RESEARCH LETTER

Analysis of the role of the two flagella of *Bradyrhizobium japonicum* in competition for nodulation of soybean

Maria Julia Althabegoiti, Julieta M. Covelli, Julieta Pérez-Giménez, Juan Ignacio Quelas, Elías J. Mongiardini, Maria Florencia López, Silvina L. López-García & Aníbal R. Lodeiro

Departamento de Ciencias Biológicas, Instituto de Biotecnología y Biología Molecular (IBBM), Facultad de Ciencias Exactas, Universidad Nacional de La Plata y CCT La Plata-CONICET, La Plata, Argentina

Correspondence: Aníbal R. Lodeiro, Departamento de Ciencias Biológicas, Instituto de Biotecnología y Biología Molecular (IBBM), Facultad de Ciencias Exactas, Universidad Nacional de La Plata y CCT La Plata-CONICET, Calles 47 y 115 (1900) La Plata, Argentina. Tel.: +54 221 425 0497; fax: +54 221 422 6947; e-mail: lodeiro@biol.unlp.edu.ar

Present address: Elías J. Mongiardini, Laboratori Genètica Molecular Bacteriana, Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

Received 1 December 2010; revised 23 March 2011; accepted 24 March 2011.
Final version published online 26 April 2011.

DOI:10.1111/j.1574-6968.2011.02280.x

Editor: Juan Imperial

Keywords
flagellin; soil; water content; swimming; symbiosis.

Abstract

Bradyrhizobium japonicum has two types of flagella. One has thin filaments consisting of the 33-kDa flagellins FliCI and FliCII (FliCI-II) and the other has thick filaments consisting of the 65-kDa flagellins FliC1, FliC2, FliC3, and FliC4 (FliC1-4). To investigate the roles of each flagellum in competition for nodulation, we obtained mutants deleted in *fliCI-II* and/or *fliC1-4* in the genomic backgrounds of two derivatives from the reference strain USDA 110: the streptomycin-resistant derivative LP 3004 and its more motile derivative LP 3008. All mutations diminished swimming motility. When each mutant was co-inoculated with the parental strain on soybean plants cultivated in vermiculite either at field capacity or flooded, their competitiveness differed according to the flagellin altered. Δ*fliCI-II* mutants were more competitive, occupying 64–80% of the nodules, while Δ*fliC1-4* mutants occupied 45–49% of the nodules. Occupation by the nonmotile double mutant decreased from 55% to 11% as the water content of the vermiculite increased from 85% to 95% field capacity to flooding. These results indicate that the influence of motility on competitiveness depended on the water status of the rooting substrate.

Introduction

The symbiotic nitrogen fixation between legumes and rhizobia is unique in the sense that plants can satisfy all of their nitrogen requirements without resorting to soil nitrogen. Because this nutrient is often limited, for decades, legume crops are inoculated with rhizobia of high nitrogen-fixing performance (Ben-Rebah *et al.*, 2007). The repeated inoculation of soybean with selected strains of their symbionts *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* led to their establishment in the local soil populations (Barcellos *et al.*, 2007). However, once established in the soil, these strains can no longer be selected for high nitrogen fixation. Therefore, they enter into an uncontrolled genetic diversification and gene exchange with the soil microbiota, which, after several years, may affect their initial symbiotic performance (Provorov & Vorobyov, 2000; Itakura *et al.*, 2009).

To achieve the nitrogen-fixing state, the rhizobia need to infect and nodulate the legume roots (Patriarca *et al.*, 2004). However, the availability of infection sites and the total number of nodules formed are limited. Normally, a soybean rhizosphere is colonized by 10^5–10^7 soybean-nodulating rhizobia, but only 10^1–10^2 nodules are formed in a root (Reyes & Schmidt, 1979; Moawad *et al.*, 1984). Therefore, $< 0.01\%$ of all the rhizobia that are in close contact with a single root can finally occupy the nodules. This situation leads to strong competition between the soil population and

the inoculated rhizobia. Thus, the identification of conditions that are a determinant for competitiveness of the inoculated rhizobia is an important goal. We proposed that the position of rhizobia in the soil profile in relation to the roots and the rhizobial motility in the soil might be two of these conditions (López-García *et al.*, 2002). Further studies by Kanbe *et al.* (2007) and Althabegoiti *et al.* (2008) indicated that *B. japonicum* possesses two different flagella. One is peritrichous, with a thin filament consisting of the 33-kDa flagellins FliCI-II, and the other is subpolar, with a filament consisting of the 65-kDa flagellins FliC1-4.

To obtain a strain with increased motility, we applied a simple selection procedure to *B. japonicum* LP 3004 (spontaneous streptomycin-resistant derivative from USDA 110) and obtained the derivative LP 3008, which has higher motility in a semi-solid medium, higher expression of the thin flagellum, and higher competitiveness to nodulate soybean in field trials, promoting higher grain yield (Althabegoiti *et al.*, 2008; López-García *et al.*, 2009). Later, the same procedure was applied to the strain E 109, derived from USDA 138. As a result, we obtained a derivative similar to LP 3008, which also promoted higher soybean grain yield in field trials (Lodeiro *et al.*, 2009). Therefore, this procedure has the advantages of simplicity, the robustness of results in different strains, and the avoidance of gene manipulation, whereby the improved strains may be safely released in the field. However, although it is possible that increased expression of the thin flagellum contributed to higher motility and competitiveness, the exact genetic changes that give rise to these phenotypes are unknown. *Bradyrhizobium japonicum* possesses two different flagella, whose role in symbiosis has not yet been characterized. This knowledge is required to evaluate the effect of differential flagellum expression on competition for nodulation.

To this end, we obtained site-directed mutants in each of the flagellin-encoding gene clusters of *B. japonicum*, both in the background of the LP 3004 and LP 3008 strains, and tested their competitiveness.

Materials and methods

Bacterial strains and culture conditions

Strains are summarized in Supporting Information, Table S1. *Bradyrhizobium japonicum* was grown in Götz medium (Quelas *et al.*, 2006) or HM salts with 0.1% yeast extract, 0.1% L-arabinose, and 0.1% sodium gluconate (Kanbe *et al.*, 2007). For conjugation, PSY medium (Regensburger & Hennecke, 1983) was used. Swimming assays were performed in Götz agar (0.3% w/v) (Althabegoiti *et al.*, 2008). *Escherichia coli* was grown in Luria–Bertani (Sambrook & Russell, 2001). Antibiotics were at the following concentrations ($\mu g\,mL^{-1}$): streptomycin (Sm), 400 (*B. japonicum*) or 100 (*E. coli*); spectinomycin (Sp), 200; kanamycin, 150 (*B. japonicum*) or 25 (*E. coli*); ampicillin, 200; and gentamicin, 100 (*B. japonicum*) or 10 (*E. coli*).

Construction of *B. japonicum* mutants

Deletion mutants were obtained and checked as described (Quelas *et al.*, 2010) using the primers and plasmids indicated in Table S1. Strains LP 5843 and LP5844 (Δ*fliC1-4*) carried the *nptII* cassette in the replacement of bases 6 410 133–6 418 950, thus removing 8817 bp between *bll5843* and *bll5846* coding regions (Kaneko *et al.*, 2002). Strains LP6865 and LP 6866 (Δ*fliCI-II*) carried the Ω-Sm-Sp-interposon between bases 7 560 766 and 7 563 627, thus replacing 2861 bp of *bll6865* and *bll6866* coding regions. The double mutants LP6543 and LP 6644 had *nptII* between bases 6 410 133 and 6 418 950 of LP6865 and LP 6866, respectively.

Flagellin preparation and analysis

Rhizobia grown in liquid HM salts were vortexed for 5 min and centrifuged at 10 000 ***g*** for 30 min at 4 °C. The supernatant was incubated with 1.3% polyethylene glycol 6000 and 166 mM NaCl for 2 h at 4 °C. Afterwards, this suspension was centrifuged at 11 000 ***g*** for 40 min at 4 °C and the resulting pellet was resuspended in phosphate-buffered saline. For analysis, the samples were boiled in Laemmli (1970) loading buffer for 10 min and then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970).

Microscopy

Light microscopy was performed using a Nikon Eclipse E 200 microscope. Videos were recorded using a Nikon 518CU digital camera coupled to the microscope. Electron microscopy was performed as described elsewhere (Althabegoiti *et al.*, 2008).

Plant assays

Competitiveness was assayed using mixtures of LP 3004 or LP 3008 with the indicated mutant (Fig. S1). Each strain was at a concentration of approximately 10^6 rhizobia mL^{-1} in a modified N-free Fåhraeus plant nutrient solution contained in vermiculite pots (Lodeiro *et al.*, 2000a; López-García *et al.*, 2001, 2002). The pots were allowed to drain the excess solution through holes at the bottom to achieve 100% field capacity, and one plantlet was aseptically planted in each pot. Fifteen plants were used for each pair of inoculated strains under competition. Unless otherwise indicated, pots were irrigated every 3–4 days with sterile-distilled water. The water status of each pot was assessed gravimetrically by weighing the pots before and after watering and draining. Flooded pots were treated in the same way, except that no

holes were placed in the pots; thus, all irrigating water was retained. Control pots without bacteria or with each strain inoculated individually were run in parallel. After 20 days, the strain occupying each nodule was identified with selective antibiotics (López-García *et al.*, 2001).

Statistical analysis

Results were analyzed using the χ^2 test. The null hypothesis was that 60% of nodules contained bacteria with the antibiotic marker of the mutant and 40% of nodules contained bacteria with the antibiotic marker of the parental strain. To obtain the expected values, we multiplied the total number of nodules of each plant by the fraction corresponding to the null hypothesis. With these values and the observed values from each plant, we calculated the χ^2 values, which were compared against tabulated χ^2 values.

Results

Motility of *B. japonicum* LP 3004 and LP 3008 mutants

The main characteristics of the mutants are summarized in Table 1. Each mutant lacked the desired flagellin, as indicated by its electrophoretic motility, which matched that previously identified by Althabegoiti *et al.* (2008) as FliCI-II or FliC1-4 (Fig. 1). The loss of flagellins led to the loss of corresponding flagellar filaments (Fig. S2).

Phase-contrast microscopy showed that, while LP 5843 and LP5844 (Δ*fliC1-4*) tumbled more frequently than the wild type, LP6865 and LP 6866 (Δ*fliCI-II*) swam more straight, while LP6543 and LP6644 (Δ*fliCI-II*Δ*fliC1-4*) did not swim, corroborating previous observations by Kanbe *et al.* (2007). In addition, we recorded the rotation sense of 57 tethered cells. In 16 videos recorded from Δ*fliCI-II* mutants, we observed clockwise rotation in 18 cells and counterclockwise rotation in another 18 cells (a total of 36 tethered cells of this mutant were observed), suggesting that the thick flagellum rotates in both directions with no bias. In contrast, all 21 cells observed in 11 videos from Δ*fliC1-4* mutants rotated in the clockwise direction. Because the rotation observed in tethered cells was in the opposite direction to flagellar rotation, these observations indicate that the thin flagellum rotates only in the counterclockwise direction.

In agreement with our previous findings, swimming halos produced in Götz 0.3% agar by LP 3008 were wider than those of LP 3004 (Fig. 2). Furthermore, mutants lacking the thick or the thin flagellum produced smaller halos than their respective parental strains. In the

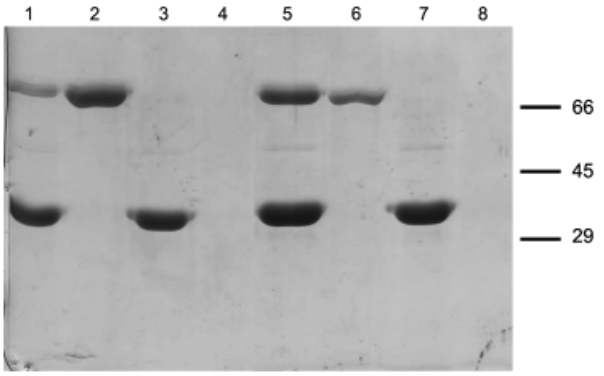

Fig. 1. Electrophoretic profiles of *Bradyrhizobium japonicum* flagellins. In lanes 1–4, strains in the genomic background of LP 3004 are shown, while in lanes 5–8 strains in the genomic background of LP 3008 are shown. Lane 1, LP 3004; lane 2, LP 6865 (Δ*fliCI-II*); lane 3, LP 5843 (Δ*fliC1-4*); lane 4, LP 6543 (Δ*fliCI-II*Δ*fliC1-4*); lane 5, LP 3008; lane 6, LP 6866 (Δ*fliCI-II*); lane 7, LP 5844 (Δ*fliC1-4*); lane 8, LP 6644 (Δ*fliCI-II*Δ*fliC1-4*). To the right of the gel, the position of the molecular weight markers is shown. Rhizobia were cultured in HM salts. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was run with 10% acrylamide in the running gel, and the gel was stained with Coomassie Brilliant Blue.

Table 1. Flagellins detected and types of flagellar filaments observed in the flagellin deletion mutant *Bradyrhizobium japonicum* strains

			Flagellins detected in SDS-PAGE		Flagellar filaments observed in TEM		
Strain	Flagellin genes deleted*	Parental strain	In HM salts	In Götz medium†	In HM salts	In Götz medium†	Antibiotic resistance
LP 3004	None	USDA 110	FliC1-4, FliCI-II	FliC1-4	Thick and thin	Thick	Sm
LP 3008	None	LP 3004	FliC1-4, FliCI-II	FliC1-4, FliCI-II	Thick and thin	Thick and thin	Sm
LP 5843	*bll5843-bll5846*	LP 3004	FliCI-II	NA	Thin	NA	Sm, Km
LP 5844	*bll5843-bll5846*	LP 3008	FliCI-II	NA	Thin	NA	Sm, Km
LP 6865	*bll6865-bll6866*	LP 3004	FliC 1-4	NA	Thick	NA	Sm, Sp
LP 6866	*bll6865-bll6866*	LP 3008	FliC 1-4	NA	Thick	NA	Sm, Sp
LP 6543	*bll5843-bll5846*; *bll6865-bll6866*	LP 6865	None	NA	None	NA	Sm, Km, Sp
LP 6644	*bll5843-bll5846*; *bll6865-bll6866*	LP 6866	None	NA	None	NA	Sm, Km, Sp

*According to Rhizobase annotation (http://genome.kazusa.or.jp/rhizobase).

†With mannitol as the sole C source.

TEM, transmission electron microscopy; NA, not assayed; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

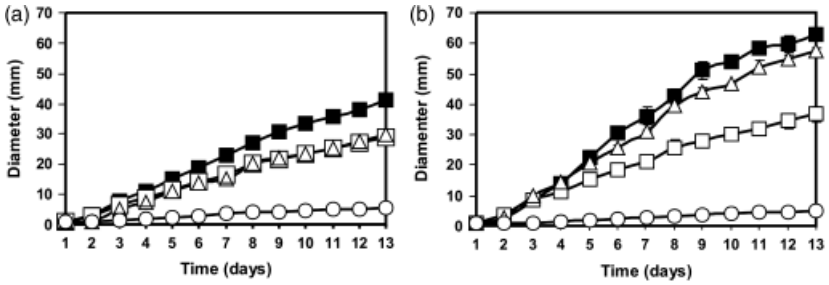

Fig. 2. Swimming motility of *Bradyrhizobium japonicum* quantified by the diameter of swimming halos produced at different times after plate inoculation. (a) Strains in the genomic background of LP 3004. (b) Strains in the genomic background of LP 3008. Filled squares: wild-type LP 3004 and LP 3008; empty squares: LP 6865 and LP 6866 (Δ*fliCI-II*); triangles: LP 5843 and LP 5844 (Δ*fliC1-4*); circles: LP 6543 and LP 6644 (Δ*fliCI-II*Δ*fliC1-4*). Diameters represent averages ± SDs from four swimming halos measured at each time point (where error bars are not visible, they are smaller than the symbol).

background of LP 3004, both mutants lacking one flagellum produced halos of similar size; in contrast, in the background of LP 3008, LP 5844 (Δ*fliC1-4*) produced wider halos than LP 6866 (Δ*fliCI-II*). By comparing any mutant in the LP 3004 background with the same mutant in the LP 3008 background, the latter always produced a wider swimming halo, with the exception of double mutants, whose colonies expanded only as a consequence of growth.

Competition for nodulation

When inoculated individually, nodulation of each mutant was similar to the parental strains. To evaluate competition for nodulation, we inoculated soybean plants with mixtures containing each parental strain together with each derived mutant, and identified the bacterial strains occupying each nodule by their antibiotic resistances. In these experiments, an Sm-resistant parental strain competed against mutant derivatives that were also resistant to Sm plus another antibiotic (Table 1). Therefore, the antibiotic resistances observed from a nodule where both competitor strains were present simultaneously (double occupation) are the same as from a nodule occupied solely by the mutant. To take into consideration the proportion of nodules with double occupation, we took into account our previous experience with different strains, where we observed an average ± [2 × SEM] of 15.1 ± 4.4% double occupation (Lodeiro *et al.*, 2000b; López-García *et al.*, 2001, 2002). Thus, to avoid underestimation of wild-type competitiveness, we took the upper limit and assumed 20% double occupation for the χ^2 analysis. Hence, we postulated as null hypothesis that 60% of nodules contained bacteria expressing the antibiotic markers of the mutant and the wild type, and the remaining 40% contained rhizobia that express only the wild-type marker. The results are shown in Table 2.

When vermiculite was at field capacity, each flagellin made a different contribution to competitiveness. The strains LP 6865 and LP 6866, which expressed only the thick flagellum, being less motile than their parental strains, were more competitive for nodulation, while mutants LP 5843 and LP 5844, which expressed only the thin flagellum, were less competitive than the parental strains. Surprisingly, mutants LP 6543 and LP 6644, devoid of both flagella, occupied around 50% of the nodules. Differences of statistical significance among competitions of double mutants against LP 3004 or LP 3008 might reflect that both the χ^2 values calculated were close to the threshold of significance for the tabulated χ^2 value. Nevertheless, the trend was clear in that none of the nonmotile double mutants was completely displaced by the wild-type parental strain. To investigate whether this high competitiveness of nonmotile mutants was related to the water contents of pots, we co-inoculated LP 3004 and LP 6543 (nonmotile, lacking both flagella) in vermiculite pots maintained in one of three watering regimens: regularly watered, watered with a double frequency, and flooded. Between days 3 and 12 after inoculation, which is the period where initial nodulation occurs, there was a significant difference in the water status between pots irrigated normally and pots irrigated with double frequency (Fig. S3). In regularly watered pots, the nodule occupation by the nonmotile mutant (plus double occupation) was 53.1%, while in pots watered with double frequency, it was 44.3%, and in flooded pots, it was 16.3% (all significantly different from the null hypothesis), indicating that there was a negative correlation between the competitiveness of the nonmotile mutant and vermiculite water content. Hence, we evaluated the competition for nodulation of all strains in the flooded condition. As shown in Table 2, the behavior of the mutants carrying one flagellum was similar as in field capacity (except LP 6866, which, although occupied 64.3% of nodules, did not deviate significantly from the null hypothesis due to higher experimental variability). As in field capacity, LP 3008 seemed to compete better than LP 3004 against its derivative without a thin flagellum.

Table 2. Nodule occupation of the different flagellin-defective mutant strains when co-inoculated with the wild-type parental strain at the indicated cell densities on 15 soybean plants that were cultivated in vermiculite at field capacity or flooded*

Condition†	Competition	Inoculum size (10^6 CFU mL^{-1})		Nodules occupied by wt : mutant‡ (%)	Total nodules per plant (average ± SD)	Significance of χ_c^2 (compared with χ_t^2) ($P < 0.05$)§
		Wild type	Mutant			
Field capacity	LP 3004/LP 6865	3.55	3.72	28.9 : 71.1	14.9 ± 5.3	Yes
	LP 3008/LP 6866	2.00	2.02	34.3 : 65.7	18.3 ± 4.8	Yes
	LP 3004/LP 5843	3.55	3.15	52.4 : 47.6	16.9 ± 4.0	Yes
	LP 3008/LP 5844	2.00	7.15	53.7 : 46.3	17.9 ± 2.7	Yes
	LP 3004/LP 6543	3.55	3.68	49.4 : 50.6	16.2 ± 3.8	Yes
	LP 3008/LP 6644	2.00	2.80	44.3 : 55.7	19.4 ± 3.5	No
Flooding	LP 3004/LP 6865	1.84	3.40	20.0 : 80.0	13.8 ± 6.6	Yes
	LP 3008/LP 6866	1.75	3.14	35.7 : 64.3	9.0 ± 6.5	No
	LP 3004/LP 5843	1.84	2.02	50.8 : 49.2	15.4 ± 5.3	No
	LP 3008/LP 5844	1.76	3.37	55.0 : 45.0	11.6 ± 6.9	Yes
	LP 3004/LP 6543	1.84	3.63	87.4 : 12.6	17.7 ± 8.9	Yes
	LP 3008/LP 6644	1.84	2.54	89.0 : 11.0	21.4 ± 8.3	Yes

*Uninoculated controls yielded no nodules; controls where a single strain was inoculated contained only this antibiotic-resistant strain in all the nodules tested.

†Refers to the water content of the pots (for details, see Materials and methods).

‡The percentage of the nodules occupied by the mutant may include double occupation by both the mutant and the wild-type (wt) strains.

§Deviation from the null hypothesis was considered statistically significant when the calculated χ^2 values (χ_c^2) were higher than the tabulated χ^2 values (χ_t^2) with $P < 0.05$. The null hypothesis was that 60% of nodules are occupied by bacteria that express the mutant antibiotic resistance (includes double occupation), while 40% of nodules are occupied by bacteria that express the antibiotic resistance of the wild type, but not that of the mutant.

Meanwhile, the nonmotile double mutants were again significantly less competitive than in field capacity.

Discussion

Bacterial swimming may be observed in semi-solid agar plates as a colony expansion a few millimeters below the agar surface, and must not be confused with swarming, which occurs in plates of more concentrated agar where colonies of differentiated cells move on the surface (Harshey, 1994, 2003). Indeed, rhizobia mutants able to produce swimming halos, but swarming colonies were not described (Braeken *et al.*, 2007; Nogales *et al.*, 2010).

Our results showing swimming in 0.3% agar indicated that the thin flagellum of *B. japonicum* is actively used for this motion, because LP 5844 (Δ*fliC1-4*, producing only the thin flagellum) formed the widest swimming halo of all mutants. In addition, this strain tumbled more frequently than the wild type. In agreement with our results, Wolfe & Berg (1989) also reported that the swimming halo rate of expansion increases with tumble frequency. Thin flagellum derepression in LP 3008 may also cause its faster spread in 0.3% agar; however, it does not explain why the LP 3008 mutant lacking this flagellum still formed wider swimming halos than the corresponding mutant in the LP 3004 background. In 0.3% agar, the consumption of nutrients and release of other chemicals by the rings of bacteria moving inside the medium creates a chemoattractant gradient (Adler, 1966). Thus, the higher chemotaxis of LP 3008 (Althabegoiti *et al.*, 2008) may also contribute to its higher displacement.

After characterizing the motility provided by each flagellum, we assessed their roles in the competition for nodulation in vermiculite. Although all mutants moved less than the parental strains in swimming plate assays, they were differently affected in their competitiveness for nodulation, which also depended on the water status of the vermiculite. While mutants lacking the thin flagellum were, in general, more competitive than the parental strains both at field capacity and in the flooded environment, the mutants lacking the thick flagellum were less competitive. By contrast, the mutants lacking both flagella behaved differently according to the water status of the vermiculite: they had a rather high competitiveness at field capacity, but were almost completely displaced in the flooded vermiculite by the wild type. These results, which are in agreement with observations of competition for root colonization, where mutants lacking the thin flagellum were equally competitive as the parental strains, while mutants lacking the thick flagellum or both were less competitive (Althabegoiti *et al.*, 2010), suggest a complex role of flagellins in competitiveness. On the one hand, the effects of motility on competitiveness depended on the water status of the rooting substrate, and on the other, mutants devoid of the thin flagellum indicated that flagellin activities unrelated to motility might have exerted an influence.

Flagellins are pathogen-related molecular patterns able to elicit plant defense responses (Nicaise *et al.*, 2009). However,

the active portion is a 22-amino acid peptide near the N-terminus called flg22, which is not conserved in rhizobial flagellins (Gómez-Gómez & Boller, 2002) including FliCI-II or FliC1-4 (J. Pérez-Giménez, unpublished data). Another possible role related to competitiveness might be in bacterial adhesion to roots; however, studies in *Rhizobium leguminosarum* indicated that flagellin is not an adhesin (Smit *et al.*, 1989). Furthermore, flagellin expression in the vermiculite is unknown. Thus, more studies are required to evaluate the nature of flagellin activities in *B. japonicum*.

In soils at field capacity, rhizobial motility may be scarce (Madsen & Alexander, 1982; Liu *et al.*, 1989; McDermott & Graham, 1989; López-García *et al.*, 2002; Horiuchi *et al.*, 2005), because chemoattractant diffusion is slower due to the lower water potential, paths are impaired due to the tortuosity and size of the soil pores, and bacterial movement is retarded due to attachment/detachment to and from soil particles (Watt *et al.*, 2006; Tufenkji, 2007). Our results with the nonmotile double mutants are in agreement with these observations, indicating that the effect of swimming on competition for nodulation would be restricted to situations of water saturation of the soil pores (which, in field crops, occur after irrigation or rainfall). However, much work still remains to be carried out to understand the different performances of each flagellum in laboratory and field experiments. Among the main factors that may play a role in the field situation are the physiological state of the rhizobia at the time of inoculation, the expression of each flagellum in the environment, their activities apart from motility, and the influence of soil factors such as micro- and macrobiota, organic matter, porosity, structure, and climate, all of which are absent in the lab system. Nevertheless, our results underscore the importance of inoculant application methods in field crops to benefit from rhizobial motility in the competition for nodulation (López-García *et al.*, 2002, 2009; Althabegoiti *et al.*, 2008).

Acknowledgements

We are grateful to Paula Giménez, Abel Bortolameotti, and Bernabé Castillo for their excellent technical assistance. Supported by ANPCyT, Argentina. M.J.A., J.P.-G., J.I.Q., and M.F.L. are fellows of CONICET, Argentina. J.M.C. is a fellow of ANPCyT. S.L.L.-G. and A.R.L. are members of the scientific career of CONICET.

References

Adler J (1966) Chemotaxis in bacteria. *Science* **153**: 708–716.

Althabegoiti MJ, López-García SL, Piccinetti C, Mongiardini EJ, Pérez-Giménez J, Quelas JI, Perticari A & Lodeiro AR (2008) Strain selection for improvement of *Bradyrhizobium japonicum* competitiveness for nodulation of soybean. *FEMS Microbiol Lett* **282**: 115–123.

Althabegoiti MJ, Covelli JM, Rivilla R, Martín M & Lodeiro AR (2010) Colonization of soybean roots by *Bradyrhizobium japonicum* expressing each one of its flagellar systems. Abstr. *XIII National Meeting of the Spanish Society of Nitrogen Fixation-II Portuguese-Spanish Congress on Nitrogen Fixation, Zaragoza, Spain, pp. 129–130* (abstract S3–09). http://digital.csic.es/handle/10261/25138

Barcellos FG, Menna P, da Silva Batista JS & Hungria M (2007) Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium* (*Ensifer*) *fredii* and *Bradyrhizobium elkanii* in a Brazilian Savannah soil. *Appl Environ Microb* **73**: 2635–2643.

Ben Rebah F, Prévost D, Yezza A & Tyagi RD (2007) Agro-industrial waste materials and wastewater sludge for rhizobial inoculant production: a review. *Bioresource Technol* **98**: 3535–3546.

Braeken K, Daniels R, Vos K, Fauvart M, Bachaspatimayum D, Vanderleyden J & Michiels J (2007) Genetic determinants of swarming in *Rhizobium etli*. *Microb Ecol* **55**: 54–64.

Gómez-Gómez L & Boller T (2002) Flagellin perception: a paradigm for innate immunity. *Trends Plant Sci* **7**: 251–256.

Harshey RM (1994) Bees aren't the only ones: swarming in Gram-negative bacteria. *Mol Microbiol* **13**: 389–394.

Harshey RM (2003) Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* **57**: 249–273.

Horiuchi J, Prithiviraj B, Bais HP, Kimball BA & Vivanco JM (2005) Soil nematodes mediate positive interactions between legume plants and *Rhizobium* bacteria. *Planta* **222**: 848–857.

Itakura M, Saeki K, Omori H *et al.* (2009) Genomic comparison of *Bradyrhizobium japonicum* strains with different symbiotic nitrogen-fixing capabilities and other Bradyrhizobiaceae members. *ISME J* **3**: 326–339.

Kanbe M, Yagasaki J, Zehner S, Göttfert M & Aizawa SI (2007) Characterization of two sets of subpolar flagella in *Bradyrhizobium japonicum*. *J Bacteriol* **189**: 1083–1089.

Kaneko T, Nakamura Y, Sato S *et al.* (2002) Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA 110. *DNA Res* **9**: 189–197.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Liu R, Tran VM & Schmidt EL (1989) Nodulating competitiveness of a nonmotile Tn*7* mutant of *Bradyrhizobium japonicum* in nonsterile soil. *Appl Environ Microb* **55**: 1895–1900.

Lodeiro AR, González P, Hernández A, Balagué LJ & Favelukes G (2000a) Comparison of drought tolerance in nitrogen-fixing and inorganic nitrogen-grown common beans. *Plant Sci* **154**: 31–41.

Lodeiro AR, López-García SL, Vázquez TEE & Favelukes G (2000b) Stimulation of adhesiveness, infectivity, and competitiveness for nodulation of *Bradyrhizobium japonicum*

by its treatment with soybean seed lectin. *FEMS Microbiol Lett* **188**: 177–184.

Lodeiro AR, Althabegoiti MJ, Piccinetti C, Covelli JM, Mongiardini EJ, Pérez-Giménez J, Quelas JI, López-García SL & Perticari A (2009) Cepas con mayor movilidad para la mejora de la fijación de N_2 en soja. Abstr. II *Jornadas Bonaerenses de Microbiología de Suelos para una Agricultura Sustentable, Azul, Argentina*, pp. 14–15 (abstract).

López-García SL, Vázquez TEE, Favelukes G & Lodeiro AR (2001) Improved soybean root association of N-starved *Bradyrhizobium japonicum*. *J Bacteriol* **183**: 7241–7252.

López-García SL, Vázquez TEE, Favelukes G & Lodeiro AR (2002) Rhizobial position as a main determinant in the problem of competition for nodulation in soybean. *Environ Microbiol* **4**: 216–224.

López-García SL, Perticari A, Piccinetti C *et al.* (2009) In-furrow inoculation and selection for higher motility enhances the efficacy of *Bradyrhizobium japonicum* nodulation. *Agron J* **101**: 357–363.

Madsen EL & Alexander M (1982) Transport of *Rhizobium* and *Pseudomonas* through soil. *Soil Sci Soc Am J* **46**: 557–560.

McDermott TR & Graham PH (1989) *Bradyrhizobium japonicum* inoculant mobility, nodule occupancy, and acetylene reduction in the soybean root system. *Appl Environ Microb* **55**: 2493–2498.

Moawad HA, Ellis WR & Schmidt EL (1984) Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. *Appl Environ Microb* **47**: 607–612.

Nicaise V, Roux M & Zipfel C (2009) Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiol* **150**: 1638–1647.

Nogales J, Domínguez-Ferreras A, Amaya-Gómez CV, van Dillewijn P, Cuéllar V, Sanjuán J, Olivares J & Soto MJ (2010) Transcriptome profiling of a *Sinorhizobium meliloti fadD* mutant reveals the role of rhizobactin 1021 biosynthesis and regulation genes in the control of swarming. *BMC Genomics* **11**: 157.

Patriarca EJ, Tatè R, Ferraioli S & Iaccarino M (2004) Organogenesis of legume root nodules. *Int Rev Cytol* **234**: 201–262.

Provorov NA & Vorobyov NI (2000) Population genetics of rhizobia: construction and analysis of an 'infection and release' model. *J Theor Biol* **205**: 105–119.

Quelas JI, López-García SL, Casabuono A, Althabegoiti MJ, Mongiardini EJ, Pérez-Giménez J, Couto A & Lodeiro AR (2006) Effects of N-starvation and C-source on *Bradyrhizobium japonicum* exopolysaccharide production and composition, and bacterial infectivity to soybean roots. *Arch Microbiol* **186**: 119–128.

Quelas JI, Mongiardini EJ, Casabuono A, López-García SL, Althabegoiti MJ, Covelli JM, Pérez-Giménez J, Couto A & Lodeiro AR (2010) Lack of galactose or galacturonic acid in *Bradyrhizobium japonicum* USDA 110 exopolysaccharide leads to different symbiotic responses in soybean. *Mol Plant-Microbe In* **23**: 1592–1604.

Regensburger B & Hennecke H (1983) RNA polymerase from *Rhizobium japonicum*. *Arch Microbiol* **135**: 103–109.

Reyes VG & Schmidt EL (1979) Population densities of *Rhizobium japonicum* strain 123 estimated directly in soil and rhizospheres. *Appl Environ Microb* **37**: 854–858.

Sambrook J & Russell D (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Habor Laboratory Press, Cold Spring Harbor.

Smit G, Kijne JW & Lugtenberg BJ (1989) Roles of flagella, lipopolysaccharide, and a Ca^{2+}-dependent cell surface protein in attachment of *Rhizobium leguminosarum* biovar *viciae* to pea root hair tips. *J Bacteriol* **171**: 569–572.

Tufenkji N (2007) Modeling microbial transport in porous media: traditional approaches and recent developments. *Adv Water Res* **30**: 1455–1469.

Watt M, Silk WK & Passioura JB (2006) Rates of root and organism growth, soil conditions, and temporal and spatial development of the rhizosphere. *Ann Bot* **97**: 839–855.

Wolfe AJ & Berg H (1989) Migration of bacteria in semisolid agar. *P Natl Acad Sci USA* **86**: 6973–6977.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Scheme of the experiments of competition for nodulation.
Fig. S2. Transmission electron micrographs of *Bradyrhizobium japonicum*.
Fig. S3. Water contents of vermiculite pots under different irrigation procedures.
Table S1. Primers, plasmids, and bacterial strains used in this study.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.