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A Catalogue of Molecular, Physiological and Symbiotic Properties of Soybean-Nodulating Rhizobial Strains from Different Soybean Cropping Areas of China

J. Thomas-Oates[1], J. Bereszczak[1], E. Edwards[1], A. Gill[1], S. Noreen[1], J. C. Zhou[2], M. Z. Chen[2], L. H. Miao[2], F. L. Xie[2], J. K., Yang[2], Q. Zhou[2], S. S. Yang[3], X. H. Li[3], L. Wang[3], H. P. Spaink[4], H. R. M. Schlaman[4], M. Harteveld[4], C. L. Díaz[4], A. A. N. van Brussel[4], M. Camacho[5], D. N. Rodríguez-Navarro[5], C. Santamaría[5], F. Temprano[5], J. M. Acebes[6], R. A. Bellogín[6], A. M. Buendía-Clavería[6], M. T. Cubo[6], M. R. Espuny[6], A. M. Gil[6], R. Gutiérrez[6], A. Hidalgo[6], F. J. López-Baena[6], N. Madinabeitia[6], C. Medina[6], F. J. Ollero[6], J. M. Vinardell[6], and J. E. Ruiz-Sainz[6]

[1] Department of Chemistry, University of York, Heslington, York, U.K.
[2] Department of Microbiolgy, Huazhong Agricultural University, Shi Zi Shan Street, Wuhan, People's Republic of China
[3] Department of Microbiology, China Agricultural University, Beijing, People's Republic of China
[4] Leiden University, Institute of Molecular Plant Sciences, Leiden, The Netherlands
[5] Centro de Investigación y Formación Agraria "Las Torres y Tomejil", Sevilla, Spain
[6] Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain

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Summary

We have analysed 198 fast-growing soybean-nodulating rhizobial strains from four different regions of China for the following characteristics: generation time; number of plasmids; lipopolysaccharide (LPS), nodulation factors (LCOs) and PCR profiles; acidification of growth medium; capacity to grow at acid, neutral, and alkaline pH; growth on LC medium; growth at 28 and 37 °C; melanin production capacity; Congo red absorption and symbiotic characteristics. These unbiased analyses of a total subset of strains isolated from specific soybean-cropping areas (an approach which could be called "*strainomics*") can be used to answer various biological questions. We illustrate this by a comparison of the molecular characteristics of five strains with interesting symbiotic properties. From this comparison we conclude, for instance, that differences in the efficiency of nitrogen fixation or competitiveness for nodulation of these strains are not apparently related to differences in Nod factor structure.

Key words: Soybean-nodulating rhizobia – Nod factors – plasmid profiles – PCR analyses – symbiotic characteristics

Introduction

Until 1982, soybeans were believed to be nodulated only by slow-growing bacteria belonging to the genus *Bradyrhizobium*. Since then, however, new groups of soybean rhizobia have been isolated and classified, making it clear that soybeans are nodulated by a wide variety of rhizobia. Soybean nodulating rhizobia are distributed in different species belonging to three different genera. *Bradyrhizobium japonicum*, *B. elkanii,* and *B. liaoningense*: slow-growing bacteria, with generation times over 6 hours; *Mesorhizobium tianshanense*, generation time is variable; and *Sinorhizobium fredii and S. xinjiangense*: fast-growers, generation time between 1.5 and 4 hours [10, 44, 45]. We will use the acronyms FSR and SSR to designate fast- and slow-growing rhizobia, respectively. Fast- and slow-growing bacteria that nodulate soybean will be collectively called soybean-rhizobia.

Fast-growing soybean bacteria were first isolated in the early 1980s [25] and were clustered into the species

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Abbreviations: FSR, fast-growing soybean-nodulating rhizobia; GC-MS, gas chromatography mass spectrometry; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; IGS-PCR, intergenic spacer PCR; MAS, most abundant strain; OD, optical density; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSR, slow-growing soybean-nodulating rhizobia

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Sinorhizobium fredii. These first soybean isolates are able to form nitrogen-fixing nodules on Asiatic soybean (*Glycine max*) cultivars but fail to nodulate, or are very poorly effective, with the modern cultivars from North America [2, 5, 25]. Later, the isolation of new *S. fredii* strains that were able to form nitrogen-fixing nodules with both Asiatic and American soybean cultivars was reported [15]. In addition to their marked soybean cultivar specificity, *S. fredii* strains show a very broad host range, being able to nodulate at least 79 different genera of legumes [39]. Most of the well-studied strains of *S. fredii* are from China [10, 15, 25], other fast-growing soybean microsymbionts have also been isolated from Panama [21], Malaysia [49], Taiwan [31], and Vietnam [11].

Taxonomic studies have shown that *S. fredii* is closely related to the alfalfa microsymbiont *Sinorhizobium meliloti* [13], although their host-ranges and nodulation factor structures (Nod factors or LCOs) are different [14, 18]. They are also related to *Sinorhizobium teranga* and *Sinorhizobium saheli*, two species isolated from nodules of West African trees, and to *S. medicae* [13, 45].

S. fredii strains are potentially valuable because it should be easier to produce inoculants of fast growing than of slow-growing rhizobia. Also, to carry out genetic analyses of fast-growing rhizobia is much easier than that of slow-growers. These facts, in combination with their exceptionally broad host range, make *S. fredii* a very attractive subject for experimental manipulation aimed at the acquisition of basic knowledge and also for practical purposes.

Field experiments carried out in Spain showed that seed yields of 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 are valid soybean inoculants [7]. 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 *S. fredii* inoculants tested are particularly effective in alkaline soils [7], it remains to be demonstrated that these soybean inoculants are also effective in other types of soil.

Until very recently, most of the basic and agronomic knowledge on *S. fredii* strains was concentrated on a small number of strains that were isolated from just a few areas of China so that the total number of strains that were available for scientific or practical purposes was very limited.

Recently, a report has shown that soil samples from Honghu county (Hubei province), Zheng Zhou county (Henan province), Wei Fang county (Shan Dong province) and Chang Ji county (Xinjiang province) contained FSR and SSR soybean rhizobia [48]. Plasmid and lipopolysaccharide (LPS) profiles and PCR-RAPD analyses showed that cultivar Jing Dou 19 inoculated with Xinjiang soil trapped a diversity of FSR strains while most FSR isolates from cultivar Heinong 33 inoculated with the same soil sample appeared to be very similar or identical. In addition, comparisons between the estimated soybean rhizobia populations by the most probable technique (MPN) using different soybean cultivars led to the conclusion that cultivar specificity can affect the estimation of total soybean rhizobia populations indigenous to the soil, and can also affect the diversity of soybean rhizobial strains isolated from soybean nodules.

Very recently, a study of 33 FSR strains isolated from Honghu describing different physiological and genetic characteristics allowed the construction of a phenetic dendrogram [8]. None of the strains studied showed identical characteristics, demonstrating the extraordinarily high diversity of *S. fredii* strains present in a particular region of China and the interest in continuing the isolation of new strains.

From the experiments carried out to determine soybean-rhizobia populations in soil samples from Hubei, Shan Dong, Henan, and Xinjiang [48], we have isolated several hundred fast- and slow-growing bacteria that nodulate soybean from Asiatic and Western soybean cultivars inoculated with soil samples from four different regions of China. A collection of 203 soybean-rhizobia isolates (198 FSR and 5 SSR strains) was characterised by studying different physiological, genetic and symbiotic traits. Here we present a summary of our data that have been compiled in the first extensive catalogue of fast-growing rhizobia that nodulate soybeans.

Materials and Methods

Soybean cultivars, bacterial strains, plasmid and media

Glycine max cultivars Heinong 33, Jing Dou 19 (Asiatic) and Williams (American) were used as trapping-hosts to isolate soybean rhizobia from soil samples from four different locations in China: Honghu county (Hubei province), Zheng Zhou county (Henan province), Wei Fang county (Shan Dong province) and Chang Ji county (Xinjiang province). In addition, the American soybean cultivars Kochi and Osumi were used in some competition experiments. Bacterial strains used in this study were isolated from soybean nodules as previously reported [7, 50] and are listed in Table 1.

S. fredii strains USDA205 [25], HH103 [15] and its streptomycin-resistant derivative HH103-1 [6], were used as control strains in different experiments.

Plasmid R68.45 [20] was introduced into *S. fredii* USDA205 by conjugation (*E. coli* 1230 R68.45 × USDA205) as described by Buchanan-Wollaston et al. [4]. Strain USDA205 (R68.45) was included as a control in all plasmid agarose-gel electrophoretic analyses that were carried out in this study.

The following media were used in the characterization of the bacteria: TY medium [3], yeast mannitol broth (YMB) medium [47], B^- medium [46], and LC medium which is a variation of LB medium [42] containing NaCl at a final concentration of 8 g L^{-1} and 1 mM Tris. When required, YMB media was solidified with agar (YMA).

Physiological analyses

Generation time of each bacterial isolate was determined in 4 ml YMB bacterial cultures growing on a rotary shaker at 28 °C. The generation time was estimated from OD_{540} measurements during the bacterial log-phase and is given to the closest quarter of an hour. Bacterial growth on solid YMB and LC media at 28 °C, or 37 °C, was scored 2, 7 and 14 days after inoculation.

Acidification of the growth medium was tested in non-buffered liquid YMB medium after 6 days of bacterial growth at

28 °C. Bacterial capacity to grow at different pH values was assayed in YMB medium supplemented with the appropriate buffer: 20 mM MES (2-[N-Morpholino]ethanesulphonic acid) for pH 5.5 and 20 mM Tris for pH 9. YMB at pH 7 was not buffered. The approximate size of the inoculum was 4×10^5 bacteria. Results were scored after 6 days of bacterial growth. Melanin production assays were carried out as described by Cubo et al. [12].

Plasmid profiles

Plasmid profiles were obtained by agarose gel electrophoresis as described by Espuny et al [16]. DNA bands that migrated just below the linearised chromosome were not considered for the determination of the number of indigenous plasmids, although in some cases they could be real plasmids.

Cell surface analyses

SDS/polyacrylamide gel electrophoresis (PAGE) of crude bacterial extracts was performed to determine lipopolysaccharide (LPS) profiles. Bacterial cultures were grown on solid TY medium. Bacterial cells were resuspended in 0.9% NaCl and pelleted by centrifugation. The bacterial pellet was resuspended in 125 µl of 60 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 1 mM EDTA, lysed by heating at 100 °C 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. [27]. The electrophoresis of crude bacterial extracts was performed on 16.5% (w/v) polyacrylamide gels with the Tricine buffer system described by Lesse et al. [30]. For the detection of LPS, gels were stained with silver as described by Kittelberger and Hilbink [26]. Congo red absorption was scored after 7 days of growth at 28 °C on solid YMB medium supplemented with the dye at a final concentration of 25 µg ml^{-1}.

PCR analyses

For random amplified polymorphic DNA (RAPD) analyses the following primers were used: P5, 5′-TCGGAGTGGC-3′; P17, 5′-GTTAGCGGCG-3′; P30, 5′-CCGAAGCCCT-3′; P64, 5′-CCAGGCGCAA-3′. PCR reactions were performed in a final volume of 25 µl containing the following components: 2.5 µl of 10x reaction buffer (10 mM Tris-ClH, 50 mM $MgCl_2$), 50 ng of the DNA primer, 2.5 µl of 15 mM $MgCl_2$, 2.5 µl of 2 mM dNTPs, 0.5 µl of Taq DNA-polymerase (5 U $µl^{-1}$), 1 µl (25 ng) of DNA template, and 15 µl ddH_20. DNA amplification was carried out in a DNA thermal cycler by using the following profiles: 1 cycle at 95 °C for 3 min; 35 cycles at 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 1 min; 1 cycle at 72 °C for 8 min.

For 16S rDNA PCR analyses the set of primers used were: pA, 5′-AGAGTTTGATCCTGGCTCAG-3′ and pH, 5′-AAGGAGGTCATCCAGCCGCA-3′. PCR reactions were prepared in a final volume of 50 µl as described above. DNA amplification was carried out as follows: 1 cycle at 94 °C for 3 min; 28 cycles at 94 °C for 50 s, 54 °C for 45 s, and 72 °C for 90 s; 1 cycle at 72 °C for 8 min.

For 16S–23S rDNA Intergenic Spacer (IGS) PCR the primers were: pHr, 5′-TGCGGCTGGATCACCTCCTT-3′ and p23SR01, 5′-GGCTGCTTCTAAGCCAAC-3′. PCR reactions were performed in a final volume of 100 µl. DNA amplification was carried out as follows: 1 cycle at 95 °C for 1 min; 35 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; 1 cycle at 72 °C for 7 min.

16 rDNA and 16S–23S rDNA IGS PCR-Restriction Fragment Length Polymorphism (RFLP) analyses were carried out by digesting the respective PCR products with the endonucleases *Hinf*I, *Hae*III, or *Msp*I. DNA fragments resulting from PCR reactions, treated or untreated with endonucleases, were electrophoresed in 1.5% (w/v) agarose gels for 2.5 hours and visualised by ethidium bromide staining.

Table 1. List of bacterial strains isolated from soybean nodules used to construct the catalogue of soybean-rhizobia strains.

Province of origin	Strains	Soybean cultivar used as trapping host	Remarks[1]
Xinjiang			
	B1–B20, B46–B50	Jing Dou 19	B41 to B50 are MAS (10^{-5})
	B21–B45	Heinong 33	
	B51–B55	Williams	B51–B55 are SSR strains
Henan			
	HH1–HH30	Heinong 33	
	HHG31–HHG35	Heinong 33	MAS (10^{-3} or 10^{-4})
	WH1–WH10	Williams	
	WHG11–WHG15	Williams	MAS (10^{-3} or 10^{-4})
Shan Dong			
	HW1–HW30	Heinong 33	
	HWG31–HWG35	Heinong 33	MAS (10^{-3} or 10^{-4})
	WW1–WW10	Williams	
	WWG11–WWG15	Williams	MAS (10^{-3})
Hubei			
	S1–S30, S41	Jing Dou 19	
	S31–S40, S42, S43, S51	Williams	
	S44–S47	Jing Dou 19	MAS (10^{-5})
	S48, S49, S50	Williams	MAS (10^{-5})

[1] MAS: <u>M</u>ost <u>A</u>bundant <u>S</u>trains. They were isolated from soybean plants inoculated with the highest dilution of the soil samples (indicated in brackets) that produced nodulated soybeans [48].

The population of indigenous soybean-rhizobia in the four areas studied ranged from 10^4 to 10^6 bacteria per gram of soil, as determined by the Most Probable Number technique [48].

Nod factor (LCO) isolation and profiling

Induction of LCO production by bacterial cultures in the presence of sterile seed exudate of *Glycine max* cv. Bragg, their extraction and analyses by thin layer chromatography (TLC) on silica-60 (Merck) and reversed phase-C18 (Merck) HP-TLC plates were carried out as previously described [48]. Production of LCOs by bacterial cultures in the presence of the inducer genistein (3.7 µM) and ^{14}C N-acetyl glucosamine, their extraction and analyses using reverse phase-C18 (Merck) HP-TLC plates were carried out as described by Spaink et al. [43].

Nod factor (LCO) production and purification for structural analyses

For the production of Nod factors, the rhizobial strains were grown in B^- medium to a total volume of 3 L containing 3.7 µM genistein. Nod factors were extracted from the cultures with n-butanol (300 ml l^{-1}) and dried. Samples were redissolved in acetonitrile: water (60:40 v/v) at 200 rpm overnight. Pre-purification was performed by passing the crude extract over a C_{18} column (Baker, 1 mg sorbent) from which LCOs were eluted using 60% acetonitrile. The 60% acetonitrile fractions were diluted to 20% acetonitrile and submitted to reversed phase HPLC on a Jasco PV-980 HPLC pump with a flow rate of 1 ml min^{-1} and Jasco MD-910 photodiode array detection (200–600 nm). 4 ml of sample was injected on to a C_{18} Sephasil column (5 µm, 4 × 250 mm, Pharmacia) and elution was performed using a stepwise gradient of mixtures of acetonitrile:water. The gradient employed was: 20% acetonitrile for 20 minutes; 30% for 30 minutes; 40% for 20 minutes; 42% for 20 minutes; 60% for 20 minutes and 80% for 10 minutes. 3-minute fractions were collected, pooled and dried.

Structural determination of Nod factors (LCOs) by mass spectrometry

For analysis of Nod factors by matrix assisted laser desorption/ionisation (MALDI-TOF) mass spectrometry, 1 ml aliquots of Nod factor-containing HPLC fractions were evaporated to dryness and redissolved in 100 µl of a solution containing 1:1 (v/v) acetonitrile:water incorporating 0.1% formic acid. A 1 µl aliquot of this solution was mixed with 1 µl of alpha cyano-4 hydroxycinannmic acid matrix (saturated in 50% aqueous acetonitrile:0.1% triflouroacetic acid). 0.7 µl of the sample and matrix mixture was spotted on to a stainless steel target and allowed to air dry. Positive ion MALDI-TOF mass spectrometry was performed essentially as described (36). Positive ion mode electrospray mass spectrometry (ES-Q-oTOF) and collision induced dissociation (CID) tandem mass spectrometry of Nod factors was performed essentially as described (36).

Carbohydrate composition analysis of Nod factors (LCOs)

For analysis using GC-MS, 2 ml aliquots of the various HPLC fractions, and 50 nmol aliquots of a range of monosaccharide standards were evaporated to dryness and composition analysis carried out essentially as described (32), except that analysis of the resulting TMS glycosides was carried out using GC-MS, (HP5890 MSD, Hewlett Packard).

For composition analysis using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) (Dionex), 1 ml aliquots of the various HPLC fractions and 5 µl aliquots of standard solutions (0.01 mg Ml^{-1}) of authentic monosacharide standards were evaporated to dryness and hydrolysis carried out in 500 µl of 2 M TFA, at 120 °C for 1 h. The solutions were evaporated to dryness and 30 µl of water was added. HPAEC-PAD analysis was carried out using a Carbopac PA-100 column. An isocratic elution was carried out using 16% 100 mM NaOH, a GP50 Gradient Pump and a flow rate of 1 ml min^{-1}. A 5 µl injection volume was used. Detection was carried out using an ED50 electrochemical detector. Monosaccharides released from the LCOs were identified on comparison of their retention times with those of the individually injected monosaccharide standards.

Linkage analysis of Nod factors (LCOs)

1 ml aliquots of the relevant HPLC fractions were evaporated to dryness and linkage analysis was carried out essentially as described (32), except that analysis of the resulting TMS glycosides was carried out using GC-MS, (HP5890 MSD, Hewlett Packard).

Plant tests

Nodulation tests on *Glycine max* were carried out on at least one Asiatic (Jing Dou 19 or Heinong 33) and the American soybean cultivar Williams, as previously described [6, 48].

Competition experiments between FSR strains on *Glycine max* cultivars Jing Dou 19, Williams, Kochi or Osumi and the determination of nodule occupancy were carried out as previously described [48]. Nodule occupancy in competition studies between the reference strain *S. fredii* HH103-1 (Str^R) and the different FSR strains used was determined by plating bacterial nodule-isolates on YMA media supplemented with streptomycin (400 µg ml^{-1}). Those isolates being able to grow in the presence of the antibiotic were considered as HH103-1. For the competition experiment between strains B15 and HH17, a spontaneous streptomycin-resistant derivative of the former was used.

Results

Rhizobial strains

Serial dilutions of watery suspensions of soil samples collected from soybean fields from Wei Fang (Shan Dong province, Central-East China), Zheng Zhou (Henan province, Central-East China), Honghu (Hubei province, Central China) and Chang Ji (Xinjiang province, Western China) counties were used as inoculants of Asiatic (Heinong 33 and Jing Dou 19) and Western (Williams) soybean cultivars.

Bacteria were isolated from nodules of all plants and stored in 40% (w/v) glycerol at –80 °C. A total of 203 nodule isolates, representing each region and trapping-soybean cultivar, were chosen for further characterization. These strains are listed in Table 1. All of them, except for five strains from Xinjiang province (isolates B51 to B55), were FSR on solified YMB media.

Construction of a catalogue of soybean-rhizobia strains

Different bacterial traits were studied to characterise the bacteria: plasmid content, surface properties (lipopolysaccharide profiles and Congo red absorption), growth characteristics (generation time, acidification of YMB medium, ability to grow at pH 5.5, 7.0 and pH 9.0, capacity to grow on LC medium and capacity to grow at 28 and 37 °C on solified YMB and LC media), melanin production, PCR finger-printing analyses [16S rDNA

PCR; 16S rDNA PCR-Restriction Fragment Length Polymorphism (RFLP); 16-23S rDNA Intergenic Spacer (IGS)-PCR; 16S–23S rDNA IGS-PCR-RFLP and Randomly Amplified Polymorphic DNAs (RAPD)], symbiotic properties (ability to nodulate Asiatic and Western soybean cultivars, competition capacity to nodulate soybeans), and Nod factor (LCO) analysis. All these aspects will be discussed in more detail below.

Plasmid profiles

Plasmid agarose-gel electrophoresis showed a wide variability in the number of indigenous plasmids that are present in the 198 FSR strains analysed (Table 2). Although the number of plasmids can range from one to six, bacterial strains carrying two, three or four plasmids are predominant. Figure 1 shows examples of plasmid

Table 2. Number of plasmids, growth characteristics, melanin production and Congo Red absorption of FSR isolated from soil samples from soybean cropping areas of Xinjiang, Henan, Shan Dong and Hubei Chinese provinces.

Bacterial trait		Number of strains analysed	Origin of the soybean nodule isolate				Total (%)
			Xinjiang	Henan	Shan Dong	Hubei	
Number of plasmids	1	198	4	1	19	1	25 (12.6)
	2		9	4	16	13	42 (21.2)
	3		9	23	3	24	59 (29.8)
	4		25	14	10	10	59 (29.8)
	5		1	6	2	1	10 (5.1)
	6		0	2	0	1	3 (1.5)
Acidification of YMB medium [A]	+	186	11	9	27	47	116 (62.4)
	±		23	19	17	1	60 (32.3)
	–		9	0	0	1	10 (5.4)
Growth at different pH [B]	pH 5.5	192	1	1	6	5	13 (6.8)
	pH 7.0		43	50	50	49	192 (100)
	pH 9.0		9	30	16	6	61 (31.8)
Growth on LC medium at 28 °C[C]	++	198	0	8	1	2	11 (5.6)
	+		9	2	1	4	16 (8.1)
	±		4	4	4	3	16 (8.1)
	–		34	36	44	41	155 (78.3)
Growth on YMB medium at 37 °C[C]	++	196	0	5	0	0	5 (2.6)
	+		0	1	0	1	2 (1.0)
	±		0	1	1	1	3 (1.5)
	–		48	41	49	48	186 (94.9)
Growth on LC medium at 37 °C[C]	++	196	0	7	1	1	9 (4.6)
	+		0	0	3	1	4 (2.0)
	±		0	2	1	1	4 (2.0)
	–		48	39	45	47	179 (91.3)
Melanin production[D]	+	198	44	30	5	35	114 (57.6)
	–		4	20	45	15	84 (42.4)
Congo red absorption[E]	+	198	1	2	1	2	6 (3.0)
	±		5	24	16	11	56 (28.3)
	–		42	24	33	37	136 (68.7)
Generation time	1.5–2.5 h	193	14	8	3	14	39 (20.2)
	2.5–3.5 h		33	41	38	32	144 (74.6)
	3.5–4.5 h		1	1	6	2	10 (5.2)
	4.5–5.0 h		0	0	1	1	2 (1.0)

[A] + pH shift higher than 0.5 pH units; ± pH shift of 0.1–0.5 pH units; – pH shift of 0–0.1 pH units. Results were scored after 6 days of incubation.

[B] Numbers after pH values indicate the number of bacterial cultures that became more turbid than the uninoculated controls after 6 days of incubation.

[C] ++ growth after 2 days; + growth after 7 days; ± intermediate growth after 7 days; – no growth after 7–14 days.

[D] + bacterial patches produced a diffusible dark-brown pigment; – bacterial patches did not change their colour.

[E] + colonies accumulated Congo red; ± weak or very weak Congo read accumulation; – no accumulation.

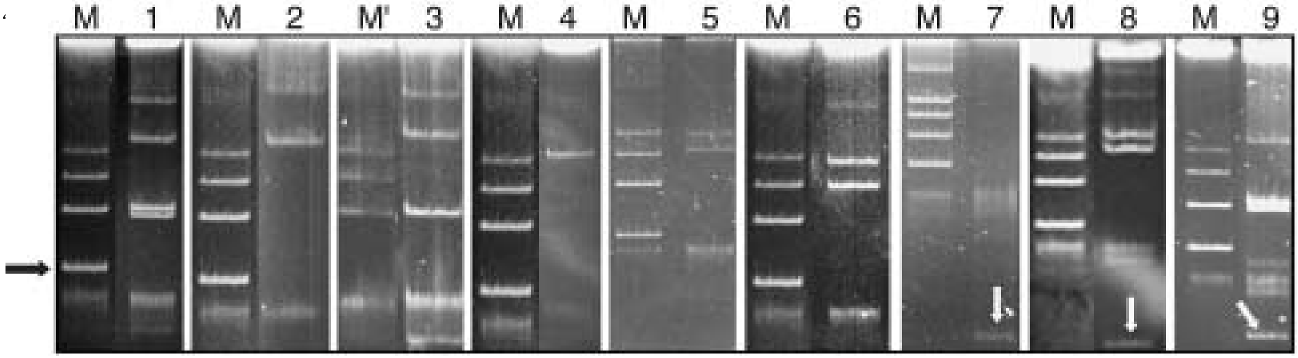

Fig. 1. Plasmid profiles of FSR strains isolated from soil samples of Xinjiang, Henan, Shan Dong and Hubei Chinese provinces. Lanes: 1) B33; 2) HH1; 3) HH17; 4) WW10; 5) HWG35; 6) S30; 7) WHG14; 8) HH12; 9) B40; M, *S. fredii* USDA205 carrying plasmid R68.45; M', *S. fredii* USDA205. Plasmid R68.45 is indicated by a black arrow. The low molecular weight plasmids of strains WHG14, HH12, and B40 are indicated by white arrows.

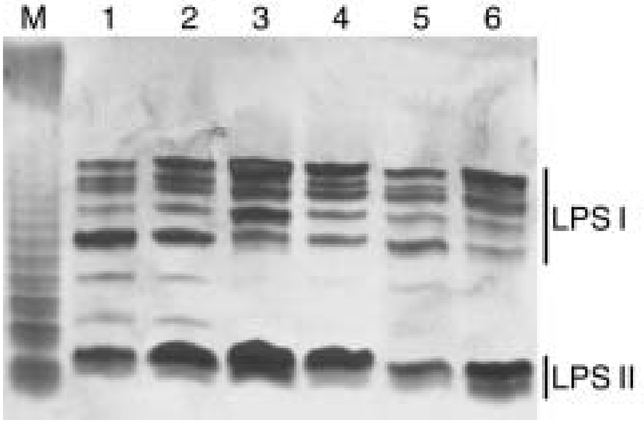

Fig. 2. Lipopolysaccharide profiles of FSR strains isolated from soybean cropping areas of Xinjiang, Henan, Shan Dong and Hubei Chinese provinces. Lanes: 1) HH1; 2) HH17; 3) B33; 4) S30; 5) WW10; 6) HWG35. M, *Salmonella typhimurium* LPS (Sigma) was used as a reference.

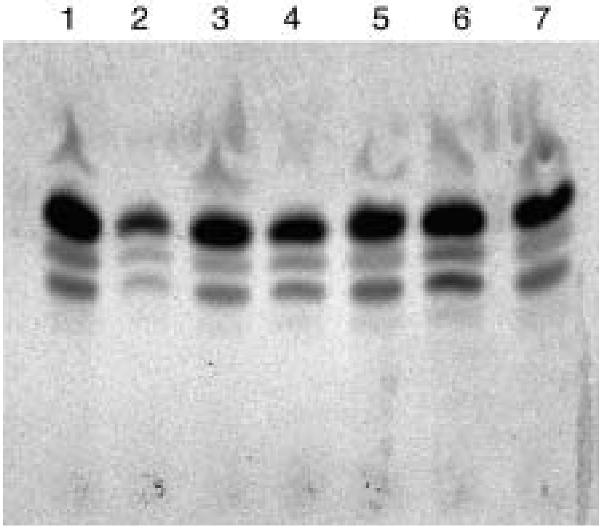

Fig. 3. LCO profiles of different FSR strains isolated from soybean cropping areas of Xinjiang, Henan, Shan Dong and Hubei Chinese provinces. Lanes: 1) *S. fredii* HH103; 2) HH1; 3) WW10; 4) HH17; 5) S30; 6) B33; 7) HWG35.

profiles of different strains. Some strains, such as WHG14, HH12 or B40, harbour small plasmids that migrate faster than the linearized chromosome (observable as a broad band) (Fig. 1, lanes 7, 8 and 9).

Surface characteristics

The LPS profiles shown by most of the isolates from soil samples of Hubei, Henan and Shan Dong provinces are very similar to those previously reported for other *S. fredii* isolates from Hubei province [8] and for *S. fredii* HH103, another isolate from the same province [19]. This profile consists of a ladder with variable numbers of bands which probably correspond to the complete LPS molecules (LPS-I region, as defined by Kannenberg et al. [24] and one or two faster migrating bands that could correspond to the lipid-A plus core and one, or zero, O-antigen subunits (LPS-II region), respectively. Although most of these strains share this LPS electrophoretic pattern, subtle differences in the mobility of the stained bands can be observed (Fig. 2). Isolates from Xinjiang showed two different LPS patterns, as previously described [48]. Basically, Xinjiang strains isolated from nodules of soybean cultivar Heinong 33 showed typical *S. fredii* LPS profiles (Fig. 2, lane 3), while those isolated from cultivar Jing Dou 19 have the tendency of producing a large smearing spot, or poor staining, in the LPS-I and/or LPS-II regions [48].

Congo red dye has been found to bind many types of bacterial polysaccharides and it has been widely used as an easy marker for the detection of differences among bacterial polysaccharides [9]. Only six isolates of the investigated collection showed positive Congo red absorption, whereas many strains weakly bound the colorant (Table 2).

Physiological characteristics

Most of the isolates showed generation times ranging between $2\frac{1}{2}$ and $3\frac{1}{2}$ hours (Table 2). All rhizobia grew on YMB medium at 28 °C. Bacterial growth on LC medium at 28 °C was found in 27 strains, 13 of them (such as, for instance, S16, HH2 or HH28) being able to grow on LC medium, at 37 °C (Table 2). The growth speed of several strains (such as HH28) was even faster on LC medium than on YMB medium.

Most of the bacterial cultures in YMB medium (initial pH value of 7.0) showed a final pH ranging from 5.0 to 6.8 (Table 2), meaning that they acidified the growth medium. Only a few strains (such as HHG34 and HW29) produced a final pH that was lower than 5.0.

Many bacterial strains were able to grow at pH values of 4.5 and/or 9.0 if the YMB medium was not buffered (data not shown). When the YMB medium was buffered with 20 mM Tris at pH 9.0, only one third (61) of the strains tested were able to grow at this alkaline pH (Table 2). Half of the strains (30) that were able to grow at pH 9 in buffered YMB were isolated from Henan province. Only a few strains (13) were able to grow at pH 5.5 in YMB buffered with 20 mM MES. Interestingly, six of these strains were also able to grow at pH 9.0 (such as S40, HH28 and WW10).

Melanin producers were found among isolates from the four regions analysed (Table 2). Although 57% of the analysed isolates produced the pigment, this characteristic was rare (10%) among isolates from Shan Dong.

The catalogue that it is available at http://www.soybeanrhizobia.net also shows additional data that are not presented in this report (such as carbon and nitrogen utilisation and intrinsic antibiotic resistance). This information should be considered as "preliminary data that requires further confirmation" and, for this reason, has not been included in the present report.

PCR analyses

16S rDNA and 16S–23S rDNA Intergenic Spacer (IGS) PCR analyses of all strains from Shan Dong and Henan and those tested from Xinjiang and Hubei provinces showed the amplification of a 1.5 kb or a 2.0 kb band, respectively (Fig. 87 of the catalogue is an example). Three enzymes, *Hae*III, *Msp*I and *Hinf*I were used to carry out restriction-fragment length polymorphism (RFLP) of DNA fragments amplified by 16S rDNA PCR or 16S–23S rDNA IGS-PCR. All isolates from Henan and Shan Dong, and those tested from Hubei soil samples showed no observable differences in their 16S rDNA-PCR RFLP, and it was that predicted from the 16S rDNA sequence reported for *S. fredii* USDA205^T [38]. Although most of the bacterial isolates from Xinjiang also showed the characteristic 16S rDNA-PCR RFLP of *S. fredii* strains, some of them (such as strain B7 with *Hinf*I and *Hae*III and B11 with *Hinf*I and *Msp*I) gave RFLP patterns that were different from those predicted from the 16S rDNA sequences reported [30] for *S. fredii* and *S. xinjiangense* strains (the accession numbers for the *S. fredii* and *S. xinjiangense* 16S rDNA sequences are x67231 and AF250354**)**. Interestingly, strain B11 is able to use dulcitol as the only carbon source, a capacity that, to our knowledge, is absent from all fast-growing soybean rhizobia studied so far [10, 40].

RFLP of the 16S–23S rDNA IGS-PCR fragments showed differences between some strains. For instance, strain WW10 was different from strains B33, HH1, HH17, HWG35, and S30 when the endonucleases *Hae*III and *Msp*I were used. No differences among the strains were found when the endonuclease *Hinf*I was used (see Fig. 87 of the catalogue).

Randomly Amplified Polymorphic DNA analyses (RAPD) using different primers showed differences in RAPD profiles among strains isolated from the four regions analysed (see catalogue). There are clear differences in RAPD between strains from Hubei (S-strains) and those from Xinjiang (B-strains) and also among strains from the same region (see Figs. 88 and 89 of the catalogue).

Symbiotic characteristics

Most of the isolates from Henan were able to form nitrogen-fixing nodules with the Asiatic soybean cultivar Heinong 33 and/or the American cultivar Williams (76% and 80%, respectively), regardless of whether they were isolated from nodules of Asiatic or American cultivars. Similarly, bacterial strains from Henan that are poorly effective on Heinong 33 or Williams were originally isolated from both Heinong 33 and Williams cultivars. Similar results were obtained with isolates from Shan Dong, most of them being effective with Heinong 33 (86%) and Williams (92%). Only two strains from Shan Dong soil samples were ineffective with both soybean cultivars. All isolates from Hubei formed nitrogen-fixing nodules with

Table 3. Nodulation tests of isolates from Henan, Shan Dong, and Hubei provinces with the American soybean cultivar Williams and the Asiatic cultivars Heinong 33 or Jing Dou 19.

Province of origin	Soybean cultivar used as trapping host[a]	Soybean cultivar tested	Symbiotic phenotype	
			Fix$^+$	Fix$^-$
Henan	Heinong 33 (35)	Heinong 33	27	8
		Williams	29	6
	Williams (15)	Heinong 33	11	4
		Williams	11	4
Shan Dong	Heinong 33 (35)	Heinong 33	31	4
		Williams	31	4
	Williams (15)	Heinong 33	12	3
		Williams	15	0
Hubei	Jing Dou 19 (34)	Jing Dou 19	34	0
		Williams[b]	28	3
	Williams (16)	Jing Dou 19[c]	16	0
		Williams[d]	12	1

[a] Number in brackets refer to the total number of strains isolated from that particular cultivar that were subsequently tested on soybean cultivars Heinong 33 (or Jing Dou 19) and on cultivar Williams.

[b] Strains S44, S45, and S47 were not tested with cultivar Williams.

[c] Strain S49 was not tested with cultivar Williams.

[d] Strains S48, S50 and S51 were not tested with cultivar Williams.

Table 4. Competition experiments among FSR strains to nodulate different soybean cultivars.

Pairs of competitors (A/B)[a]	Soybean cultivar	Number of nodules tested	Nodule occupancy (%)[b]	
			A	B
B15/HH103-1	Jing Dou 19	125	50	50
B15 Str^R/HH17	Jing Dou 19	123	39	61
HH103/HH17	Jing Dou 19	125	72	28
S18/HH103-1	Williams	146	34	66
S18/HH103-1	Kochi	150	45	55
S18/HH103-1	Osumi	145	79	21
S30/HH103-1	Williams	125	46	54
S30/HH103-1	Osumi	143	71	29
S30/HH103-1	Kochi	148	80	20

[a] The ratio of competitors was 1:1, which approximately corresponds to 2×10^8 cells per competitor.

[b] Numbers indicate the percentage of nodules occupied by each competitors.

Nodule occupancy was determined 6–7 weeks after inoculation by assessing the presence of the streptomycin-resistance marker. Strains B15 Str^R and HH103-1 are streptomycin-resistant derivatives of B15 and HH103, respectively.

Jing Dou 19. The degree of effectiveness of those strains that were able to form nitrogen-fixing nodules varied from "poor" to "highly effective", as judged by plant dry-weight and/or colour of leaves. Only four strains out of 44 tested on soybean Williams were ineffective with this cultivar. Nodulation ability of isolates from Xinjiang has already been reported [48]. Table 3 summarises the results obtained in plant tests with bacterial isolates from Henan, Shan Dong, and Hubei.

Forty-three different competition experiments were carried out between different FSR strains, between FSR strains and SSR strains, or between FSR strains and the reference strain *S. fredii* HH103 (results are listed in the catalogue presented at the Web address given above). Table 4 illustrates two examples from which conclusions can be obtained: a) although strains B15 and HH103 were equally competitive for nodulation of cultivar Jing Dou 19 and the former was less competitive than strain HH17, strain HH103 outcompeted strain HH17. The results showed that it is not possible to predict how a particular strain will compete with a second strain from the results of competition experiments between the first and a third or the second and the third strains. b) soybean cultivar specificity not only exerted a strong influence on the

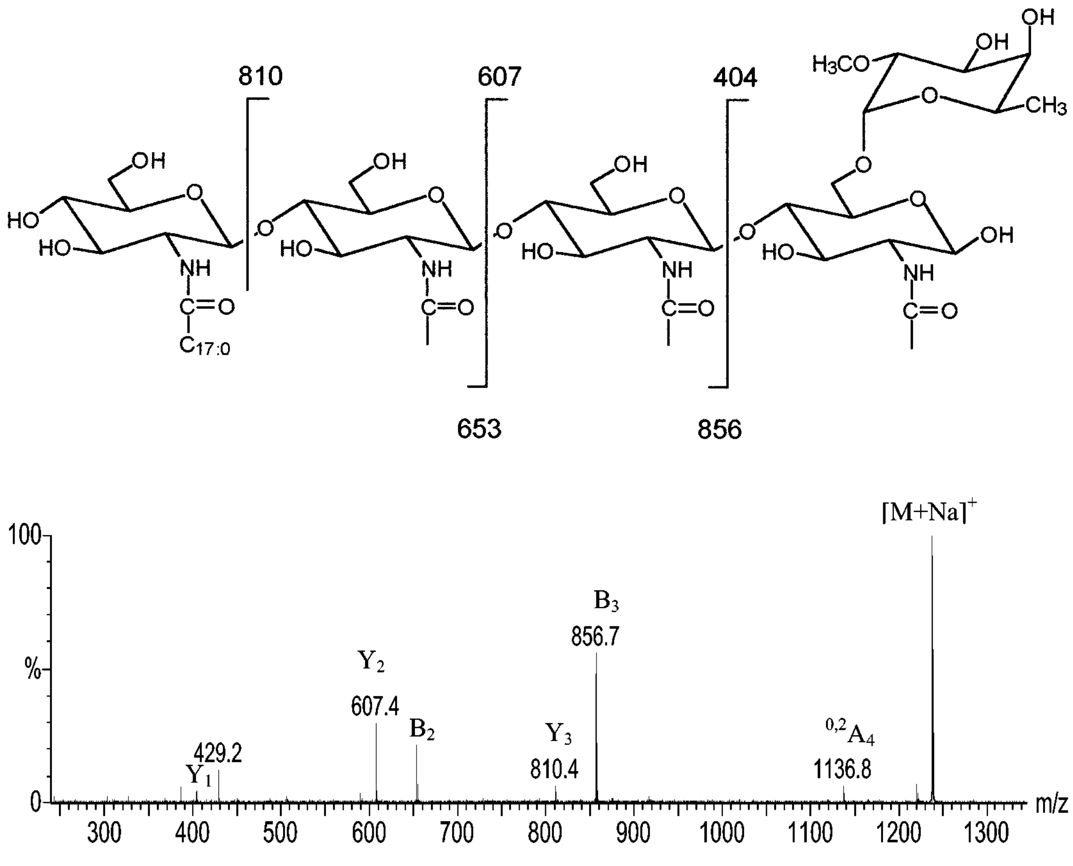

Fig. 4. Fragmentation scheme and ES-Q-oTOF collision induced dissociation product ion spectrum of LCO IV($C_{18:0}$,MeFuc) produced by strain B33. The ion at m/z 429 arises as a result of B_nY_n double cleavage.

outcome of competition experiments between FSR and SSR (as reported by Yang et al. [48]), but also between FSR strains. For instance, the reference strain HH103 (FSR) was more competitive than S18 (FSR) for nodulation of Williams, equally competitive with cultivar Kochi, but less competitive in cultivar Osumi. Another FSR strain, S30, was as competitive as HH103 in Williams but it outcompeted the reference strain in cultivars Kochi and Osumi.

Lipochitin-oligosaccharides (LCOs)

LCO profiles of all strains in the whole bacterial collection were analysed on silica (TLC) and reversed-phase-C18 thin layer chromatography (RP-TLC). It was very surprising to observe that LCO profiles of all strains were indistinguishable from that of the control strain *S. fredii* HH103 (Fig. 3). From the slow-growing strains B51 to B55 and strain HW30 LCO production was not detected under the experimental conditions used.

Strains B33, WW10 and HH1 (high nitrogen-fixing capacity in greenhouse experiments), and HWG35 and HH17 (high competition ability versus SSR strains) were selected for further study of their LCO structures. The selected strains were isolated from three different regions: Xinjiang (B33), Shan Dong (HWG35, WW10) and Henan (HH1 and HH17). LCO samples were produced and purified using reversed-phase HPLC with photodiode array detection. The structure of each of the LCO components in each of the fractions from each of the strains was determined using MALDI-TOF, ES-Q-oTOF and CID-tandem mass spectrometry. In this experiment, electrospray-generated ions may be isolated and activated on collision with nitrogen, causing the ion to fragment in a structurally-diagnostic manner. The product ions that result from this collision induced dissociation allow the structure of the LCO, including the length of the carbohydrate backbone, the type of lipid and the nature and location of the backbone substituents to be assigned (e.g. Fig. 4). Furthermore, the carbohydrate composition of the Nod factors was determined and linkage analysis was performed. The LCO structures identified from the combined results of these analyses, from each strain are summarised in Table 5. From these data it is very noticeable that strain HH17 produces a very much wider range of LCO structures than the other strains examined in this way. Because such a large number of different structures was somewhat surprising, the culture of strain HH17 was repeated, and LCOs were once again extracted and fractionated using reversed-phase HPLC. The new HPLC fractions were once again analysed using MALDI-TOF, ES-Q-oTOF and CID-tandem mass spectrometry. The LCO structures identified from the newly-produced fractions from strain HH17 were almost indistinguishable from those identified from the first LCO preparation from this strain, demonstrating that this strain reproducibly produces a wider range of LCO structures under the culture and isolation conditions used in our study.

From the results, it is clear that most LCOs identified as being produced by these five selected strains have structures in line with those expected to be produced by strains of *S. fredii* [18]. However, strain HH17 showed two interesting features in its LCO production ability. Firstly, this strain produces a large number (39) of different LCO structures, most of which are variants of the structures normally produced by strains of *S. fredii*. Secondly, it produces LCOs carrying N-methyl substituents on the non-reducing *N*-acetylglucosamine residue and also LCOs bearing an acetyl substituent, in place of the fucosyl residue, on the reducing *N*-acetylglucosamine residue. Such structures have not, to our knowledge, previously been reported as being produced by strains of *S. fredii*.

Discussion

This work summarises physiological, genetic, structural and symbiotic traits of 198 fast-growing soybean rhizobia (FSR) isolated from soil samples from four different soybean-cropping areas of China. The data obtained in the characterization of this collection are compiled in a catalogue that is available on internet at the address given above.

Table 5. Structural assignment for LCOs from Chinese fast-growing soybean rhizobial strains.

Strain	LCO Structure assigned
B33	V($C_{18:1}$,MeFuc); V($C_{18:0}$,MeFuc); IV($C_{18:1}$, MeFuc); IV($C_{18:0}$, MeFuc); III($C_{18:1}$, MeFuc); III($C_{18:0}$, MeFuc)
HWG35	V($C_{18:1}$, MeFuc); V($C_{18:0}$, MeFuc); IV($C_{18:1}$, MeFuc); IV($C_{18:0}$, MeFuc); IV($C_{18:1}$, Fuc); III($C_{18:1}$, MeFuc)
WW10	V($C_{18:1}$, MeFuc); V($C_{18:0}$, MeFuc); IV($C_{18:1}$, MeFuc); IV($C_{18:0}$, MeFuc); III($C_{18:1}$, MeFuc)
HH1	V($C_{18:1}$, MeFuc); V($C_{18:0}$, MeFuc); IV($C_{18:1}$, MeFuc); IV($C_{18:1}$, Fuc)
HH17	V ($C_{18:1}$, NMe, MeFuc); V ($C_{20:1}$, MeFuc); V ($C_{16:1}$, MeFuc); V ($C_{16:1}$, Fuc); IV ($C_{20:0}$, MeFuc); IV ($C_{18:1}$ NMe, MeFuc); IV ($C_{18:1}$ NMe, Fuc); III ($C_{18:0,}$ NMe, MeFuc); III ($C_{18:0}$, MeFuc);III ($C_{18:1}$, NMe, MeFuc); IV ($C_{16:1}$, MeFuc); IV ($C_{16:1}$, Fuc); III ($C_{16:1}$, MeFuc); III ($C_{16:1}$, Fuc); III ($C_{16:0}$, MeFuc);V ($C_{16:0}$, MeFuc); V ($C_{16:0}$,Fuc); V ($C_{18:0}$ NMe, MeFuc); V ($C_{18:1}$, MeFuc); V ($C_{18:1}$, Fuc); V($C_{18:1}$); IV ($C_{16:0}$, MeFuc); IV ($C_{16:0}$, Fuc); IV ($C_{18:1}$, Ac); V ($C_{18:1}$, MeFuc); IV ($C_{18:1}$, MeFuc); IV ($C_{18:1}$, Fuc); IV ($C_{18:1}$, MeFuc); IV($C_{18:1}$); III ($C_{18:1}$, MeFuc); III ($C_{18:1}$, Fuc); V ($C_{18:0}$, MeFuc); V ($C_{18:0}$, Fuc); IV ($C_{18:0}$, MeFuc); IV ($C_{18:0}$, NMe, MeFuc); IV ($C_{18:0}$, NMe); IV ($C_{18:0}$, Fuc); IV ($C_{20:3}$, Ac); III ($C_{18:1}$, MeFuc)

Ac – acetate ; Fuc – fucose; MeFuc – methyl-fucose.

Fast-growing bacteria that nodulate soybean (FSR strains) have been isolated from soil samples from soybean cropping areas of four different regions, indicating that FSR strains are widely present in Chinese fields dedicated to soybean cultivation, regardless of their geographical location. The 16S rDNA RFLP pattern of the strains analysed from Henan, Shan Dong and Hubei provinces was that expected for *S. fredii*. Surprisingly, none of the strains investigated from Xinjiang region gave the 16S rDNA RFLP (*Msp*I) pattern that would be predicted from the 16S rDNA sequence reported [38] for *S. xinjiangense*, another fast-growing soybean nodulating bacterium isolated from soils of Xinjiang province [10]. Instead, most of them presented the pattern of *S. fredii*, the exception being some strains, such as B7 and B11, that showed 16S rDNA RFLP patterns that were distinct from those of both *S. fredii* and *S. xinjiangense*. Although most of the strains investigated appear to belong to *S. fredii*¸ RFLP of DNA fragments amplified by 16S–23S rDNA IGS-PCR and RAPD analyses proved to be efficient techniques for discriminating among strains, even if they were isolated from the same soil sample. Although all the strains grew much faster than those belonging to the genus *Bradyrhizobium* (generation time of strains B51 to B55 varied from 8 h to 8 h 45 min), there are clear differences in their generation times, that range from 2 h to 4 h 45 min. Most of the isolates analysed show generation times between 2 h 30 min and 3 h 30 min which is in line with data presented in previous reports on fast-growing soybean nodulating rhizobia isolated in China [15, 25] but slower than the generation time of strain SMH12 (1 h 30 min), a soybean-nodulating baterium isolated from Vietnamese soils [40].

The ability to grow on LB medium at 37 °C had been already reported in some strains belonging to *S. meliloti* [35] and *R. tropici* [34]. Strains able to grow at 37 °C on LC medium (which it is very similar to LB medium) were found in soil samples from Henan, Shan Dong and Hubei but not from Xinjiang region.

The ability to grow at pH 5.5 in buffered YMB was observed in only 13 strains, which is in accordance with a previous report showing that the percentage of fast-growing soybean rhizobial strains able to grow at pH 5.5 is low [8]. Six of them also grew at pH 9.0. Only one strain (HH28) was able to grow in the pH range of 5.5–9.0 and also at 37 °C. The data presented here show that these pH and temperature conditions usually reduce or inhibit the growth of fast-growing soybean rhizobia. Hence, strain HH28 might be an interesting potential soybean inoculant in those environmental conditions that could pose a pH or temperature stress for the bacterial inoculant albeit that its competitiveness still should be tested.

Previous reports on *S. fredii* strains have shown that they carry at least one plasmid and that, only with the exception of *S. fredii* USDA208, at least one of their indigenous plasmids hybridises to *nod* and/or *fix* probes [1, 6]. None of the FSR isolates investigated in this work was devoid of indigenous plasmids. Four strains (B50, S5, S44, and HWG35) were investigated for the presence of symbiotic plasmids. All of them contained a plasmid that complemented a pSym-cured derivative of *S. fredii* USDA193 for soybean nodulation and nitrogen fixation (data not shown). Hence, the presence of symbiotic plasmids in the bacterial collection appears to be the general rule.

Twelve strains (such as WHG14, HH12 and B40) harbour plasmids that, according to their mobility in agarose gels, have molecular weights clearly lower than those currently reported for rhizobial strains [22]. Two other *S. fredii* strains carrying small plasmids have already been reported [8]. Hence, at least in *S. fredii* strains the presence of small plasmids is not an extremely rare phenomenon. Some of them, such as those found in strains WHG14 and HH12 (Fig. 1, lanes 7 and 8) might become suitable rhizobial cloning vectors. It is reasonable to expect that cloning vectors based on indigenous rhizobial plasmids could be highly stable in these bacteria.

It was surprising to observe that none of the LCO profiles investigated on TLC, or RP-TLC, showed any clear difference from that of the control strain *S. fredii* HH103. However, the determination of LCO structures produced by five different strains that were selected for their capacity to fix nitrogen or to compete for nodulation on soybeans, showed that strain HH17 produced 39 different LCOs. To our knowledge, this is the largest number of different LCOs produced by any *Sinorhizobium* strain reported. Most of the LCOs produced by HH17 (and all the LCOs determined for the other four strains analysed) have been already described for the wild-type strain, or mutant derivatives of *S. fredii* HH103 [18, 29, 33]. In addition, HH17 produces new LCOs never described before for *S. fredii*, such as those carrying N-methyl substituents on the non-reducing glucosamine residue or those bearing an acetyl substituent (instead of the fucosyl residue) on the reducing *N*-acetylglucosamine residue. An N-methyl substituent on the non-reducing glucosamine residue is due to the expression of the *nodS* gene [17, 23] which is inactive in the only *S. fredii* strain (USDA257) in which this gene has been isolated and sequenced [28]. Transfer of the *Rhizobium* sp. NGR234 *nodS* gene to *S. fredii* USDA257 produced transconjugants that had gained the ability to nodulate *Leucaena leucocephala* [28]. Since strain HH17 failed to nodulate *Leucaena leucocephala* (our own unpublished results), we conclude that only the presence of a N-methyl substituent on the non-reducing glucosamine residue of the LCOs produced by strain HH17 is not sufficient to confer nodulation ability on this legume.

In addition, the presence of acetyl residues directly linked to the reducing terminal glucosamine residue of the LCOs from strain HH17 suggests the presence of a nodulation gene having the same function as *nodX* (encoding an acetyltransferase), a nodulation gene only reported in *R. leguminosarum* strains [37].

The first report on *S. fredii* [25] described that only one out of 14 isolates was able to form nitrogen fixing nodules on American cultivars. Although the isolation of effective *S. fredii* strains was later reported [8, 15, 41], it was generally believed that most of the *S. fredii* strains were generally unable to establish nitrogen-fixing associations with advanced soybean cultivars. Here we show that 87.5% of the bacterial isolates studied in this work form nitrogen-fixing nodules with soybean cultivar Williams. These results clearly show that although it is easy to isolate *S. fredii* strains that fail in effectively nodulating American soybean cultivars they represent the exception rather than the general rule, at least in the areas investigated in this report. However, very few of the investigated *S. fredii* strains fix nitrogen at a level comparable to *B. japonicum* USDA110, a highly effective strain with American soybean cultivars [7, 41].

S. fredii strains show marked soybean-cultivar specificity [5, 15, 25, 48]. Results presented here show that this cultivar specificity is also reflected in the bacterial ability to compete for nodule formation in different soybean cultivars. Plant tests carried out on different soybean cultivars led to the identification of different bacterial strains that were highly effective for a particular cultivar, such as Jing Dou 19 or Williams. However, due to the cultivar specificity phenomenon, it is probable that those strains showing high nitrogen fixation with a particular soybean cultivar will not be the best inoculant in the whole collection for another soybean cultivar. Strain S18 is a clear example: it is highly effective with soybean cultivar Williams and a mediocre nitrogen-fixer with cultivar Osumi (our own unpublished results).

Since at present it is not possible to predict which strain out of the whole collection is the best nitrogen-fixer with any particular soybean cultivar, the whole collection of rhizobia should be tested for symbiotic nitrogen fixation with any new soybean cultivar. This time-consuming task is however, worthwhile, since, once a subset of strains has been identified as promising inoculants for a specific soybean cultivar, information on many different bacterial characteristics that could be relevant to their use as inoculants, or for carrying out further investigations, is already available. Additionally, strains presented in the catalogue that show peculiar characteristics among soybean-nodulating bacteria, such as the ability to grow on high salt media, such as LC medium, at 37 °C, could be helpful for different research purposes. At present we are carrying out an EU-funded project that will allow the isolation of soybean rhizobia from another ten traditional soybean-cropping regions of China, and also from locations where it is expected that soybean will be cultivated for the first time. The catalogue has been constructed in such a way that allows the creation of updated versions containing new information on the existing strains as well as the addition of new soybean-nodulating bacteria that may be isolated.

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References

1. Appelbaum, E. R., Johansen, E., Chartrain, N.: Symbiotic mutants of USDA191, a fast-growing *Rhizobium* that nodulates soybeans. Mol. Gen. Genet. 201, 454–461 (1985).
2. Balatti, P. A., Pueppke, S. G.: Identification of North American soybean lines that form nitrogen-fixing nodules with *Rhizobium fredii* USDA257. Can. J. Plant Sci. 72, 49–55 (1992).
3. Beringer, J. E.: R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84, 188–198 (1974).
4. Buchanan-Wollaston, A. V., Beringer, J. E., Brewin, N. J., Hirsch, P. R., Johnston, A. W. B.: Isolation of symbiotically defective mutants in *Rhizobium leguminosarum* by insertion of the transposon Tn*5* into a transmissible plasmid. Mol. Gen. Genet. 178, 185–190 (1980).
5. Buendía-Clavería, A. M., Ruiz-Sainz, J. E.: Isolation of mutants of fast-growing soybean strains that are effective on commercial soybean cultivars. Physiol. Plant. 64, 507–512 (1985).
6. Buendía-Clavería, A. M., Chamber, M., Ruiz-Sainz, J. E.: A comparative study of the physiological characteristics, plasmid content and symbiotic properties of different *Rhizobium fredii* strains. Syst. Appl. Microbiol. 12, 203–209 (1989).
7. Buendía-Clavería, A. M., Rodríguez-Navarro, D. N., Santamaría-Linaza, C., Ruiz-Sainz, J. E. Temprano-Vera, F.: Evaluation of the symbiotic properties of *Rhizobium fredii* in European soils. Syst. Appl. Microbiol. 17, 155–160 (1994).
8. Camacho, M., Santamaría, C., Temprano, F., Rodríguez-Navarro, D. N., Daza, A., Espuny, R., Bellogín R., Ollero F.J., Lyra M. C. C. P., Zhou, J., Li., F.D., Mateos, C., Velázquez, E., Vinaldell, J. M., Ruiz-Sainz, J.E.: Soils of the Chinese Hubei province show a very high diversity of *Sinorhizobium fredii* strains. System. Appl. Microbiol. 25, 592–602 (2002).
9. Campbell, G. R. O., Rehus, B. L., Walker, G. C.: Different phenotypic classes of *Sinorhizobium meliloti* mutants defective in synthesis of K antigen. J. Bacteriol. 180, 5432–5436 (1998).
10. Chen, W. X., Yang, G. H., Li, J. L.: Numerical taxonomy study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. 38, 392–397 (1988).
11. Cleyet-Marel, J.-C.: Dynamique des populations de *Rhizobium* et de *Bradyrhizobium* dans le sol et la rhizosphere. These d'Etat. University Claude Bernard, Lyon, France (1987).
12. Cubo, M. T., Buendía-Clavería, A. M., Beringer, J. E., Ruiz Sainz, J. E.: Melanin production by *Rhizobium* strains. Appl. Environ. Microbiol. 54, 1812–1817 (1988).
13. De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M. D., Dreyfus, B., Kersters, K., Gillis, M.: Polyphasic taxonomy of rhizobia: emendiation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int. J. Syst. Bacteriol. 44, 715–733 (1994).
14. Dénarié, J., Debellé, F., Promé, J.-C.: *Rhizobium* lipo-chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. 65, 503–535 (1996).
15. Dowdle, S. F., Bohlool, B. B.: Predominance of fast-growing *Rhizobium japonicum* in a soybean field in the People´s Republic of China. Appl. Environ. Microbiol. 33, 990–995 (1985).

16. Espuny, M. R., Ollero, F. J., Bellogín, R. A. , Ruiz-Sainz, J. E., Pérez-Silva, J.: Transfer of the *Rhizobium leguminosarum* biovar *trifolii* pRtr5a to a strain of *Rhizobium* sp that nodulates on *Hedysarum coronarium*. J. Appl. Bacteriol. 63, 13–20 (1987).
17. Geelen, D., Leyman, B., Mergaert, P., Klarskov, K., van Montagu, M., Geremia, R. A., Holsters, M.: NodS is an *S*-adenosyl-L-methionine-dependent methyltransferase that methylates chitooligosaccharides deacetylated at the non-reducting end. Mol. Microbiol. 17, 387–397 (1995).
18. Gil-Serrano, A. M., Franco-Rodríguez, G., Tejero-Mateo, P., Thomas-Oates, J., Spaink, H. P., Ruiz-Sainz, J. E., Megías, M., Lamrabet, Y.: Structural determination of the lipo-chitin oligosaccharide nodulation signals produced by *Rhizobium fredii* HH103. Carbohydr. Res. 303, 435–443 (1997).
19. Gil-Serrano, A. M., Rodríguez-Carvajal, M. A., Tejero-Mateo, P., Espartero, J. E., Thomas-Oates, J., Ruiz-Sainz, J. E., Buendía-Clavería, A. M.: Structural determination of a 5-*O*-methyl-deaminated neuraminic acid (Kdn)-containing polysaccharide isolated from *Sinorhizobium fredii*. Biochem. J. 334, 585–594 (1998).
20. Haas, D., Holloway, B. W.: R factor variance with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol Gen Genet. 144, 234–251 (1976).
21. Hernández, B. S., Focht, D. D.: Invalidity of the concept of slow growth and alkali production in cowpea rhizobia. Appl. Environ. Microbiol. 48, 206–210 (1984).
22. Hynes, M. F., Finan, T. M.: General genetic knowledge, pp 24–43. In: *The Rhizobiaceae*. (H. P. Spaink, A. Kondorosi, P. J. J. Hooykaas, eds.) Kluwer Academic Publishers, Dordrecht, The Netherlands 1998.
23. Jabbouri, S., Fellay, R., Talmont, F., Kamalaprija, P., Burger, U., Relič, B. Promé, J.-C., Broughton, W. J.: Involvement of *nodS* in *N*-methylation and *nodU* in 6-*O*-carbamoylation of *Rhizobium* sp. NGR234 Nod factors. J. Biol. Chem. 270, 22968–22973 (1995).
24. Kannenberg, E. L., Reuhs, B. L., Forsberg, L. S., Carlson, R. W.: Lipopolysaccharides and K-antigens: their structures, biosynthesis, and functions, pp. 119–154. In: *The Rhizobiaceae*. (H. P. Spaink, A. Kondorosi, P. J. J. Hooykaas, eds.). Kluwer Academic Press, Dordrecht, The Netherlands 1998.
25. Keyser, H. H., Hu, T. S., Bohlool, B. B., Weber, D. F.: Fast-growing rhizobia isolated from root nodules of soybean. Science 215, 1631–1632 (1982).
26. Kittelberger, R., Hilbink, F.: Sensitive silver-staining detection of bacterial lipopolysaccharides in polyacrylamide gels. J. Biochem. Biophys. Methods 26, 81–86 (1993).
27. Köplin, R., Wang, G., Hötte, B., Priefer, U. B., Pühler, A.: A 3.9-kb DNA region of *Xanthomonas campestris* pv. *campestris* that is necessary for lipopolysaccharide production encodes a set of enzymes involved in the synthesis of the dTDP-rhamnose. J. Bacteriol. 175, 7786–7792 (1993).
28. Krishnan, H. B., Lewin, A., Fellay, R., Broughton, W. J. Pueppke, S. G.: Differential expression of *nodS* accounts for the varied abilities of *Rhizobium fredii* USDA257 and *Rhizobium* sp. strain NGR234 to nodulate *Leucaena* spp. Mol. Microbiol. 6, 3321–3330 (1992).
29. Lambaret, Y., Bellogín, R. A., Cubo, T., Espuny, R., Gil, A., Krishnan, H. B., Megías, M., Ollero, F. J., Pueppke, S. G., Ruiz-Sainz, J. E., Spaink, H. P., Tejero-Mateo, P., Thomas-Oates, J., Vinardell, J. M.: 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 (1999).
30. Lesse, A. J., Campagnari, A. A., Bittner, W. E., Apicella, M. A.: Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecylsulphate-polyacrylamide gel electrophoresis. J. Immunol. Methods 126, 109–117 (1990).
31. Lim, S. T., Ong, K. K.: A periplasmic cyclic 3,5′-adenosine monophosphate phosphodiesterase from *Sinorhizobium fredii* MAR-1. Microbios 77, 207–216 (1994).
32. López-Lara, I. M., van den Berg, J. D. J., Thomas-Oates, J. E., Glushka, J., Lugtenberg, B. J. J., Spaink, H. P.: Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti*. Mol. Microbiol. 15, 627–638 (1995).
33. Madinabeitia, N., Bellogín, R. A., Buendía-Clavería, A. M., Camacho, M., Cubo, T., Espuny, M. R., Gil Serrano, A. M., Lyra, M. C. C. P., Moussaid, A., Ollero, F. J., Soria-Díaz, M. E., Vinardell, J. M., Zeng, J., Ruiz-Sainz, J. E.; *Sinorhizobium fredii* HH103 has a truncated *nolO* gene due to a –1 frameshift mutation that is conserved among other geographically distant *S. fredii* strains. Mol. Plant-Microbe Interact. 12, 207–217 (2002).
34. Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P., Pardo, M. A.: *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. 41, 417–426 (1991).
35. Oke, V., Rushing, B. G., Fisher, E. J.: Identification of the heat-shock sigma factor RpoH and a second RpoH-like protein in *Sinorhizobium meliloti*. Microbiology 147, 2399–2408 (2001).
36. Olsthoorn, M. M. A., Stokvis, E., Haverkamp, J., Spaink, H. P., Thomas-Oates, J.E.: Growth temperature regulation of host specific modifications of rhizobial lipochitin oligosaccharides: a function of *nodX* is temperature regulated. Mol. Plant-Microbe Interact. 13, 808–820 (2000).
37. Ovtsyna, A. O., Rademaker, G. J., Esser, E., Weinman, J., Rolfe, B. G., Tikhonovich, I. A., Lugtenberg, B. J. J., Thomas-Oates, J. E., Spaink, H. P.: Comparison of characteristics of the *nodX* genes from various *Rhizobium leguminosarum* strains. Mol. Plant-Microbe Interact. 12, 252–258 (1999).
38. Peng, G. X., Tan, Z. Y., Wang, E. T., Reinhold-Hurek, B., Chen, W. F., Chen, W. X.: Identification of isolates from soybean nodules in Xinjiang region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii* Int. J. Syst. Evol. Micr. 52, 457–462 (2002).
39. Pueppke, S. G., Broughton, J.: *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. Mol. Plant-Microbe Interact. 12, 293–318 (1999)
40. Rodríguez-Navarro, D. N., Ruiz-Sainz, J. E., Buendía-Clavería, A. M., Santamaria, C., Balatti, P. A., Krishnan, H. B., Pueppke, S.: Characterizarion of fast-growing rhizobia from nodulated soybean [*Glycine max* (L.) Merr.] in Vietnam. System. Appl. Microbiol. 19, 240–248 (1996).
41. Rodríguez-Navarro, D. N., Bellogín, R., Camacho, M., Daza, A., Medina, C., Ollero, F. J., Santamaría, C., Ruiz-Sainz, J. E., Vinardell, J. M., Temprano, F. J.: Field assessment and genetic stability of *Sinorhizobium fredii* strain SMH12 for commercial soybean inoculants. Europ. J. Agronomy 19, 301–311.
42. Sambrook, J., Fritsch, E. F., Maniatis, T.: Molecular Cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA 1989.
43. Spaink, H. P., Aarts, A., Stacey, G., Bloemberg, G. V., Lugtenberg, B. J. J., Kennedy, E. P.: Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. Mol. Plant-Microbe Interact. 5, 72–80 (1992).

44. Tan, Z.-Y., Xu, X.-D., Wang, E.-T., Gao, J.-L., Martínez-Romero, E., Chen, W.-X.: Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. Int. J. Syst. Bacteriol. 47, 874–879 (1997).
45. van Berkum, P., Eardly, D.: Molecular evolutionary systematics of the *Rhizobiaceae*, pp. 1–24. In: The *Rhizobiaceae*, Molecular Biology of Model Plant-associated Bacteria (H. P. Spaink, A. Kondorosi, P. J. J. Hooykaas, eds.) Kluwer Academic Press, Dordrecht, The Netherlands 1998.
46. van Brussel A. A. N., Tak, T., Wetselaar, A., Pees, E., Wijffelman C. A.: The wall of *Rhizobium leguminosarum* in bacteroid and free-living forms. J. Gen. Microbiol. 101, 51–56 (1977).
47. Vincent, J.M.: A Manual for the Practical Study of Root-Nodule Bacteria, p. 3. Blackwell Scientific Publications, Oxford, United Kingdom 1970.
48. Yang, S. S., Bellogin, R. A., Buendía, A., Camacho, M., Chen, M., Cubo, T., Daza, A., Díaz, C. L., Espuny, M. R., Gutiérrez, R., Harteveld, M., Li, X. H., Lyra, M. C. C. P., Madinabeitia, N., Medina, C., Miao, L., Ollero, F. J., Olsthoorn, M. M. A., Rodríguez, D. N., Santamaría, C., Schlaman, H. R. M., Spaink, H. P., Temprano, F., Thomas-Oathes, J. E., van Brussel, A. A. N., Vinardell, J. M., Xie, F., Yang, J., Zhang, H. Y., Zhen, J., Zhou, J., Ruiz-Sainz, J. E.: Effect of pH and soybean cultivars on the quantitative analyses of soybean rhizobia populations. J. Biotechnol. 91, 243–255 (2001).
49. Young, C. C., Chang, J. Y., Chao, C. C.: Physiological and symbiotic characteristics of *Rhizobium fredii* isolated from subtropical-tropical soils. Biol. Fertil. Soils 5, 350–354 (1988).
50. Zhang, X. X., Zhou, X. X., Zhang, Z. M., Chen, H. K., Li, F. D.: Behaviour of plasmid pJB5JI in *Rhizobium huakuii* under free-living and symbiotic conditions. Curr. Microbiol. 31, 97–101 (1995).

Corresponding author:
J. E. Ruiz-Sainz, Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Apdo. 1095, 41080 Sevilla, Spain
Tel.: ++34-95-455-7121; Fax: ++34-95-455-7830;
e-mail: rsainz@us.es