de Lajudie et al. (1994)
S. terangae bv. *sesbaniae*	*Sesbania grandiflora*, *S. pubescens*, *S. rostrata*	de Lajudie et al. (1994)
S. terangae bv. *acaciae*	*Acacia senegal*, *A. tortilis* subsp. *raddiana*, *A. nilotica*, *Leucaena leucocephala*	de Lajudie et al. (1994)
S. xinjiangense	*Glycine max*	Chen et al. (1988)
Ensifer adhaerens Former name: *Sinorhizobium adhaerens*	*Sesbania grandiflora*, *Leucaena leucocephala*, *Pithecellobium dulce*, *Medicago sativa*	Casida (1982)
Ensifer garamanticus	*Argyrolobium uniflorum*, *Medicago sativa*	Merabet et al. (2010)
Ensifer mexicanus	*Acacia*	Lloret et al. (2007)
Ensifer numidicus	*Argyrolobium uniflorum*, *Lotus creticus*	Merabet et al. (2010)

Table 2
Symbiotic phenotype of *S. fredii* HH103 with different legumes.

Legume tribe	Name of the species	Symbiotic phenotype	Reference
Aeschynomeneae	*Arachis hypogaea*	Nod$^-$	Our own unpublished results
Desmodieae	*Desmodium canadense*	Fix$^+$	Crespo Rivas et al. (2007)
	Kummerowia stipulacea	Fix$^+$	Buendía-Clavería et al. (1989)
Galegeae	*Glycyrrhiza uralensis*	Fix$^+$	Crespo-Rivas et al. (2009)
Indigofereae	*Indigofera tinctorea*	Fix$^+$	Buendía-Clavería et al. (1989)
Ingeae	*Albizia lebbeck*	Fix$^+$	Buendía-Clavería et al. (1989)
Millettieae	*Tephrosia vogelii*	Nod$^-$	Dr. F. Temprano (personal communication)
Phaseoleae	*Glycine max*	Fix$^+$	Dowdle and Bohlool (1985)
	Canavalia ensiformis	Nod$^-$	de Lyra et al. (2006)
	Cajanus cajan	Fix$^+$	Buendía-Clavería et al. (1989)
	Erythrina variegata[a]	Fix$^+$	de Lyra et al. (2006)
	Lablab purpureus	Nod$^-$	Our own unpublished results
	Macroptilium atropurpureum	Fix$^+$	Buendía-Clavería et al. (1989)
	Macrotyloma axyllare	Fix$^+$	Buendía-Clavería et al. (1989)
	Neonotonia wightii	Fix$^+$	Buendía-Clavería et al. (1989)
	Psophocarpus tetragonolobus	Fix$^+$	Buendía-Clavería et al. (1989)
	Vigna unguicula	Fix$^+$	Buendía-Clavería et al. (1989)
	Vigna radiata	Fix$^+$	Buendía-Clavería et al. (1989)
Robinieae	*Sesbania marginata*	Nod$^-$	de Lyra et al. (2006)
Sophoreae	*Sophora tomentosa*	Fix$^+$	Our own unpublished results

[a] Nitrogen fixing nodules are only formed by HH103 mutants affected in the T3SS.

3.1. Indigenous plasmids

Plasmid agarose electrophoresis revealed that *S. fredii* HH103 carries 5 different indigenous plasmids (Fig. 2A). Genome sequencing analyses confirmed the presence of these five plasmids (see below). Fig. 2B shows the plasmid profiles of *S. fredii* HH103 and three other rhizobial strains for which the complete genome sequence is available. The electrophoretic mobilities of the two largest plasmids of HH103, called pSfrHH103e and pSfrHH103d, were similar to those of *Rhizobium* sp. NGR234 plasmids, whose sizes are 2.4 and 0.5 Mb, respectively (Schmeisser et al., 2009). Preliminary analysis of the draft genome sequence indicated that the HH103 largest plasmid (pSfrHH103e) carries genes for the production of diverse surface polysaccharides, such as exopolysaccharides (EPS), lipopolysaccharides (LPS), and capsular polysaccharides (KPS). The second largest plasmid (pSfrHH103d) is a typical symbiotic plasmid (pSym), on which nodulation and nitrogen fixation genes are located. The electrophoretic mobility of pSfrHH103c indicates that the size of this plasmid is slightly lower than 0.15 Mb. Preliminary computer analyses of the draft sequence of plasmids pSfrHH103b (61880 bp) and pSfrHH103a (24036 bp) showed the presence of numerous transposable elements. For instance, each

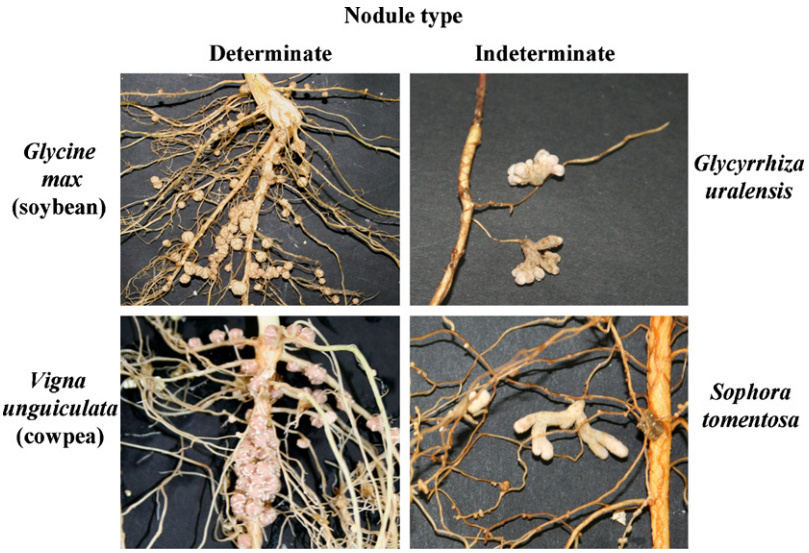


Fig. 1. Determinate and indeterminate nodules induced by *S. fredii* HH103.

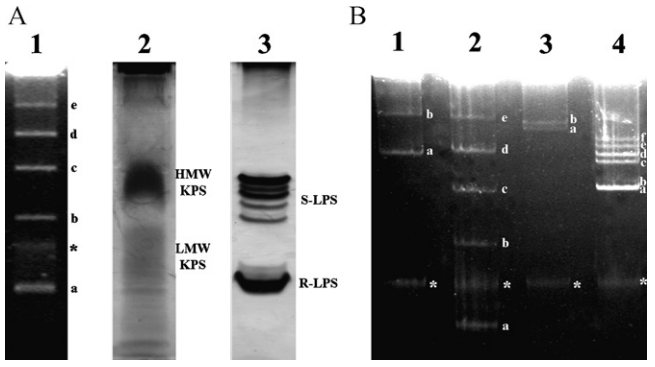


Fig. 2. (A) Plasmid (lane 1), capsular KPS (lane 2), and LPS (lane 3) electrophoretic profiles of *Sinorhizobium fredii* HH103. HMW and LMW KPS correspond to high- and low-molecular weight KPS. S-LPS and R-LPS corresponds to "smooth" and "rough" lipopolysaccharide forms. (B) Plasmid profiles in agarose gel electrophoresis of different rhizobial strains for which the complete genome sequence is known. Plasmid sizes are given between brackets. Lane 1, *Rhizobium* sp. NGR234 (two plasmids, *b* and *a*, of 2.43 and 0.54 Mb); lane 2, *S. fredii* HH103 [five plasmids of approximate sizes of 24 kb (a), 62 kb (b), 0.15 Mb (c), 0.60 Mb (d), and 2.4 Mb (e)]; lane 3, *S. meliloti* 1021 (two plasmids, b and a, of 1.68 Mb and 1.35 Mb); lane 4, *R. leguminosarum* bv. *viciae* 3841 (six plasmids, f–a of 0.87, 0.68, 0.49, 0.35, 0.15, and 0.15 Mb). The fragmented chromosome is marked with an asterisk.

of these plasmids contains one of the two HH103 copies of IS*Rf*1, an insertion sequence that, up to now, has only been found in this *S. fredii* strain (Vinardell et al., 1997). Strikingly, various ORFs located in these two HH103 plasmids are highly identical to genes contained in plasmids pSmeSM11a and pSmeSM11b of *S. meliloti* SM11 (Stiens et al., 2006, 2007). Plasmid pSfrHH103a could be useful as cloning vector if it is appropriately adapted for this purpose. One of the advantages of using such a modified plasmid is that it should be very stable in sinorhizobial strains. The cloning vectors currently used in genetic studies of *S. fredii* HH103 are frequently lost if the bacteria are subcultured in the absence of the antibiotic(s) that select for the presence of the plasmid (Vinardell et al., 1993; Vinardell et al., 2004a). Since antibiotics cannot be used in nodulation experiments to counter-select the spontaneous loss of the plasmid, this precarious stability of cloning vectors makes it difficult, or even impossible, to carry out reliable experiments in which genes that are not absolutely necessary to nodulate are investigated for their possible influence in the symbiotic interaction.

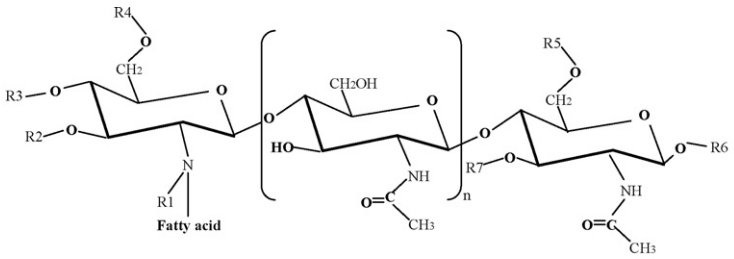
3.2. Nodulation genes

Nodulation genes (*nod*, *nol*, and *noe*) are defined as those rhizobial genes which play a role in nodulation or which are coordinately regulated with such genes (Downie, 1998). *S. fredii* HH103 nodulation genes (Lamrabet et al., 1999; Madinabeitia et al., 2002), genes involved in the synthesis or secretion of nodulation outer proteins (de Lyra et al., 2006; López-Baena et al., 2009) or acting as regulatory genes (Vinardell et al., 2004a,b) have been already reported. All these genes, except *nolR* are placed in the pSym plasmid (pSfrHH103d). The regulatory *nolR* gene is located in the bacterial chromosome. The distribution and order of the *S. fredii* HH103 *nod* genes is equal to that described for *Rhizobium* sp. NGR234 (Freiberg et al., 1997). For instance, there are two copies of *nodD* (*nodD1* and *nodD2*) and the clusters *nolK-noeL-nodZ-noeK-noeJ-nodD1* and *nodA-nodB-nodC-nodI-nodJ-nolO-noeI-noeE* are almost identical in both bacteria. The *nolO* gene, however, is functional in NGR234 but truncated in HH103 due to a −1 frameshift mutation (Madinabeitia et al., 2002).

3.3. Nodulation factors

Rhizobia produce lipo-chitin oligosaccharide signal molecules, called LCOs or Nod factors, in response to plant-produced flavonoids (Fisher and Long, 1992). The most abundant flavonoids produced by soybean roots are the isoflavones daidzein, genistein, and coumestrol (D'Arcy-Lameta, 1986; Kosslak et al., 1987). The interaction of plant-flavonoids with the bacterial NodD protein provokes the transcriptional activation of rhizobial nodulation genes. Expression of nodulation genes ultimately results in the production of Nod factors, which in turn induce root hair curling and nodule meristem initiation in the plant (Gage, 2004; Jones et al., 2007).

The chemical structure of Nod factors produced by different *S. fredii* strains, including HH103, has been reported (Bec-Ferte et al., 1994; Gil-Serrano et al., 1997; Thomas-Oates et al., 2003). This structure consist of a backbone of three to five β-(1→4)-linked *N*-acetyl-glucosamine (GlcNAc) residues bearing an amide bound fatty acyl residue, saturated or unsaturated, on the non-reducing terminal GlcNAc residue. The most common substitution is located at the C6 position of the reducing terminal GlcNAc and consists of a fucosyl or methyl-fucosyl residue. The most abundant Nod factors produced by *S. fredii* HH103 consist of a cocktail of tri-, tetra-, and pentasaccharides carrying a saturated or unsaturated



Rhizobia	Fatty acid	R1	R2	R3	R4	R5	R6	R7	n
S. fredii HH103	$C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$	H	H	H	H	2-*O*-MeFuc Fuc	H	H	1, 2, 3
Rhizobium sp. NGR234	$C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$,	Me	Cb H	Cb H	Cb H	3-*O*-S-2-*O*-MeFuc 3-/4-*O*-Ac-2-*O*-MeFuc	H	H	3
B. japonicum USDA110	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$	H	H	H	H Ac	2-*O*-MeFuc	H	H	3
B. elkanii USDA61	$C_{16:0}$, $C_{18:1}$	H Me	Ac Cb	H Ac Cb	H Ac Cb	2-*O*-MeFuc Fuc	H Gro	H	2, 3

Ac: acetyl; Cb: *O*-carbamoyl; Fuc: fucosyl; Gro: glycerol; Me: methyl; S: sulfate ester.

n: number of internal GlcNAc residues. This table is based on: 15, 20 and 25.

Fig. 3. . Nodulation factors produced by *Sinorhizobium fredii* HH103, *Rhizobium* sp. NGR234, *Bradyrhizobium japonicum* USDA110, and *B. elkanii* USDA61.

C16 or C18 fatty acyl residue (Fig. 3). The main structural differences between Nod factors produced by *S. fredii* HH103 and those produced by slow-growing soybean rhizobia (*B. japonicum* or *B. elkanii*) are located in the non-reducing terminal GlcNAc residue. In slow-growers this hexose can carry methyl, acetyl, and/or carbamoyl substitutions (D'Haeze and Holsters, 2002). In contrast, Nod factors produced by *S. fredii* HH103 do not have these modifications (Gil-Serrano et al., 1997).

3.4. Surface polysaccharides

In addition to *nod* genes and Nod factors, different rhizobial surface polysaccharides are usually required for successful nodulation. Cyclic glucans (CG), exopolysaccharides (EPS), lipopolysaccharides (LPS), and capsular polysaccharides (KPS or K-antigens) are the main rhizobial polysaccharides investigated for their role in symbiosis. In rhizobial strains, KPS has only been reported in *S. meliloti*, *S. fredii*, and *Rhizobium* sp. NGR234 (Kannenberg et al., 1998; Rodríguez-Carvajal et al., 2005). Fig. 2A shows the *S. fredii* HH103 KPS (lane 2) and LPS (lane 3) electrophoretic profiles in PAGE experiments as described by Parada et al. (2006). The chemical structures of the KPS and cyclic glucans (CG) produced by *S. fredii* HH103 have been determined (Crespo-Rivas et al., 2009; Gil-Serrano et al., 1999).

3.4.1. Cyclic glucans

S. fredii HH103 produce CG that only contain glucosyl residues linked by β-(1,2) glycosidic bonds. The number of glucosyl residues varies from 18 to 24 and 1-phosphoglycerol is the only substituent (Crespo-Rivas et al., 2009). An *S. fredii* HH103 mutant affected in the *cgs* gene (cyclic glucan synthase, formerly called *ndvB*) does not produce CG and fails to induce the formation of nitrogen-fixing nodules in any of the legumes tested, regardless of whether it formed determinate (*G. max* and *Vigna unguiculata*) or indeterminate (*Glycyrrhiza uralensis*) nodules. This *S. fredii* HH103 *cgs* mutant (SVQ562) shows other phenotypic alterations, such as an increase in the amount of EPS, an increase in the molecular weight of the EPS produced, and loss of mobility. SVQ562 grows slower in the hypoosmotic GYM medium than its parental wild type strain, suggesting that the mutation has caused sensitivity to hypoosmotic conditions. However, the fact that SVQ562 is able to survive in distilled water indicates that strain HH103 does not require cyclic glucans to stand severe hypoosmotic conditions.

3.4.2. Exopolysaccharides

Although the chemical structure of the *S. fredii* HH103 EPS has not been determined yet, it is already known that it carries acetyl and pyruvyl substituents (Crespo-Rivas et al., 2009). The absence of succinyl substituents is corroborated by the fact that the *exoH* gene is not present in the genome of *S. fredii* HH103. As in *S. meliloti* 1021 and *Rhizobium* sp. NGR234 (Capela et al., 2001; Schmeisser et al., 2009), the *exo* cluster of *S. fredii* HH103 is located on the largest plasmid (pSfrHH103e). Preliminary computational analyses indicate that the regulatory genes *exoR*, *exoS*, *mucR1*, and *mucR2* are placed on the chromosome. A mutant (SVQ530) unable to produce EPS (by mutation in the *exoA* gene) does not show any reduction of its symbiotic capacity with soybean, indicating that this polysaccharide does not play a relevant role in the *S. fredii*-soybean symbiosis (Parada et al., 2006).

3.4.3. K-antigens (KPS)

S. fredii HH103 produces a KPS consisting of a homopolymer of a pseudaminic acid derivative (Gil-Serrano et al., 1999). Rhizobial genes involved in KPS biosynthesis were first described in *S. meliloti* Rm41. These genes, called *rkp*, are distributed in three different clusters called *rkp-1*, *rkp-2*, and *rkp-3* (for a review, see Becker et al., 2005). These three distinct *rkp* regions have also been identified in *S. fredii* HH103.

The *S. fredii* HH103 *rkp-1* region is located on the chromosome and contains 7 different genes: *rkpU*, *rkpA*, *rkpG*, *rkpH*, *rkpI*, *rkpJ*, *and kpsF3*. Mutants in all these *rkp* genes are unable to produce KPS. Soybean plants inoculated with *rkp* mutants showed reduced nodulation and severe symptoms of nitrogen starvation (Hidalgo et al., 2010; Parada et al., 2006).

The *S. fredii* HH103 *rkp-2* is located on the chromosome and is composed of two genes highly homologous to those (*lpsL* and *rkpK*) of the *S. meliloti* Rm41 *rkp-2* region. *S. fredii* HH103 mutants in the *rkp-2* region have not been described.

Genome sequencing of *S. fredii* HH103 shows that the *rkp-3* region is located on pSfrHH103e. In this region there are six genes,

rkpLMNOPQ, that putatively code for enzymes required for the biosynthesis of the pseudaminic acid, which is present (as a modified derivative) in the KPS repeating unit of strain HH103. These genes are also present in *S. meliloti* (Becker et al., 2005). In *Rhizobium* sp. NGR234, the *rkpP* gene is absent (Le Quéré et al., 2006). Other genes involved in KPS export (*rkpR*, *rkpS*, and *rkpT*) or in determining the length of the KPS chain (*rkpZ1*) are also present on pSfrHH103e. The *S. meliloti* Rm41 *rkpY* gene, which directs KPS synthesis to the KR5 antigen and influences lipo-oligoKdo production (Pálvölgyi et al., 2009), has not been found in HH103. Our previous results indicate that KPS, but not EPS, is relevant for the symbiotic capacity of *S. fredii* HH103 with soybeans (Parada et al., 2006).

3.4.4. Lipopolysaccharides

Lipopolysaccharides (LPS) are components of the outer leaflet of almost all Gram-negative bacteria. It is postulated that rhizobial LPS protect the bacteria from plant-derived antimicrobial compounds, are involved in plant recognition and adhesion to the plant, and appear to promote infection and nodule invasion (Kannenberg et al., 1998). The chemical structure of the *S. fredii* HH103 LPS has not been determined yet. Only two *S. fredii* HH103 mutants affected in *lps* genes have been constructed and studied (Margaret et al., 2010). Preliminary results indicated that mutants affected in *lpsB* or *lpsE* showed alterations of their LPS electrophoretic profiles and their nodulation and nitrogen fixation capacity with soybean was greatly reduced.

4. The draft sequence of the *S. fredii* HH103 genome and its synteny to the *Rhizobium* sp. NGR234 genome sequence

There are different reasons why the determination of the genome sequence of an *S. fredii* strain appears as an attractive subject, not only for the acquisition of basic knowledge but also for practical purposes. We chose strain HH103 for the determination of its complete genome sequence because of its wide symbiotic capacity and also because, to our knowledge, *S. fredii* HH103 is the best studied fast-growing soybean-nodulating strain.

The genomic draft sequence of *S. fredii* HH103 was established by a *de novo* sequencing approach applying the 454 sequencing technology. A combination of whole genome shotgun (GS FLX Titanium) and Long Paired End (GS FLX standard) sequencing was used. In total 1,478,753 reads with about 265.5 Mb sequence information were assembled with the Newbler software (454 Life Sciences, Roche Diagnostics) resulting in 241 large contigs (>500 bp) and 59 scaffolds with a total size of 7.01 Mb. The coverage of the draft genome was about 37 fold.

A plot of the number of sequencing reads to all contigs of the draft genome assembly indicated a variant coverage of the contigs. This analysis is shown in Fig. 4. Assuming that in a bacterial genome for a continuous sequence of >10 kb length the probability to constitute a repetitive sequence is very low, for contigs of this length the median read coverage was calculated. This coverage was regarded as the normal coverage of the draft genome. Therefore, a deviation of ≥1.5 fold of this median was regarded as an over-representation, a deviation of ≤0.75 as an under-representation of the respective contig. All 14 under-represented contigs exhibited less than 450 bp in length and are probably contaminations. Database comparisons of over-represented contigs with a size of >1 kb using the blastx algorithm revealed that they almost exclusively contain repetitive sequences particularly encoding transposases. Also the contig encoding the *rrn* operons was found among these contigs. The read coverage of this contig was 3.2 fold indicating that *S. fredii* HH103 like, e.g. *S. meliloti* 1021 or *Rhizobium* sp. NGR234 possesses three copies of the *rrn* operon. Interestingly, a contig carrying the genes *nifHDK* was 2.1 fold over-represented, suggesting that like in *Rhizobium* sp. NGR234 the *nifHDK* genes might also be duplicated in *S. fredii* HH103. A 2.7 fold over-represented contig encodes the conjugal transfer protein TraA, which probably is located on several plasmids of the *S. fredii* HH103 genome. Evidence that the small plasmids pSfrHH103c, pSfrHH103b and pSfrHH103a are present in more than one copy per *S. fredii* HH103 cell could not be deduced from the read coverage analysis.

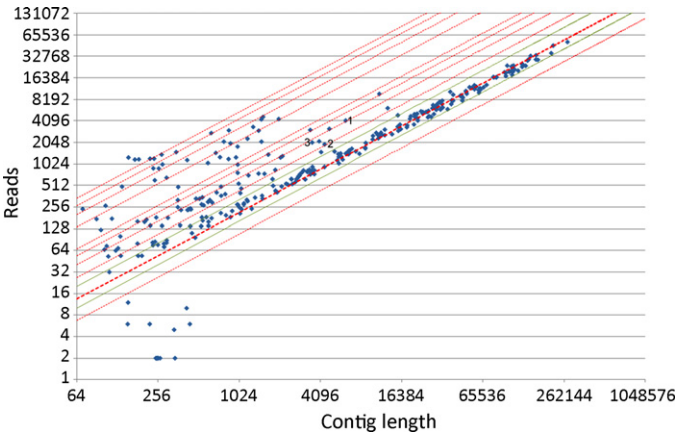


Fig. 4. Coverage of the contigs of the *S. fredii* HH103 draft genome by sequencing reads. Contigs of the assembly are illustrated by blue diamonds. The sequence length of the contigs is given on the *x*-axis. The number of sequencing reads assembled in the respective contig is shown on the *y*-axis in logarithmic scale. The bold dotted red line represents the median number of reads in contigs with >10 kb size. The parallel red lines illustrate the 2, 3, 4, 5, 10, 15, 20, 25 fold of this median, respectively. Contigs with a read coverage of ≥1.5 and ≤0.75 fold are regarded as over- and under-represented, respectively, visualized by the pale green lines. The labelled contigs carry *rrn* operons (1); *nifHDK*(2); *traA* (3).

Comparative analysis of the assembled draft genome sequence of *S. fredii* HH103 revealed high homology to other rhizobial genomes, in particular to *S. meliloti* 1021 and to *Rhizobium* sp. NGR234. The results of a comparison to *Rhizobium* sp. NGR234 using the MUMmer package (Kurtz et al., 2004) are shown in Fig. 5. The degree of synteny between these two rhizobial genomes varies for the different replicons. The chromosomes exhibit a very high synteny and almost the complete *Rhizobium* sp. NGR234 chromosome is represented by contigs of the *S. fredii* HH103 assembly. Also plasmids pSfrHH103*e* and pNGR234b (pSymB plasmids) are very syntenious and for the most part congruent. In contrast, the symbiotic plasmids pSfrHH103d and pNGR234a (pSymA plasmids) show considerably lower synteny and not all regions of pNGR234a are present in the *S. fredii* HH103 draft genome.

5. Future research aspects concerning *S. fredii* HH103

S. fredii shares with *B. japonicum* the capacity to nodulate soybeans but it is closely related to *S. meliloti* and also to *Rhizobium* sp. NGR234, which are unable to nodulate soybeans. Thus, comparative genomic studies aimed at determining what is similar and what is different among all these rhizobia look very promising.

Wild soybeans (*Glycine soja*) are important in soybean breeding programmes because they represent an excellent source of genetic material for improving desirable soybean characteristics, such as resistance to phytopathogens or to environmental stresses. Because the symbiotic relation between wild soybeans and *S. fredii* strains has not been studied, the acquisition of knowledge about this symbiosis might be relevant for the development of sustainable soybean cropping systems.

S. fredii strains, *B. japonicum* USDA110, and *Rhizobium* sp. NGR234 secrete nodulation outer proteins (Nops) through a type 3 secretion system (T3SS) in the presence of flavonoids able to induce the transcription of nodulation genes. In many cases, rhizobial Nops

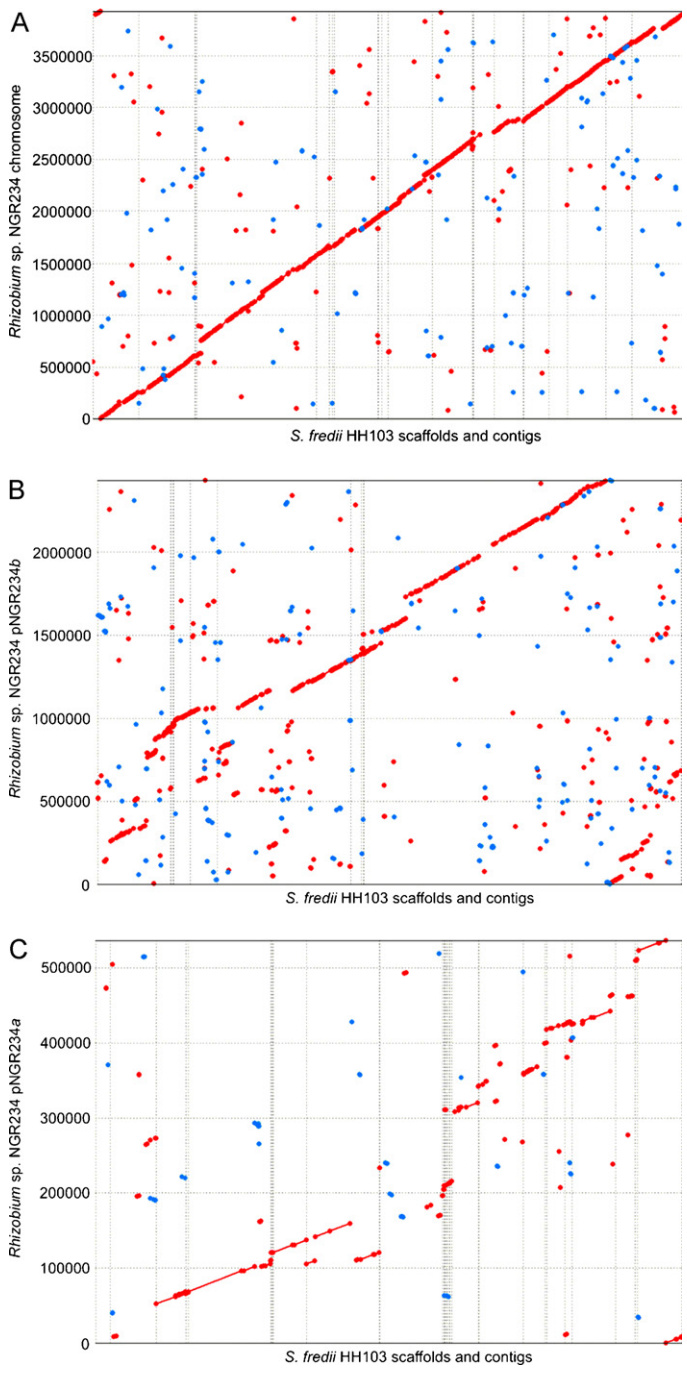


Fig. 5. Synteny plots of the *S. fredii* HH103 draft genome mapped to *Rhizobium* sp. NGR 234 as reference genome. All scaffolds and contigs of the *S. fredii* HH103 draft genome were mapped and ordered to the chromosome of *Rhizobium* sp. NGR 234 (A). The remaining scaffolds and contigs were mapped and ordered to plasmid pNGR234*b* (B) and the residual scaffolds and contigs to pNGR234*a* (C), respectively. The *S. fredii* HH103 scaffolds and contigs are laid on the *x*-axes, the reference replicons and their sizes are shown on the *y*-axes. Vertical dotted lines refer to individual contig and scaffold ends. In red forward matches are illustrated, reverse complement matches are shown in blue. The analysis was performed with the MUMmer package (Kurtz et al., 2004).

are involved in determining soybean cultivar specificity and bacterial host range of nodulation (de Lyra et al., 2006; Krause et al., 2002; López-Baena et al., 2009; Marie et al., 2001; Meinhardt et al., 1993). In *B. japonicum* USDA110, *S. fredii*, and *Rhizobium* sp. NGR234, most genes encoding the transport machinery and secreted proteins are preceded by a conserved 30-bp motif, the type-three secretion (*tts*) box (Krause et al., 2002; Zehner et al., 2008). Recently, a cluster of genes coding for a putative second T3SS has been found on plasmid pNGR234b of *Rhizobium* sp. NGR234 (Schmeisser et al., 2009). The expression of these genes is independent of the flavonoid-*nodD1*-*ttsI* regulatory cascade and its possible role in symbiosis remains to be determined. As revealed by the draft genome sequence, part of this cluster is also present on the pSfrHH103e plasmid. Comparisons between the *B. japonicum* USDA110, *Rhizobium* sp. NGR234, and *S. fredii* secretomes might provide relevant information about bacterial host-range of nodulation and cultivar-strain specificity.

S. fredii USDA257 fails to form nitrogen-fixing nodules with American soybean cultivars unless genes responsible for the bacterial T3SS machinery or for the production of nodulation outer proteins (Nop) are mutated. However, a recent report has shown that disruption of the glycine cleavage system (*gcv*THP operon) also generates USDA257 mutants that have gained the capacity to fix nitrogen with American soybean cultivars (Lorio et al., 2010). Inactivation of the *gcv*THP operon did not affect the secretion of USDA257 nodulation outer proteins, which indicates that other yet unidentified factors influence soybean cultivar specificity. This operon, which is present in other rhizobial strains (Lorio et al., 2010), is also well conserved in the chromosome of *S. fredii* HH103.

S. fredii strains show an exceptionally broad host range for nodulation, being able to establish symbiotic interactions with different determinate- and indeterminate-nodule forming legumes (Buendía-Clavería et al., 2003). This symbiotic capacity allows the identification of bacterial genes that are particularly relevant for both types of symbioses or only for one of them. *S. fredii* strains can also be used to investigate differences during the nodulation process of legumes that usually share the same bacterial symbionts. For instance, *G. max* and *V. unguiculata*, two determinate-nodule forming legumes, are commonly nodulated by *S. fredii* and *B. japonicum* strains. However, when *G. max* and *V. unguiculata* roots are inoculated with an *S. fredii* HH103 *rkpJ* mutant, only the former shows reduced nodulation (Hidalgo et al., 2010).

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