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Swarming motility in *Bradyrhizobium japonicum*

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Abstract

Flagellar-driven bacterial motility is an important trait for colonization of natural environments. *Bradyrhizobium japonicum* is a soil species that possesses two different flagellar systems: one subpolar and the other lateral, each with a filament formed by a different set of flagellins. While synthesis of subpolar flagellins is constitutive, translation of lateral flagellins was detected in rhizobia grown with L-arabinose, but not with D-mannitol as sole carbon source, independently of whether bacteria were in liquid or semisolid medium. We characterized swarming of *B. japonicum* in semisolid medium and found that this motility was faster with L-arabinose than with D-mannitol. By using mutants with deletions in each flagellin set, we evaluated the contribution of each flagellum system to swarming in semisolid culture media, and in soil. Mutants devoid of either of the flagella were affected in swarming in culture media, with this impairment being stronger for mutants without lateral flagella. In sterile soil at 100% or 80% field capacity, flagellar-driven motility of mutants able to swim but impaired in swarming was similar to wild type, indicating that swimming was the predominant movement here.

Keywords: *Bradyrhizobium*; Swimming; Swarming; Flagellum; Soil; Rhizosphere

1. Introduction

Bacterial motility is a key trait affecting fitness and survival in natural environments. Almost all species in the bacterial realm have evolved different devices for locomotion, the construction and propulsion of which consume a considerable fraction of the metabolic energy produced by the cell. Flagella are among the best characterized of these locomotion devices. Some bacteria, among them *Aeromonas* spp., *Azospirillum* spp., *Plesiomonas shigelloides*, *Rhodospirillum centenum*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus* possess two different flagellar systems in the same cell: a polar (or subpolar) flagellum and lateral flagella (Inoue et al., 1991; Jiang et al., 1998; Kawagishi et al., 1995; McClain et al., 2002; Shimada et al., 1985; Shinoda and Okamoto, 1977; Tarrand et al., 1978). It was observed that these flagellar systems are propelled by H^+ or Na^+ gradients respectively (Jiang et al., 1998; Kawagishi et al., 1995; McClain et al., 2002; Shinoda and Okamoto, 1977). Furthermore, lateral flagellar expression is upregulated by contact with surfaces or in response to impairment of polar flagellar motion (Kirov, 2003; Merino et al., 2006; Merino and Tomás, 2009).

Bradyrhizobium japonicum, the diazotrophic symbiont of soybean plants, has two different flagellar systems: a thick subpolar flagellum and thinner peritrichous flagella, which are often sinusoidal in shape (Althabegoiti et al., 2008; Kanbe et al., 2007). Morphologically, these flagella are similar to the respective polar and lateral flagella of *P. shigelloides* (Inoue et al., 1991), *R. centenum* (McClain et al., 2002) and *V. parahaemolyticus*, one of the best-characterized dual-flagella systems (Bardy et al., 2003). However, in *B. japonicum*, both

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flagella are powered by an H^+ proton gradient and are expressed in agitated liquid AG medium (Althabegoiti et al., 2008; Kanbe et al., 2007).

The flagellins that constitute the filament of the *B. japonicum* subpolar flagellum, which is constitutively expressed in liquid medium, are encoded in four genes, named *fliC1*, *fliC2*, *fliC3*, and *fliC4* (locus tags bll5843, bll5844, bll5845, and bll5846, respectively). In addition, two genes named *fliCI* and *fliCII* (locus tags bll6866, and bll6865, respectively) encode the structural flagellin of the peritrichous flagellum, which is produced in liquid AG complex medium but not in Götz minimal medium (Althabegoiti et al., 2008, 2011; Kanbe et al., 2007). Due to their homology with other *fliC* genes, Kanbe et al. (2007) termed the latter two genes subpolar flagellins, according to the *Salmonella* model, which has only one flagellum type. However, we agree with other authors that these flagellins are part of a lateral flagellum (Merino and Tomás, 2009) in view of the morphology of the *B. japonicum* peritrichous flagellum and the response of its flagellin synthesis to different culture conditions. Thus, in this work, we will refer to bll6866 and bll6865 as *lafA1* and *lafA2*, respectively.

Swarming is a kind of flagellar-driven movement that, in contrast to swimming into a liquid, can be observed on the surface of medium (0.5–0.8%) to hard (1.0–2.0%) agar. It looks like a colony expanding with branches or tendrils that radiate from the colony center toward the border, where cells are often enlarged and multiflagellated (Butler et al., 2010; Harshey, 2003). This social movement was reported in some rhizobial species, namely *Ensifer meliloti* (Nogales et al., 2010, 2012; Soto et al., 2002), *Rhizobium etli* (Braeken et al., 2008; Daniels et al., 2006) and *Rhizobium leguminosarum* bv. *viciae* (Tambalo et al., 2010), which are evolutionarily distant from *B. japonicum* and have only one flagellar system. Furthermore, it is believed that swarming might be required for bacterial motility in soils or in the rhizosphere. Because the role of *B. japonicum* motility in soil and in relation to competitiveness for soybean root infection and nodulation is not clear (Althabegoiti et al., 2008, 2011; Liu et al., 1989; López-García et al., 2002; McDermott and Graham, 1989), it is important to study the possible contributions of the two flagella to this type of motility and how it might impact *B. japonicum*'s ability to thrive in its natural environment. Kanbe et al. (2007) suggested that, as in other species with dual flagella systems, the *B. japonicum* subpolar flagellum is used for swimming and the lateral flagellum is used for swarming. However, studies of swarming in this species have not yet been addressed. Therefore, in this paper, we characterize *B. japonicum* swarming and evaluate the expression of the lateral flagellin and the role of each flagellum in swarming. We further use a laboratory approach to estimate the contributions of swimming and swarming to bacterial dispersal in soil.

2. Materials and methods

2.1. Bacterial strains and culture conditions

B. japonicum strains used here are listed in Table 1 and were described by López-García et al. (2002) and Althabegoiti et al. (2008, 2011). These strains are LP 3004 (a spontaneous streptomycin-resistant derivative from USDA 110), LP 3008 (selected for higher swimming motility from LP 3004) and flagellin deletion mutants derived from these backgrounds, as follows. From LP 3004 we obtained LP 5843 (Δ*fliC1234*), LP 6865 (Δ*lafA12*) and LP 6543 (Δ*fliC1234*Δ*lafA12*). From LP 3008 we obtained LP 5844 (Δ*fliC1234*), LP 6866 (Δ*lafA12*) and LP 6644 (Δ*fliC1234*Δ*lafA12*). The rhizobia were grown in Götz minimal medium (Götz et al., 1982) with 5.0 g l^{-1} carbon source or in AG medium (Sadowsky et al., 1987) with appropriate antibiotics (Althabegoiti et al., 2011). Conditions for growth in liquid medium were 28 °C and 180 rpm. Antibiotics were used at the following concentrations (μg ml^{-1}): streptomycin, 400; spectinomycin, 200; kanamycin, 150; gentamicin, 100; and cycloheximide, 100.

2.2. Reverse transcription PCR

Rhizobial suspensions cultured in Götz medium with the different carbon sources, as described above, were centrifuged at 11,000 × *g* at 4 °C for 40 min and washed twice with 1 M NaCl. Then the cells were disrupted with lysozyme in TE buffer, pH 8.0. Total RNA was obtained with Trizol (Invitrogen, Buenos Aires, Argentina) following the manufacturer's instructions and then treated with DNase I at 37 °C for 15 min. Complementary DNA (cDNA) was synthesized using random primers with M-MLV reverse transcriptase (Invitrogen, Buenos Aires, Argentina) following the manufacturer's instructions. After reverse transcription, PCR was performed as described by Quelas et al. (2010) using the following primers: for *fliC1*, fliC1Fw: 5′-CGATGGCACCACCGTACTGT-3′ and fliC1Rv: 5′-ACCGCGGTTCCCTCATAGA-3′; for *fliC2(34)*, fliC2(34)Fw: 5′-CGGTCCTTTGCAAGCACT-3′ and fliC2(34)Rv: 5′-TTCACGGTCAGCGTATCGC-3′; for *lafA1*, lafA1Fw: 5′-CCTCACCAACTCGTCTGCAA-3′ and lafA1Rv: 5′-CCGTGTTCAGAGCGGTGTATT-3′; for *lafA2*, lafA2Fw: 5′-GGTTACATCGCGCAGGTCA-3′ and lafA2Rv: 5′-GGGTGGACTCCTGGTTCATGT-3′, and for *sigA* (internal control, Hauser et al., 2006): sigAFw: 5′-CTGATCCAGGAAGGCAACATC-3′ and sigARv: 5′-TGGCGTAGGTCGAGAACTTGT-3′. The following reaction controls were employed: 1) reaction without template to test for contamination in the reagents, 2) reaction with a product obtained by omitting the retrotranscription step to test for DNA remaining after DNAse treatment, and 3) reaction with genomic DNA as

Table 1
Strains used in this study (Althabegoiti et al., 2011).

Strain	Parental strain	Flagellin genes deleted	Flagellar filaments produced
LP 3004	USDA 110	None	Subpolar and lateral
LP 3008	LP 3004	None	Subpolar and lateral
LP 5843	LP 3004	*fliC1234*	Lateral
LP 5844	LP 3008	*fliC1234*	Lateral
LP 6865	LP 3004	*lafA12*	Subpolar
LP 6866	LP 3008	*lafA12*	Subpolar
LP 6543	LP 6865	*fliC1234*; *lafA12*	None
LP 6644	LP 6866	*fliC1234*; *lafA12*	None

template to test accuracy of primers and amplicon size. The results from reverse-transcription PCR reactions were analyzed only when controls 1) and 2) produced negative results and control 3) produced a positive result.

2.3. Flagellin preparation and analysis

To obtain flagellin, rhizobia grown in a liquid medium were vortexed for 5 min and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was collected and incubated with 1.3% polyethylene glycol (6000) and 166 mM NaCl for 2 h at 4 °C. Next, this suspension was centrifuged at 11,000 × *g* for 40 min at 4 °C. The pellet was resuspended in phosphate-buffered saline (PBS).

For analysis, the samples were boiled in Laemmli (1970) loading buffer for 10 min and then separated by electrophoresis in a 10% (w/v) polyacrylamide SDS–PAGE gel. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250.

To obtain anti-LafA1-2 polyclonal antibodies, bands containing this flagellin were recovered from SDS–PAGE and then used to immunize Balb/c mice with three injections at 0, 14 and 28 days. The first injection was performed with complete Freund's adjuvant in a volume ratio of 1:1 (final volume: 100 µl), and the other two injections were performed with incomplete Freund's adjuvant at the same volume ratio. Seven days after the last injection, the mice were bled to recover the antibody. Western blots were performed as previously described (Lodeiro et al., 2000a).

2.4. Swarming assays

Swarming plates were prepared with culture medium containing 0.5% agar (w/v). The method employed to form a carbon source gradient inside the plates is depicted in Supplementary Fig. 1. Petri dishes were elevated 0.5 cm on one side, and 25 ml of melted Götz minimal medium with the required carbon source at 5.0 g l^{-1} concentration was dispensed. After cooling and air-drying for 15 min in laminar flow, the agar surface remained inclined. The plates were then leveled and completed with an additional 25 ml of melted Götz minimal medium without the carbon source. After cooling and solidification of the culture medium, the plates were incubated overnight at 28 °C before inoculation. The strains to be tested were grown in liquid AG medium without antibiotics at 28 °C to an OD_{600} of 1.5 (late logarithmic phase). Aliquots of 2 µl culture suspensions were drop-inoculated at 2 cm from the edge of the plate at the side of the carbon source limitation and air-dried in laminar flow. The inoculated plates were wrapped with parafilm and incubated for the required time at 28 °C in an upright position.

2.5. Preparation of root exudates and root extracts

Soybean seeds cv. DonMario 4800 were surface-sterilized and germinated as described (Althabegoiti et al., 2008). To obtain root exudates, germinated seedlings (3–4 cm in length) were aseptically transferred to a steel mesh and soaked in plant nutrient solution (Lodeiro et al., 2000b) for four days. To obtain root extracts, the roots were ground in liquid nitrogen and suspended in plant nutrient solution. Root exudates or root extracts were centrifuged and the supernatants were filter-sterilized and mixed in 1:1 volume proportion with 2× Götz medium (with D-mannitol as carbon source) for swarming assays.

2.6. Electron microscopy

For examination of bacteria taken from swarming colonies, a modification of the method reported by Kirov et al. (2002) was followed. The grids were wetted on drops of piperazine-*N*, *N*′-bis[2-ethanesulfonic acid] (PIPES) buffer, pH 7.3. Then, the grids were applied directly to bacterial growth on the agar surface either at the edges or the center of the swarming colonies and fixed with a drop of 0.5% glutaraldehyde in 0.1 M PIPES for 2 min. The grids were then washed three times for 10 s each in PIPES buffer and negatively stained with 2% (w/v) phosphotungstic acid for 10–30 s. Microscopy was performed with a JEM 1200 EX (JEOL, Japan Electron Optics Laboratory Co., Ltd.) at the Central Microscopy Service of the School of Veterinary Sciences, National University of La Plata (UNLP), Argentina.

2.7. Observation of motility in soil

The method previously reported by Vilain et al. (2006) with some modifications was followed. Petri dishes were prepared with 30 ml AG medium agarized at 1.5%. Holes of 1.4 cm in diameter were created in the AG medium and the medium from inside the holes was removed. Holes were then filled with grounded sterile soil obtained from Cavanagh (Province of Córdoba, Argentina), whose chemical and physical characteristics are described elsewhere (López-García et al., 2009). Each soil sample used to fill each hole was placed in an Eppendorf tube, labeled and weighed with an analytical balance before and after filling the hole. In this way, the exact amount of soil used in each hole was determined and then a volume of sterile double-distilled water was slowly poured onto each soil sample to obtain soil tablets at the desired percentage of field capacity (as previously determined gravimetrically) surrounded by the AG solid medium. The soil tablets prepared in this way were inoculated at the center with 2 µl of rhizobia grown in AG medium to an $OD_{600} = 1.5$ as before and incubated at 28 °C in an upright position. The plates were inspected periodically until bacterial growth was noticed.

3. Results

3.1. Influence of the carbon source on growth and flagellin production

Previously, LafA proteins had been detected in SDS–PAGE of flagellin-enriched fractions from AG broths of

the wild-type *B. japonicum* strains USDA 110 and LP 3004, but not from Götz minimal medium broths (Althabegoiti et al., 2008, 2011; Kanbe et al., 2007). Since AG is a complex medium, while Götz is a minimal medium, and they have different carbon sources (L-arabinose and D-gluconate in AG or D-mannitol in Götz), we wondered whether the carbon source could be responsible for the observed differences in LafA contents.

With this aim, we first compared growth of *B. japonicum* strain LP 3004 in original Götz minimal medium with D-mannitol as the only carbon source (GM), or in modified Götz minimal medium where D-mannitol was substituted for L-arabinose (GA), which is more efficiently metabolized than D-mannitol by *B. japonicum* (Pedrosa and Zancan, 1974; Thorne and Burris, 1940). Growth kinetics was similar in GA and GM (Fig. 1A), in agreement with the observation that growth in Götz minimal medium is limited by Ca^{2+} and not by the carbon source (López-García et al., 2001).

Next, we studied transcription of the flagellin genes and production of the corresponding polypeptides in these media. Transcription was evaluated by reverse-transcription PCR using *sigA* as a constitutive standard (Hauser et al., 2006). Because the DNA sequences of *fliC2*, *fliC3*, and *fliC4* are almost identical, we could not design primers specific for each of the genes; therefore, the corresponding amplicons will be referred to as *fliC2(34)*. As shown in Fig. 1B, we obtained similar levels of cDNA amplification for *fliC1*, *fliC2(34)*, *lafA1* and *lafA2* in both carbon sources. The production of flagellin proteins was evaluated by obtaining flagellin-enriched fractions from these cultures (Althabegoiti et al., 2011), separating them by SDS–PAGE (Fig. 1C) and assessing the presence/absence of LafA1-2 using western blot with a polyclonal anti-LafA1-2 antibody (Fig. 1D). FliC1-4 were constitutively expressed in AG, GA and GM; however, LafA1-2 could be detected only from bacterial grown in AG and GA (Fig. 1C, D). Behind the LafA1-2 band in the western blot there was a faint band pattern that suggested that the antibody cross-reacted with an additional substance (Fig. 1D). This band pattern was similar to bacterial LPS (Quelas et al., 2010), possibly indicating that LafA1-2 and LPS were associated in some way, e.g., by LPS sheathing of the filament. Such sheathing by the outer membrane was described in the closely related flagellar filament of *Brucella melitensis* (Fretin et al., 2005). Nevertheless, the presence of LafA1-2 in GA and its absence in GM were still clear, in agreement with the reported lack of detection of LafA1-2 using silver-stained SDS–PAGE from GM broths (Althabegoiti et al., 2008).

3.2. Experimental conditions for the observation of swarming in B. japonicum

We explored conditions for macroscopic visualization of swarming in *B. japonicum* LP 3004 and its spontaneous derivative LP 3008, which constitutively synthesizes the lateral flagella in GM and has faster swimming motility (Althabegoiti et al., 2008).

Rhizobia were grown in liquid AG (where both flagella are synthesized) and then inoculated for swarming in Petri dishes containing GM. We tested agar concentration, incubation temperature, growth state, medium composition, iron depletion, the addition of soybean root exudates or root extracts and carbon or nitrogen source concentrations (McCarter and Silverman, 1989; Soto et al., 2002; Tambalo et al., 2010; Thampuran and Surendran, 1996). To test the carbon or nitrogen source concentrations, we prepared Petri dishes with a source gradient from 0 to 5.0 g l^{-1} for the carbon source or 0 to 1.3 g l^{-1} for the nitrogen source. Among all these trials, we succeeded with an agar concentration of 0.5%, incubation temperature 28 °C and use of a carbon source gradient.

A 2-µl inoculum of AG-grown rhizobia in late-logarithmic growth was placed at 2 cm from the 0 g l^{-1} side (Supplementary Fig. 1) and the plates were incubated at 28 °C

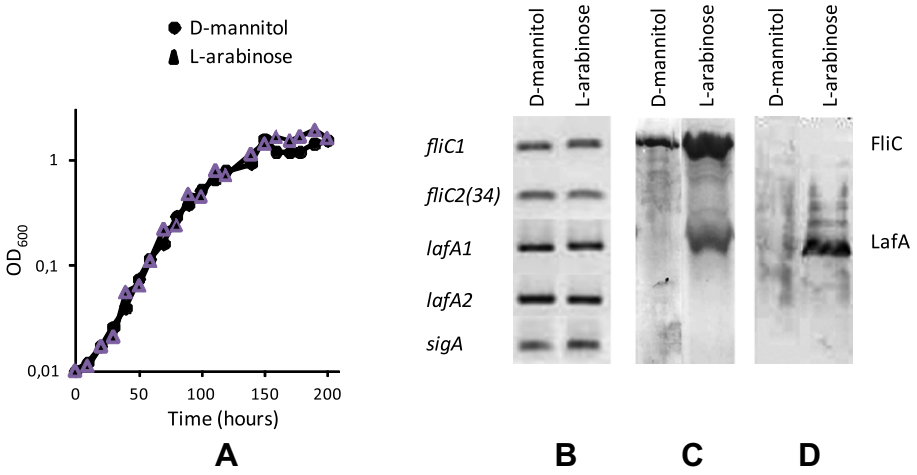

Fig. 1. Effects of D-mannitol or L-arabinose (at 5 g l^{-1}) in Götz media upon growth and flagellin production in *B. japonicum* LP 3004. **A**: Growth kinetics, optical density 600 nm. **B**: Composite image from an agarose gel of reverse-transcribed PCRs of the different flagellins; *sigA* was included as a constitutive standard. **C**: Composite image from an SDS–PAGE of flagellins stained with Coomassie Brilliant Blue. **D**: Composite image from a western blot of flagellins revealed with polyclonal anti-LafA antibody. The positions of transcripts and polypeptides are indicated.

in an upright position. A movement with tendrils in the colony border began to be observed 5 days after inoculation (DAI), especially in LP 3008. By 15 DAI, the colony had expanded to its maximum size, approximately 2–3 cm, although these diameters are difficult to calculate precisely because of irregularities in the colony borders and anisotropy of colony expansion (Fig. 2A). These observations could not be reproduced at any fixed D-mannitol concentration within the limits of the gradient.

3.3. Observations on swarming with different carbon sources

We inoculated LP 3004 and LP 3008 in Petri dishes containing Götz semisolid medium with gradients of L-arabinose (GA_{gr}) or D-mannitol (GM_{gr}). Colonies developed, and after 5 DAI began to expand on the surface and form ramifications or tendrils. Complete colony expansion, with a brownish color at the colony center and white tendrils, was observed at 10 DAI in GA_{gr} and 15 DAI in GM_{gr} (Fig. 2A and B). Considering that doubling times were approximately 20–22 h (Fig. 1A), complete colony expansion required approximately 11 doubling times in GA_{gr}, and 16 doubling times in GM_{gr}. The value obtained in GA_{gr} was similar to those observed in fast-growing rhizobia (Nogales et al., 2012), where the doubling time was approximately 2–3 h, requiring 20 h for colony expansion (7–10 doubling times), and in *Salmonella*, with a doubling time of 30 min and 3–4 h required for colony expansion (Kim and Surette, 2004), i.e., 6–8 doubling times.

To observe swarming cells, we took samples from the colony center and from the edges of tendrils, fixed them in situ, and stained them for use in transmission electron microscopy (TEM). In general, LP 3008 had more flagella than LP 3004 (Fig. 2C–F), correlating with the wider expansion of LP 3008 colonies. Both flagella were clearly observed in LP 3004 and LP 3008 with GA_{gr}. Although filament width was difficult to measure due to filament association in bundles, the sinusoidal (lateral) and relaxed (subpolar) flagella were distinguishable. The lateral flagellum was mainly observed in bundles and was more abundant in LP 3008 (Fig. 2D and F). To our surprise, few flagella were observed in LP 3004 swarming in GM_{gr}, correlating with undetectable production of lateral flagellins in liquid GM (Figs. 1C and 2C). This observation suggested that synthesis of lateral flagella was also poor in GM_{gr} swarming plates.

The morphology of bacteria taken from the edges of tendrils sharply contrasted with that of bacteria taken from the colony centers (Fig. 2G–J). These cells had fewer flagella and were, in general, not associated with one another. In contrast, bacteria from tendrils were associated in different ways. In some instances, polar associations were observed, especially notable in those grown in D-mannitol (Fig. 2C and D).

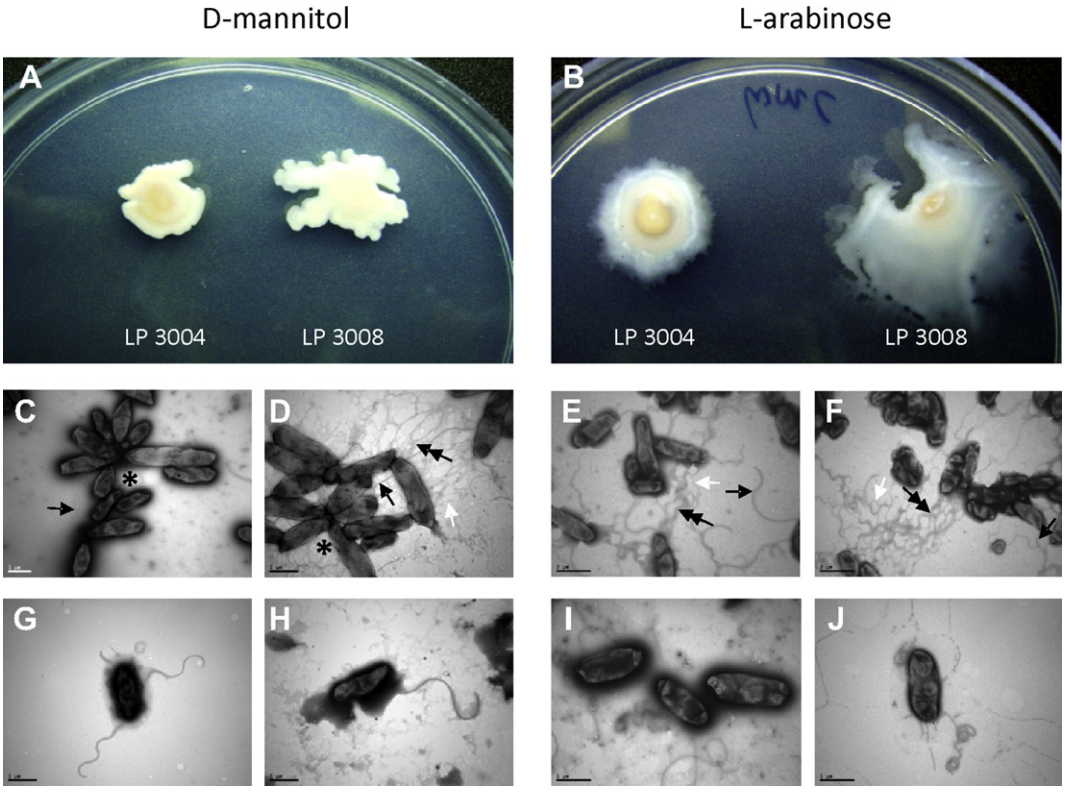

Fig. 2. Swarming of *B. japonicum* LP 3004 and LP 3008 with D-mannitol or L-arabinose. **A** and **B**: Macroscopic swarming in Petri dishes with Götz media with agar 0.5% and the indicated carbon sources. **C–J**: Electron micrographs of LP 3004 (**C**, **E**, **G**, and **I**) and LP 3008 (**D**, **F**, **H**, and **J**) taken from the border (**C–F**) or the center (**G–J**) of swarming colonies grown in Götz media with D-mannitol (**C**, **D**, **G**, and **H**) or L-arabinose (**E**, **F**, **I**, and **J**) as sole carbon source. Black arrows: subpolar flagella; white arrows: lateral flagella; double arrows: flagella associated in bundles; asterisks: polar associations of cells. Scale bars are at the bottom left corner of each micrograph.

Furthermore, the cells from tendrils tended to be twice as long as cells from colony centers (Fig. 2C–J).

3.4. Swarming of flagellin deletion mutants

Previously, we obtained deletion mutants in *fliC1234* and *lafA12* genes and corroborated the absence of the respective flagella by TEM (Althabegoiti et al., 2011). In addition, we observed that these mutants have the same growth rate in liquid Götz medium as the parental strains, and in solid medium, the time to the onset of colonies as well as their growth rate were also similar. Here we used these mutants to observe the possible requirement of each flagellum for swarming in GA_{gr}.

As shown in Fig. 3, there were clear differences between each mutant and its parental strain. The double mutants lacking both flagella did not move at all (Fig. 3A and D), while any mutant with only one flagellum always moved less than its parental strain (Fig. 3B, C, E, and F). In particular, mutants LP 6865 and LP 6866 (Δ*lafA12*), which lacked the lateral flagellum, showed almost no movement (Fig. 3B and E).

By comparing these results with our previous observations of the same mutants swimming in 0.3% agar, we observed that swarming was more severely affected by these mutations. In fact, mutants lacking the lateral flagellum produced swimming halos with diameters approximately 60% that of the wild-type, while those lacking the subpolar flagellum produced swimming halos 70–90% that of the wild-type size (Althabegoiti et al., 2011). Thus, these mutants might allow partial dissection of swarming and swimming in soil.

3.5. Contribution of swarming to displacement in soil

In view of the results obtained in the preceding section, we used parental strain LP 3008 and its derivatives to assess the contribution of swarming to rhizobial movement in soil, since visualization of swarming in LP 3008 was easier, leading to more pronounced differences in swarming with the mutants (Fig. 3). In this study, we included mutants LP 6644 (Δ*fliC1234*Δ*lafA12*), disabled for both swimming and swarming, LP 6866 (Δ*lafA12*), which showed 60% swimming in soft agar with respect to LP 3008 and was impaired in swarming, and LP 5844 (Δ*fliC1234*), which showed 90% swimming in soft agar with respect to LP 3008 and was defective in swarming (Althabegoiti et al., 2011, Fig. 3).

We evaluated the movement in soil through a modification of a method previously reported (Vilain et al., 2006). We prepared Petri dishes with solid AG (1.5% agar) into which 1.4-cm-diameter holes were punched. The holes were filled with known amounts of sterile soil, which was watered either at 100% or 80% field capacity with sterile double-distilled water (for further details, see Materials and methods). Then, 2 µl of bacteria from a late-logarithmic liquid AG culture was applied to the center of the soil sample and incubated at 28 °C in an upright position. Bacterial growth at the point of inoculation on the soil was noticeable at 6 DAI, while growth was not observed in uninoculated control soil tablets (Fig. 4). Flagellar-driven movement from the site of inoculation at the center of the soil tablet to its edges was demonstrated by growth observed at the soil-AG interface with all strains except LP 6644 (Δ*fliC1234*Δ*lafA12*). Growth at 6 DAI in the soil–AG interface produced by motile rhizobia was observed in soil at 80% and 100% field capacity. In both field capacities, LP 3008 and LP 5844 (Δ*fliC1234*) grew to similar extents at the soil–AG interface, while LP 6866 (Δ*lafA12*) grew less well at 6 and 7 DAI (Fig. 4). Growth at the soil–AG interface was more noticeable at 9 DAI (Fig. 4). The similar abilities of LP 3008 (swimming- and swarming-proficient) and LP 5844 (swimming-proficient and swarming-defective) to reach the soil–AG interface, as well as the capacity of LP 6866 (swimming mildly deficient and swarming impaired) to do the same suggested that swimming was the predominant movement in soil at both water contents.

4. Discussion

In the present work, we observed that LP 3004 and LP 3008, two *B. japonicum* strains derived from USDA 110,

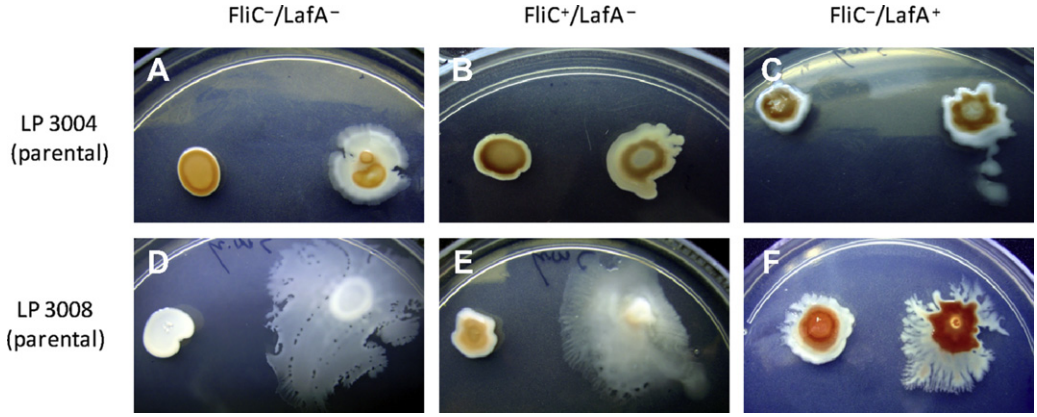

Fig. 3. Requirement of each flagellum for swarming in Götz medium with L-arabinose as sole carbon source. Upper panel: LP 3004-derived mutants LP 6543 (**A**), 6865 (**B**) and 5843 (**C**) at the left of each plate; parental strain LP 3004 at the right of each plate. Lower panel: LP 3008-derived mutants LP 6644 (**D**), 6866 (**E**) and 5844 (**F**) at the left of each plate; parental strain LP 3008 at the right of each plate. The flagellar phenotypes are indicated at the top of each series of photographs.

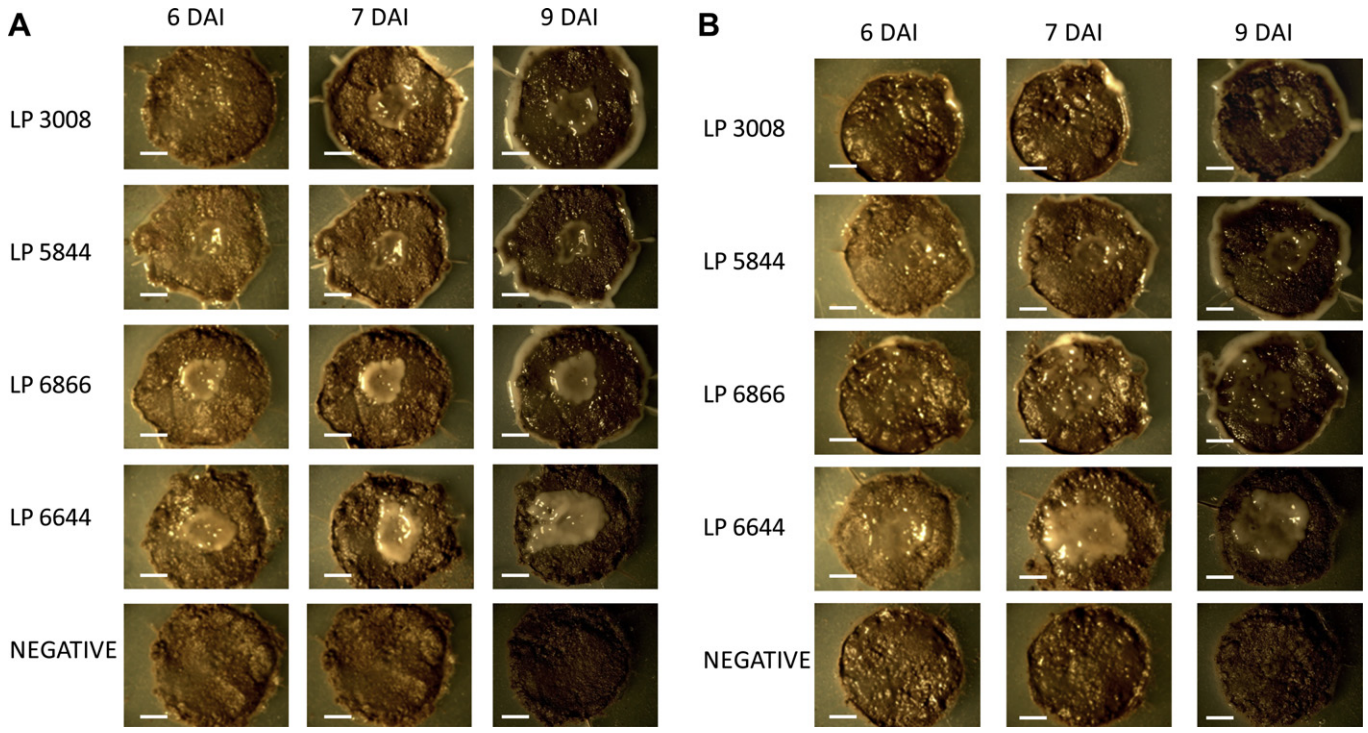

Fig. 4. Motility of LP 3008 and derived mutants LP 5844 (FliC$^-$/LafA$^+$), LP 6866 (FliC$^+$/LafA$^-$), and LP 6644 (FliC$^-$/LafA$^-$) in soil at 80% (**A**) or 100% (**B**) field capacity. The soil tablets included in solid (1.5% agar) AG medium were inoculated with 2 μl of bacteria in the center of each tablet and growth was registered at the indicated DAI. Note growth at the center of all inoculated soil tablets and growth at the AG–soil interfaces only in soil tablets inoculated with LP 3008, LP 5844 and LP 6866. Negative: non-inoculated controls. Scale bars: 3 mm. Supplementary Fig. 2 shows another set of Petri dishes without magnification, to appreciate the general aspect of the experiment.

moved on 0.5% agar surfaces producing ramified colonies in which bacterial cells were associated in groups, were larger in size and had more flagella than planktonic cells. In addition, mutants lacking flagella were non-motile in these conditions. Taken together, these results indicate that the type of movement observed here is swarming, as was previously characterized in other rhizobial species (Braeken et al., 2008; Daniels et al., 2006; Nogales et al., 2010, 2012; Soto et al., 2002; Tambalo et al., 2010). Interestingly, our swarming colonies often developed a brownish color at their centers, which was never observed in plate colonies in 1.5% agar. This pigmentation seemed to indicate a different physiological state at the colony centers than in the white tendrils. Kim and Surette (2004) demonstrated that different physiological requirements exist for swarming initiation and expansion in *Salmonella*, with initiation being the most stringent step.

The rate of swarming colony expansion could not be explained by an influence of the carbon source on growth rate, but did correlate with the influence of a carbon source on lateral flagella production. These results suggest that the lateral flagella play a major role in swarming, as expected from the proposed function of these flagella in other species (Kirov, 2003; Merino et al., 2006; Merino and Tomás, 2009). Results from mutant strains deleted in one or another flagellin support the role of LafA1-2 in swarming, but show that the subpolar flagella are also required, as observed in *fliC1234* deletion mutants. These results are similar to those reported by McClain et al. (2002), who found that *R. centenum* requires both flagella for swarming.

In contrast to most species that possess dual flagellar systems, *B. japonicum* lateral flagella may be synthesized in response to the carbon source either in agitated liquid or semisolid medium, but did not seem to be stimulated by cell contact with a surface in the presence of the non-inducing carbon source. Since *lafA1* and *lafA2* mRNAs were detected in GA and GM, but the corresponding polypeptides were observed only in GA, control exerted by the carbon source upon LafA1-2 synthesis seems to be at the level of translation. In *B. melitensis* and *Caulobacter crescentus*, translation of flagellin genes is regulated by FlbT, which interacts with the 5′ untranslated region of flagellin mRNA (Anderson and Gober, 2000; Ferooz et al., 2011). However, the role of FlbT seems contrary in these two species: it is an activator of flagellin mRNA translation in *B. melitensis* (Ferooz et al., 2011) and an inhibitor in *C. crescentus* (Anderson and Gober, 2000). The lateral flagellum operons of *B. japonicum* are evolutionarily related to *B. melitensis*, while those of the subpolar flagellum are akin to *C. crescentus* (Liu and Ochman, 2007). Since two copies of putative *flbT* exist in the *B. japonicum* genome (Kanbe et al., 2007), it is plausible that each copy controls translation of each set of flagellins, but we cannot anticipate the putative role of each *B. japonicum* FlbT as an activator or inhibitor, nor the possible influence of the carbon source in this control.

Both carbon sources tested here are chemoattractants and are present in plant root exudates (Badri and Vivanco, 2009; Barbour et al., 1991). Thus, there is no obvious advantage in differentially

regulating LafA1-2 synthesis in response to one of these carbon sources. On the other hand, these carbon sources are catabolized by different pathways in *B. japonicum* (Kuykendall and Elkan, 1977; Pedrosa and Zancan, 1974) leading to different respiration rates, with L-arabinose being more efficient than D-mannitol (Thorne and Burris, 1940). Since lateral flagella are moved by an H^+ proton gradient (Kanbe et al., 2007), a proton-motive force that is shared with ATP synthase, it is plausible that H^+ availability instead of the carbon source itself might be the signal for LafA1-2 translation, although more work is required to experimentally support this possibility.

After several trials, we found that the set of experimental conditions enabling observation of swarming in *B. japonicum* are very restricted, similar to previous reports in other species. This restriction argues against the robustness of the swarming phenotype (Stelling et al., 2004) and sheds doubts on its significance in nature. To evaluate the possible role of swarming in soil, we inoculated the LP 3008 strain and its mutant derivatives in 1.4 cm diameter soil tablets and recorded bacterial displacement toward the tablet edges. We observed flagellum-driven movement that was more compatible with swimming than with swarming, since mutant LP 6866, impaired in swarming but capable of swimming, reached the border of soil tablets at a significant rate. Moreover, mutant LP 5844, which has only lateral flagella, reached the border of soil tablets faster than LP 6866. These observations were made both at 100% and 80% field capacity. LP 5844 cells tumble at higher frequency than LP 6866 (Althabegoiti et al., 2011), which may allow better displacement of LP 5844 cells within the tortuous water channels of the soil (Wolfe and Berg, 1989). This tortuosity increases as soil water content decreases because of loss of gravitational water from the macropores; therefore, displacement of *B. japonicum* by swimming in this environment might be favored in cells expressing the lateral flagella. In this scenario, it makes sense that the lateral flagellar expression responds to the bacterial respiration rate, since O_2 availability increases as soil water content decreases. However, a role for swarming in the establishment of biofilms on soil biotic or abiotic surfaces cannot be discarded. Further dedicated experiments are required to clarify these important aspects of the *B. japonicum* life cycle in its natural environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resmic.2012.10.014.

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