RESEARCH LETTER

Strain selection for improvement of *Bradyrhizobium japonicum* competitiveness for nodulation of soybean

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Abstract

A *Bradyrhizobium japonicum* USDA 110-derived strain able to produce wider halos in soft-agar medium than its parental strain was obtained by recurrent selection. It was more chemotactic than the wild type towards mannitol and three amino acids. When cultured in minimal medium with mannitol as a single carbon-source, it had one thick subpolar flagellum as the wild type, plus several other flagella that were thinner and sinusoidal. Root adsorption and infectivity in liquid media were 50–100% higher for the selected strain, but root colonization in water-unsaturated vermiculite was similar to the wild type. A field experiment was then carried out in a soil with a naturalized population of 1.8×10^5 soybean-nodulating rhizobia g of soil^{-1}. *Bradyrhizobium japonicum* strains were inoculated either on the soybean seeds or in the sowing furrows. Nodule occupation was doubled when the strains were inoculated in the sowing furrows with respect to seed inoculation (significant with $P < 0.05$). On comparing strains, nodule occupation with seed inoculation was 6% or 10% for the wild type or selected strains, respectively, without a statistically significant difference, while when inoculated in the sowing furrows, nodule occupation increased to 12% and 22%, respectively (differences significant with $P < 0.05$).

Introduction

Soybean is the most important legume in Argentina, being cropped in 16 million ha, 90% of which receives inoculation with *Bradyrhizobium japonicum* (González, 2007). Although this plant is not originally from South America, several rhizobacteria from soil populations are able to nodulate it (González, 2007), as was observed previously in Brazil (Da Costa Coutinho *et al.*, 1999) and Paraguay (Chen *et al.*, 2000). At the center of the cropping area, these soil populations are present in high numbers, often more than 10^4 soybean-nodulating rhizobia per gram of soil. The N_2-fixing effectiveness of these rhizobia is variable, because they may either be derived from ancient inoculants subjected to genetic drift in the soil, or belong to a number of species different from *B. japonicum* that acquired in some way (e.g. horizontal gene transfer) the ability to nodulate soybean (Barcellos *et al.*, 2007).

The ubiquitous presence of these populations precludes the occupation of soybean nodules by high-quality inoculated strains, so that inoculation often fails to produce the expected outcomes in seed yield and quality. Because soybean possesses a high demand of N, a poor N_2-fixation performance leads to depletion of soil N-fertility. This problem was observed worldwide, also with other legume crops, and is referred to as the problem of competition for nodulation (Sadowski & Graham, 1999).

This problem has been addressed in the last decades, using different approaches, but with little success. Use of genetic-engineered rhizobial strains was proposed (Toro, 1996), but application of this approach in Argentina suffers from two main inconveniences: (1) most inoculant-producing factories are small-medium enterprises (SME) whose infrastructure and capital are below the requirements for R&D genetic engineering projects, and (2) release of genetic engineered strains into field crops is controversial, and

still not entirely regulated by law (Amarger, 2002). Therefore, approaches for strain improvement toward better competitiveness for nodulation without genetic manipulation are required.

Traits and conditions able to ensure good root colonization and infection by rhizobia at early plant growth stages are considered to be good candidates for nodulation competitiveness improvement. In this context, rhizobial motility and chemotaxis are considered to play a role (Brencic & Winans, 2005), as characterized in *in vitro* experiments carried out in saturated aqueous media (Hunter & Fahring, 1980; Ames & Bergman, 1981; Gaworzewska & Carlile, 1982; Mellor *et al.*, 1987; Caetano-Anollés *et al.*, 1988a, b; Barbour *et al.*, 1991; Parco *et al.*, 1994; Pandya *et al.*, 1999; Chuiko *et al.*, 2002). However, experiments developed in water-unsaturated porous media or in soils revealed that under these conditions rhizobial motility is impaired (Madsen & Alexander, 1982; Horiuchi *et al.*, 2005). It was observed previously that this limitation in rhizobial dispersion in unsaturated porous media might put rhizobia inoculated on the seeds at a disadvantage compared with the soil population that is already evenly distributed in the soil. This distribution profile might constitute a bottleneck for the competitiveness of inoculants (Liu *et al.*, 1989; López-García *et al.*, 2002). Therefore, the improvement of rhizobial dispersion might be an important target for selection of strains with enhanced competitiveness for nodule occupation in soils with naturalized rhizobial populations.

In this work, we devised a procedure that allows selection of rhizobial strains with a higher dispersion capability to seek good competitive potential in soils. With this purpose, we used the type strain *B. japonicum* USDA 110, starting with the selection of bacteria able to produce wider halos in semisolid agar medium. This procedure, which does not involve genetic engineering manipulation, is simple and feasible to carry out with any strain at SME-sized inoculants producing factory. Here, we present our results on implementation of the method, a preliminary characterization of the obtained strain, and the evaluation of its symbiotic performance both in the laboratory and in the field.

Materials and methods

Bacterial strains and culture conditions

Bradyrhizobium japonicum LP 3004 (López-García *et al.*, 2002), LP 3008 (this work), and USDA 110 (obtained from the United States Department of Agriculture, Beltsville) were maintained in stocks in yeast extract mannitol (YM, Vincent, 1970) supplemented with 20% v/v glycerol at $-80\,^{\circ}$C and, for routine use, in YM with 1.5% w/v agar (YMA) at 4 °C. The YMA cultures were renewed every one to two months. No more than four to five subculturing rounds in YMA were performed, after which new cultures were initiated from the frozen stocks. For experimental purposes, Götz medium with 27 mM mannitol as the carbon-source (Quelas *et al.*, 2006) was used, except for chemotaxis assays, where the mannitol concentration of the reference medium was diminished to 5.4 mM (Götz *et al.*, 1982). For selection and assay of rhizobial colony spreading, soft agar (final concentration 0.3% w/v) was added to Götz medium (GSA medium), while for all the other experiments, rhizobia were grown in liquid Götz medium (LGM) at 28 °C and 180 r.p.m.

Chemotaxis assays

The method of Adler (1973) was used. The reference medium was LGM with 5.4 mM mannitol as the carbon-source, and the attractants used were: 10 mM aspartate, 1 mM lysine, 1 mM glycine, or 27 mM mannitol (Götz *et al.*, 1982). For each attractant, incubations were performed in triplicate at 28 °C for 30 min, after which the *R*-value $\pm$ SE was determined.

Polysaccharide preparations

Exopolysaccharides, capsular polysaccharides, and total cell proteins were obtained from logarithmic phase cultures grown in LGM and quantified as described (López-García *et al.*, 2001). The extracellular polysaccharide contents were then calculated on the basis of total cell protein contents.

Microscopic observations

Samples were obtained after culturing the rhizobia in LGM at 28 °C and 180 r.p.m. for 5 days [logarithmic growth phase according to López-García *et al.* (2001)] and then left without agitation before observation. Aliquots were transferred to a grid covered with a colodion film over which carbon was previously layered through vacuum evaporation. After 30 s, excess liquid was removed with a filter paper and the sample was stained with 2% potassium phosphotungstate (pH 5.2; 2% w/v KOH). The microscope used was a JEM 1200 EX (JEOL, Japan Electron Optics Laboratory Co., Ltd).

Proteins preparation and analysis

To obtain flagellin, rhizobia were grown in LGM at 28 °C and 180 r.p.m. for 5 days. Then, the cultures were centrifuged at 6000 ***g*** for 30 min at 4 °C and the cell pellet was resuspended in phosphate-buffered saline (PBS). Flagella were detached by vigorous shaking in a Beckmann omnimixer for 30 s at medium power, and the suspension was centrifuged twice at 12 000 ***g*** for 15 min at 4 °C. The flagella were recovered in the supernatant, and pelleted by ultracentifugation at 100 000 ***g*** for 2 h at 4 °C.

For the isolation of extracellular proteins, cultures were grown under the same conditions as above, vortexed for 2 min, and centrifuged twice at 14 000 ***g*** 20 min at 4 °C. The supernatant was precipitated with 5% w/v trichloroacetic acid overnight at 4 °C. Then, the extracellular proteins were pelleted by centrifugation at 14 000 ***g*** for 40 min at 4 °C, washed twice with 70% v/v ethanol and once with acetone. The pellet was air-dried and resuspended in bidistilled water (Ausmees *et al.*, 2001).

For analysis, the samples were boiled in Laemmli (1970) loading buffer for 10 min and then separated by electrophoresis in denaturing, discontinuous polyacrylamide gels [sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] with a polyacrylamide concentration of 10% (w/v) in the separation gels. After electrophoresis, gels were stained with Coomassie Brilliant Blue R250, or silver (Blum *et al.*, 1987).

For in-gel tryptic digestion and mass spectrometry identification of SDS-PAGE polypeptide bands, a procedure that had already been described was used (Watt *et al.*, 2003). The mass spectrum was obtained on a Biflex III matrix-Assisted Laser desorption ionization-time of flight-MS (MALDI-TOF-MS) (Bruker). The annotation of the peptide mass fingerprints was performed by the MASCOT software (Matrix Science). Protein scores higher than 52 were considered to be significant with $P < 0.05$ (Perkins *et al.*, 1999). The search was performed in the *Bradyrhizobium* database at RhizoBase using the following parameters: enzyme, trypsin; missed cleavages, 1; peptide mass tolerance, $\pm 150\ \mu g\ g^{-1}$; and mass values, monoisotopic.

DNA fingerprint

We performed total DNA amplification fingerprints using BoxAR-1 primers (Versalovic *et al.*, 1994). We carried out PCR reactions in 25 μL containing: 50 mM Tris, pH 8.3; 500 $\mu g\ mL^{-1}$ bovine serum albumin (BSA); 3 mM $MgCl_2$; 200 μM dNTPs; 1 U Taq polymerase (Promega Corp.); 10 μM primers; and 2 μL of template DNA, obtained previously by phenol extraction. The cycling conditions were as follows: 94 °C for 2 min, 35 cycles at 94 °C for 10 s, at 52 °C for 60 s, and at 65 °C for 8 min with a final cycle of 65 °C for 16 min. After the reaction, 10 μL of the PCR products were separated in 1.5% agarose gels containing 0.5–1 $\mu g\ mL^{-1}$ ethidium bromide and photographed.

DNA sequencing

DNA fragments encompassing bll6865 and bll6866 were amplified from total genomic LP 3004 or LP 3008 DNA using the following primers: for bll6865, Fw fla2: 5′-GA CAGGTTGGGTCCTTCACA-3′, and Rv fla2: 5′-TCCAG CAGATTGTTGAGCAG-3′. For bll6866: Fw fla1 5′-TGC CGTTCAAGATGCTGTTC-3′, and Rv fla1 5′-GGCTTG TGGCTCTGTGACTC-3′. The PCR reactions were carried out with Pfx from Invitrogen according to the manufacturer's instructions, and with the following cycling conditions: initiation, 94 °C, 2 min; 35 cycles at 94 °C 35 s, 59 °C 30 s, 68 °C 2.5 min; and final elongation, 68 °C, 3 min. Sequencing was performed at Macrogen Corp., Seoul, South Korea.

Plant assays

Don Mario 4800 soybean seeds were surface sterilized with commercial bleach and germinated in water–agar (1.5% w/v) for 4 days as described (Lodeiro & Favelukes, 1999).

Adsorption of rhizobia to soybean roots was carried out in an N-free Fåhraeus-modified plant nutrient solution (FMS, Lodeiro *et al.*, 2000a) during 4 h of incubation following a method already described (Lodeiro & Favelukes, 1999).

For infectivity, nodulation profiles were obtained by inoculating 30 plants with the indicated concentrations of rhizobia per plant in plastic growth pouches watered with FMS as described (Bhuvaneswari *et al.*, 1980). At the time of inoculation, the positions of the smallest emergent root hairs (SERH) and the root tip (RT) were marked for each plant on the surface of the plastic growth pouches. The distance between SERH and RT marks for each individual plant was taken as the relative distance unit (RDU) for that plant. After 15 days of growth in the greenhouse at 26 °C/18 °C day/night temperature, all nodule positions in the primary root of each plant were recorded in mm (± 0.5 mm) with respect to the corresponding RT mark. Nodule distances to RT were expressed in RDU to compensate for the different elongation rates and SERH/RT lengths among individual plants. Nodules appearing above the RT mark were assigned positive values, while those appearing below the RT mark received negative values (for details, see Bhuvaneswari *et al.*, 1980).

Root colonization was evaluated by incubating the rhizobia and the roots for 48 h at 28 °C in 50 mL Sarstedt test tubes containing sterile vermiculite at field capacity with FMS as described (López-García *et al.*, 2002).

Field assay

Field assays were carried out at San Antonio de Areco, Province of Buenos Aires, Argentina (34° 15′ S–59° 29′ W), in a field cropped with soybean since several years ago. The soil was a loamy vertic argiudol, with a soil water content of 70% field capacity at sowing. The assay was designed as randomized blocks in 1.14 × 7.00 m plots. Soybean Don Mario 4800 was sowed with no tillage on 20 December, 2005 with 25 seeds m^{-1} and furrows separated by 0.38 m. The most probable number of soybean-nodulating rhizobia was determined from soil samples taken at sowing,

according to Vincent (1970). The inoculations were performed with LP 3004 on seeds or in sowing furrows, or with LP 3008 on seeds or in sowing furrows. In all cases, 4×10^9 viable rhizobia kg^{-1} seed were inoculated. Each treatment was repeated in three blocks, and samples of 10 plants per block were taken at vegetative state V6. After sampling, all nodules were excised from the roots and washed extensively with distilled water to remove soil particles. Then, each individual nodule was placed in a well of multiwell plates, surface sterilized with 20% v/v commercial bleach, and washed with sterile-distilled water (López-García *et al.*, 2001). Finally, each nodule was crushed with a sterile toothpick and its contents were deposited onto the surface of YMA replica plates with or without streptomycin at a concentration of 1.0 mg mL^{-1}. Statistical analysis was carried out using ANOVA and the Tukey's test of the arc sin root square transform of the individual values.

Results

Selection and preliminary characterization of a *B. japonicum* strain able to produce wider halos in semisolid agar

For the purposes of selection and strain characterization, we used GSA and LGM minimal media, whose composition was developed previously to provide optimal culture conditions for motility and chemotaxis studies in rhizobia (Götz *et al.*, 1982).

To initiate selection, *B. japonicum* LP 3004, a streptomycin-resistant spontaneous derivative from USDA 110 (Lodeiro & Favelukes, 1999; López-García *et al.*, 2002), was sowed in the center of a GSA petri dish and incubated for 15 days at 28 °C. A sample from the edge of the halo formed was taken and sown in the center of a new plate. After 13 consecutive rounds of this selection procedure, the culture was enriched in bacteria that produced a wider halo. A colony from the final selection was purified in YMA, and inoculated on soybean plants. After 20 days in the greenhouse, a single root nodule was obtained, surface sterilized, and crushed to release its bacterial contents. These were inoculated on YMA, a colony was isolated, and assessed again in GSA. We observed that after this cycle of isolation and nodule passage, the isolate conserved its ability to produce a wider halo in this semisolid medium (Fig. 1a). We authenticated this isolate as LP 3004-derived by its genomic Box AR1 DNA fingerprint similarity to the original LP 3004 and USDA 110 strains (Fig. 1b). Thus, this isolate was named *B. japonicum* LP 3008, and used for further experiments.

To see whether the broader halo was due to a higher growth rate, we determined growth kinetics for LP 3008 and the wild type in LGM, by measuring total and viable bacteria as well as $OD_{500\,nm}$. Both strains had a 22-h doubling time as

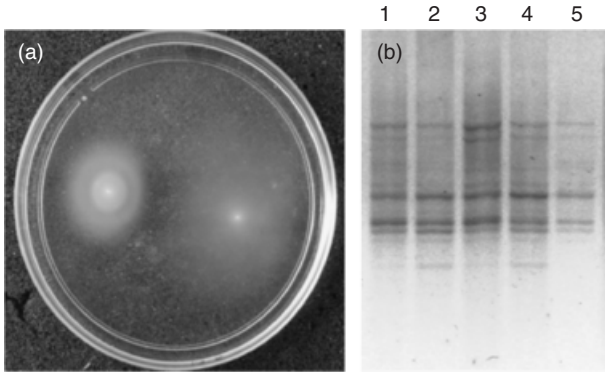

Fig. 1. Characterization of *Bradyrhizobium japonicum* LP 3008. (a) Halos in Götz-soft agar (0.3% w/v) from the wild-type LP 3004 (left) and the selected isolate LP 3008 (right). (b) Box AR-1 DNA fingerprint band pattern from LP 3004 (lanes 1–2), LP 3008 (lanes 3–4), and USDA 110 (lane 5).

Table 1. Chemotaxis of *Bradyrhizobium japonicum* LP 3004 and LP 3008 towards different attractants*

	R-value ± SE	
Attractant (conc.)	LP 3004	LP 3008
Aspartate (10 mM)	0.71 ± 0.05	4.07 ± 0.62
Lysine (1 mM)	1.55 ± 0.09	3.60 ± 0.78
Glycine (1 mM)	1.59 ± 0.04	5.47 ± 0.75
Mannitol (27 mM)	1.03 ± 0.17	5.10 ± 0.51

*Rhizobia were grown in liquid Götz with mannitol 5.4 mM as the reference medium. R-values represent the relationships of CFU recovered from a capillary tube filled with the indicated attractant dissolved in the reference medium to the CFU recovered from a capillary tube filled with reference medium alone, after 30 min incubations at 28 °C.

observed before for USDA 110 under the same conditions (López-García *et al.*, 2001; Quelas *et al.*, 2006), and in the stationary phase (14–15 days) cell numbers and biomasses were also similar. The fact that both strains grew at similar rates but LP 3008 spread faster in GSA might explain the lower density of mutant halos compared with the wild type (Fig. 1a).

We measured capsular polysaccharide and exopolysaccharide production because these molecules may indirectly modify bacterial spreading by altering the viscosity of the culture medium. In contrast with previous studies (Wei & Bauer, 1999), there was no difference between LP 3008 and the wild type (not shown).

Because wider halos could be related to differences in chemotaxis and flagellar structure, we characterized both traits in the selected strain LP 3008 in comparison with the wild type.

Chemotaxis was assessed in LGM with 5.4 mM mannitol as the carbon-source as the reference medium. LP 3008 showed significantly higher chemotaxis rates than LP 3004

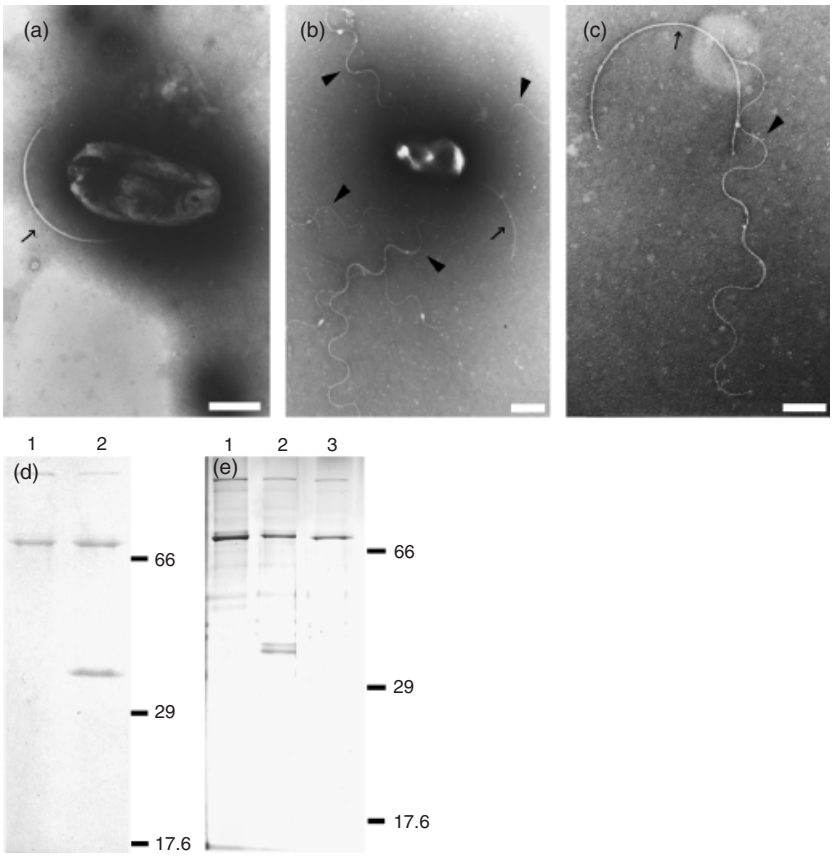

Fig. 2. Flagella morphology (a–c) and composition (d–e) in *Bradyrhizobium japonicum* LP 3004 and LP 3008. (a) A subpolar flagellum (arrow) in LP 3004. (b) Peritrichous flagella (arrowheads) and a subpolar flagellum (arrow) in LP 3008. (c) One subpolar (arrow) and one peritrichous (arrowhead) flagella detached from LP 3008. Bar scale: 500 nm. (d–e) SDS-PAGE profiles from LP 3004 (lane 1), LP 3008 (lane 2), and USDA 110 (lane 3) of (d): flagellin preparations, stained with Coommassie brilliant blue; (e): extracellular proteins, stained with silver. Molecular weight marker positions in (d–e) are indicated at the right of each gel.

towards 10 mM aspartate, 1 mM glycine, 1 mM lysine, and 27 mM mannitol, used as attractants (Table 1).

We then observed flagellar morphology and composition under a transmission electron microscope. LGM-grown LP 3004 displayed a single, thick, and relaxed subpolar flagellum (Fig. 2a), while LP 3008 showed multiple peritrichous flagella in addition to the subpolar one (Fig. 2b). These additional flagella were thinner, longer, and sinusoidal (Fig. 2c). Flagellar filament composition was analyzed in SDS-PAGE. In LP 3004 and LP 3008 preparations, a 70-kDa band was observed after Coomassie blue staining, while LP 3008 showed an additional band with 37 kDa molecular mass (Fig. 2d). The absence of the 37-kDa band in LP 3004 was confirmed by silver staining. It was also absent in SDS-PAGE profiles of extracellular proteins from LP 3004 or USDA 110 (Fig. 2e).

All polypeptide bands were analyzed by tryptic digestion, followed by MALDI-TOF-MS and identified against the *B. japonicum* USDA 110 complete proteome (Kaneko *et al.*, 2002). The 70-kDa band contained a mixture of the proteins bll5843, bll5844, bll5845, and bll5846 (scores: 193 in LP 3004 and 125 in LP 3008), while the 37-kDa band contained the flagellin bll6865 (score: 71). These observations agree with a description of *B. japonicum* flagella published recently (Kanbe *et al.*, 2007). According to the nomenclature proposed there, we will call the genes encoding high- and low-MW polypeptides as *fliC1234* and *fliCI*, respectively. Although FliCI was not observed in LP 3004 cultured with mannitol as the only carbon-source, this polypeptide was present in both LP 3004 and LP 3008 when either arabinose or gluconate was substituted for mannitol; nevertheless, the differential phenotype of LP 3008 in GSA was maintained under all these conditions (not shown).

To see whether the differences in *fliCI* expression between LP 3004 and LP 3008 could correlate with differences at the promoter region of this gene and its tandem copy *fliCII*, the DNA sequences of the Pfx-amplified 1401- and 1413-bp DNA fragments encompassing *fliCI* and *fliCII*, respectively

Table 2. Early interactions of *Bradyrhizobium japonicum* LP 3004 and LP 3008 with soybean roots*

Adsorption (%*A*)†		Infectivity (Mean distance of uppermost nodule to root tip mark)‡	
LP 3004	LP 3008	LP 3004	LP 3008
0.32 ± 0.03	0.62 ± 0.04	-1.33 ± 0.46	-0.26 ± 0.29

*Rhizobia were grown in LGM at 28 °C and rotary shaking at 180 r.p.m. to log phase.
†Adsorption was measured after 4 h incubation with 1–3 × 10^3 rhizobia mL^{-1} in liquid FMS. Adsorption index (%$A \pm$ SE; $n = 10$) is the ratio of adsorbed rhizobia on 10 soybean roots to the total CFU present in 50 mL incubation mixture.
‡Infectivity was measured in FMS-watered plastic growth pouches after 15 days incubation in the greenhouse. Inoculation was with 1 × 10^4 rhizobia per plant. One RDU is the distance between the root tip and smallest emergent root hairs marks made on pouches at the moment of inoculation for each plant. Negative values represent nodules below root tip mark. Values are means ± SE ($n = 30$).

Table 3. Colonization of soybean roots by LGM-grown *Bradyrhizobium japonicum* LP 3004 or LP 3008*

	Log_{10} CFU ($\pm$ SE, $n = 10$) per root segment					
	LP 3004			LP 3008		
Inoc.	Basal	Medial	Apical	Basal	Medial	Apical
SI	5.62 ± 0.19	5.52 ± 0.13	ND	5.19 ± 0.05	ND	ND
VI	2.89 ± 0.22	3.19 ± 0.12	2.65 ± 0.14	3.38 ± 0.14	3.21 ± 0.09	2.85 ± 0.07

*Log phase-rhizobia were either inoculated at a density of 2–6 × 10^8 CFU per plant onto the emerging cotyledons of seedlings planted in vermiculite (SI), or diluted to 1–3 × 10^6 CFU mL^{-1} FMS and poured in dry vermiculite, which was carried to field capacity before planting the seedlings (VI). Colonizing rhizobia were counted as CFU released from three separate root segments of equal length, called basal, medial and apical according to their respective positions.
ND, Not detected.

(which included 117- and 345-bp upstream regions of these respective genes), were obtained. In both Pfx amplicons, we observed 100% identity among LP 3004, LP 3008, and USDA 110.

Symbiotic characterization of LP 3008

Rhizobial adsorption to legume roots as well as early root infectivity in liquid FMS requires motility and chemotaxis (Caetano-Anollés *et al*., 1988b). Therefore, we compared LP 3008 and LP 3004 in adsorption and infectivity assays, and observed that LP 3008 was more efficient because it adsorbed better and produced the earliest nodules (Table 2).

To assess the ability of these strains to move vertically downwards to the root tip in a porous medium, we carried out inoculation onto the cotyledons of seedlings with 2 cm-long roots (hereafter referred to as seed inoculation; see López-García *et al*., 2002), which were cultivated in plastic flasks with vermiculite at field capacity with FMS. After a 48-h incubation at 28 °C, roots, which doubled in length, were carefully removed by longitudinally cutting the flasks, thus avoiding mix of vermiculite from different depths. Then, the basal, medial, and apical root thirds were excised, washed, and viable rhizobia colonizing each root third were counted (López-García *et al*., 2002). Contrary to the above results, LP 3008 was unable to colonize the medial root third in contrast to LP 3004 (Table 3). None of the strains reached the apical root portion, which would require a mean swimming speed slower than 0.3 μm s^{-1}, 100-fold less than the speed measured in liquid media *in vitro* (Kanbe *et al*., 2007). When bacteria were homogeneously distributed into the vermiculite instead of seed-inoculated, both strains colonized the three root portions at equivalent rates, thus indicating that rhizobia are able to colonize the root apical portion under this condition (Table 3). In conclusion, the increased adhesiveness of LP 3008 observed at short incubation times (Table 2) was not accompanied by a higher colonization rate.

To assess LP 3008 competitiveness for nodulation under field conditions, we performed an experiment in a field cropped previously with soybean for years. We estimated that the soil contained a population of 1.8 × 10^5 soybean-nodulating rhizobia g^{-1}. To compare LP 3004 with LP 3008, we inoculated each strain on the soybean seeds, or using a different application procedure, we inoculated each strain in the soil, at the sowing furrows. In both inoculation methods (seed or soil), the same inoculant dose of 4 × 10^9 viable rhizobia (kg of seed)$^{-1}$ was used, for the purpose of comparison. We expected that, given the low vertical spreading of these rhizobia (Table 3), soil inoculation would position the inoculated strains in a better situation than seed-inoculated ones with respect to the roots-infectable sites (Bhuvaneswari *et al*., 1980; López-García *et al*., 2002).

As often occurs in soybean-cropped soils, most of the nodules were occupied by representatives of the naturalized rhizobial population. When soybean plants were inoculated

on the seeds, LP 3004 occupied 6% of the nodules, while LP 3008 occupied 10%; these differences were not significant with $P < 0.05$. However, when inoculation was carried out in the sowing furrows, nodule occupation was 12% for LP 3004 and 22% LP 3008; these differences were significant with $P < 0.05$. In addition, inoculation in the sowing furrows yielded around double nodule occupation than seed inoculation, considering each strain individually (12% vs. 6% for LP 3004 and 22% vs. 10% for LP 3008, both significant with $P < 0.05$).

Discussion

An important challenge for agricultural sciences is optimization of plant growth-promoting rhizobacteria for sustainable grain production. In this context, improvement of rhizobia competitiveness for soybean nodulation is required to maintain high productivity without compromising soil N fertility. In this work, we attempted to enhance *B. japonicum* competitiveness based on iterative selection of rhizobial-spreading capability, which resulted in the *B. japonicum* isolate LP 3008. Contamination in the isolate was discarded because both its BoxAR-1 DNA fingerprint band pattern and its DNA sequence throughout 2814 bp in the *fliCI-fliCII* region were identical to the original USDA 110. Furthermore, LP 3008 phenotypical stability after nodule passage and subculturing was confirmed.

Next, a physiological and symbiotic characterization was carried out to assess whether the selection procedure was effective in achieving the desired strain improvement for application to soybean crops before any attempt to obtain a detailed molecular description of the selected strain genotype. Therefore, we started our preliminary physiological characterization by ruling out the possibility that the wider halos produced in GSA (Fig. 1a) were related to growth. We then studied LP 3008 flagellar structure and chemotactic behavior in comparison with the wild-type LP 3004.

We observed differences in flagellar filament production between both strains (Fig. 2), which corroborated the assignment of *fliC1234* flagellins to the thick flagellum, and *fliCI* and *fliCII* to the thin one (Kanbe *et al*., 2007). This corroboration is significant, especially for *fliC1234*, because these genes encode polypeptides very different from currently known flagellins in their high molecular mass, and lacking sequence similarity to other flagellins at their central regions. These differences may also indicate that LP 3008 possesses either derepression of *fliCI* (and *fliCII*) expression, more efficient assembly of released polypeptides, or stronger attachment of thin flagella. Although these possibilities are not mutually exclusive, the absence of FliCI and FliCII in LP 3004 extracellular polypeptide profiles observed under our conditions (Fig. 2e) argues in favor of the first possibility, despite the fact that no differences were observed in the upstream regions of these genes in comparison with the wild type.

Whether these differences in FliCI-FliCII production can fully explain the LP 3008 phenotype is uncertain. Expression of the thin filament is not constitutive, as observed after growth on different carbon-sources. However, even under conditions that allow FliCI-FliCII expression in the wild-type strain, LP 3008 continues forming wider halos in GSA. The higher chemotaxis observed in LP 3008 (Table 1) might explain this phenotype, although a possible relationship between FliCI-FliCII expression and chemotaxis is still unknown.

We then assessed the symbiotic performance of strain LP 3008. When root adsorption and infectivity were measured using well-established methods (Bhuvaneswari *et al*., 1980; Caetano-Anollés *et al*., 1988b), LP3008 showed superior behavior compared with the wild type in the sense that it adsorbed more to the roots, and nodulated earlier (Table 2). The higher adsorption observed with LP 3008 could be a consequence of its higher chemotaxis. In addition, flagellin might also act as an adhesin and, therefore, the higher adhesiveness of LP 3008 might partially be caused by a derepression of FliCI, in case this occurs in the rhizosphere environment. In turn, higher adhesiveness could have led to higher infectivity, as observed previously with soybean seed lectin rhizobial preincubation (Lodeiro *et al*., 2000b) and N-source limitation of rhizobial growth (López-García *et al*., 2001).

In both series of experiments shown in Table 2, rhizobial cells interacted with the roots in liquid media, where rhizobia were homogeneously distributed. However, when we assessed the ability of these strains to move vertically downwards to the root tip in vermiculite at field capacity, we observed that both displayed a very slow motion, in agreement with previous observations in this and other water-unsaturated porous media (Madsen & Alexander, 1982; López-García *et al*., 2002; Horiuchi *et al*., 2005). Furthermore, the wild-type LP 3004 was able to reach deeper root regions than LP 3008, thus indicating that the wider halo produced by this strain in GSA did not correlate with more vertical dispersion in unsaturated vermiculite, pointing out the differences between both kinds of media. Kanbe *et al*. (2007) argued that the thin flagellum might be used for bacterial displacement upon the roots and the thick one for swimming in the liquid phase. If this is the case, the overproduction of the thin flagellum in LP 3008 might have precluded its motion in the porous medium and/or upon the root. However, this description was derived from observations made in unrelated marine bacteria that also possess two sets of flagella, and, in the absence of direct proof of *B. japonicum* displacement on surfaces, it has to be viewed with caution. Moreover, the observation that both strains colonized the roots equally well when uniformly

distributed into the vermiculite indicates that both were able to colonize the root tip, and that the higher adsorption observed in liquid medium with LP 3008 (Table 2) might be more related to chemotaxis than to the additional thin flagellum acting as adhesin.

Given the above results, it was not surprising that soybean seeds' inoculation with LP 3008 yielded only a small, nonstatistically significant increase in nodule occupation with respect to the wild type in a field with an important soybean-nodulating bacterial population. However, inoculation in the sowing furrows yielded significant benefits. First, this inoculation method allowed duplication in nodule occupation compared with seed inoculation, irrespective of the inoculated strain. Second, when the performance of LP 3008 was compared with LP 3004, both inoculated in the sowing furrows, the nodule occupation by LP 3008 was significantly higher than that of LP 3004. Thus, decreasing the distance between growing roots and rhizobia seemed important to achieve LP 3008 higher competitiveness for nodulation in soil, perhaps by profiting from higher chemotaxis and adsorption potentials during short water saturation periods (e.g. rainfalls). It remains to be studied whether FliCI and FliCII are differentially expressed in the soil and rhizosphere environments, to assess their possible contribution to LP 3008 higher competitiveness.

In this work, competitiveness for nodulation of soybean was improved using simple selection and checking procedures available to SME-sized inoculant production factories for application to any strain. Field use of such an improved strain does not involve the risks inherent to genetically engineered strains, and is allowed by current regulations on distribution and release of biofertilizers. Work with defined mutants in LP 3004 and LP 3008 is in progress to assess the contribution of flagellar expression and chemotaxis, as well as to identify the alteration(s) occurring in LP 3008 at the molecular level.

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