Post-print Symbiosis (2015) 67: 79-90

Characterization of surface motility in *Sinorhizobium meliloti*: regulation and role in symbiosis

Lydia Bernabéu-Roda, Nieves Calatrava-Morales, Virginia Cuéllar, María J. Soto.

Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Profesor Albareda 1, 18008 Granada, Spain.

Corresponding author: María J. Soto mariajose.soto@eez.csic.es

Phone: +34-958181600. Ext. 150

Fax: +34-958181609

ACKNOWLEDGMENTS

This work was supported by grants BIO2010-18005 and BIO2013-42801-P from the Ministerio de Economía y Competitividad (Spain), the Excellence Project P08-CVI-03541 from the Consejería de Innovación, Ciencia y Empresa (Junta de Andalucía, Spain) and FEDER funds. We are grateful to J. Nogales for her participation in the construction of GR4flaAB. LBR was supported by a Junta de Andalucía contract and NCM by grant FPU12/01006 from the Ministerio de Educación, Cultura y Deporte (Spain).

Abstract *Sinorhizobium meliloti* can exhibit diverse modes of surface translocation whose manifestation depends on the strain. The mechanisms involved and the role played by the different modes of surface motility in the establishment of symbiosis are largely unknown. In this work, we have characterized the surface motility shown by two *S. meliloti* reference strains (Rm1021 and GR4) under more permissive conditions for surface spreading and analyzed the symbiotic properties of two flagella-less *S. meliloti* mutants with different behavior on surfaces. The use of Noble agar in semisolid minimal medium induces surface motility in GR4, a strain described so far as non-motile on surfaces. The motility exhibited by GR4 is swarming as revealed by the non-motile phenotype of the flagella-less *flaAB* mutant. Intriguingly, a *flgK* mutation which also abolishes flagella production, triggers surface translocation in GR4 through an as yet unknown mechanism. In contrast to GR4, Rm1021 moves over surfaces using mostly a flagella-independent motility which is highly reliant on siderophore rhizobactin 1021 production. Surprisingly, this motility is absent in a flagella-less *flgE* mutant. In addition, we found that *fadD* loss-of-function, known to promote surface motility in *S. meliloti*, exerts different effects on the two reference strains: while *fadD* inactivation promotes a flagella-independent type of motility in GR4, the same mutation interferes with the surface translocation exhibited by the Rm1021 *flaAB* mutant. The symbiotic phenotypes shown by GR4flaAB and GR4flgK, non-flagellated mutants with opposite surface motility behavior, demonstrate that flagella-dependent motility positively influences competitiveness for nodule occupation, but is not crucial for optimal infectivity.

Keywords: Rhizobium**,** swarming, flagella, nodulation, infectivity, competitiveness

1 Introduction

The ability to move provides bacteria with a competitive advantage allowing them to occupy environmental niches that are optimal for growth and survival. Bacteria can develop different types of motility, but only two require the presence of bacterial flagella: swimming and swarming. The first occurs as an individual movement in liquid media or environments with low agar concentrations (0.2-0.4%), while swarming takes place over semisolid surfaces (0.4-1.2%) as a social spreading phenomenon. Besides swarming, bacteria can also translocate over surfaces by twitching and sliding. While twitching motility is mediated by the extension and retraction of type IV pili, sliding is a passive surface translocation promoted by bacterial growth and facilitated by compounds that reduce friction between cells and surfaces (Henrichsen 1972; Harshey 2003; Kearns 2010).

Rhizobia are motile bacteria that can exist either as free-living organisms in the soil or in symbiotic association with their host legume plants in which they induce the formation of nitrogen-fixing root nodules, a process that is the result of a complex and continuous molecular dialogue between bacteria and the host plant that co-ordinates bacterial infection with nodule organogenesis (Oldroyd and Downie 2008). It is generally accepted that motility is not essential for nodulation or nitrogen fixation although it is thought to be important in the initial stages of the symbiosis by directing bacteria to the proper infection sites which could impact bacterial infectivity and competitiveness. In agreement with this, different studies performed with non-flagellated, non-motile or non-chemotactic rhizobial strains, showed that these bacteria were less competitive and less efficient in nodulation (Ames and Bergman 1981; Soby and Bergman 1983; Mellor et al. 1987; Caetano-Anollés et al. 1988, 1992; Yost et al. 1998; Fujishige et al. 2006; Miller et al. 2007). However, most of these results were obtained using genetically undefined mutants defective in swimming motility.

Sinorhizobium meliloti, the alfalfa endosymbiont, has been used as a model organism for the study of several aspects of swimming motility and chemotaxis since they substantially differ from the *Escherichia coli* paradigm, such as the mode of flagellar rotation, signal processing, and gene regulation (Scharf and Schmitt 2002). Recently, it has been shown that *S. meliloti* can translocate over semisolid surfaces by employing different mechanisms depending on the strain. Strains carrying a functional *expR* gene (encoding the transcriptional regulator of the ExpR/Sin quorum sensing system) exhibit an unusual quorum-sensing-controlled spreading (or surfing) which is mostly driven by physical/chemical effects

created by the secreted exopolysaccharide EPS II (Gao et al. 2012; Dilanji et al. 2014). ExpR-deficient strains like Rm1021 can also translocate over surfaces. Rm1021 moves over surfaces of semisolid minimal medium (0.6% purified Pronadisa agar) using at least two different types of motility. One type is flagellum-independent surface spreading or sliding, which is dependent on the siderophore rhizobactin 1021 (Nogales et al. 2010, 2012). The second type of surface translocation shown by *S. meliloti* Rm1021 is swarming, dependent on flagellar action and positively influenced by exopolysaccharides (Nogales et al. 2012). Under the same experimental conditions used to test the surface motility of Rm1021, strain GR4 which is also ExpR deficient but unable to produce rhizobactin 1021, behaves as non-motile (Soto et al. 2002). Nevertheless, inactivation of the *fadD* gene, which codes for a long-chain fatty acyl Coenzyme A ligase, promotes swarming motility on semisolid minimal medium in GR4 and increases the surface translocation exhibited by Rm1021 (Soto et al. 2002; Nogales et al. 2010). The molecular mechanism by which the lack of FadD induces surface motility is still unknown. Among environmental signals, iron has been identified as a cue playing an important role in controlling surface-associated phenotypes in *S. meliloti*. High iron conditions inhibit surface motility while promoting biofilm formation (Nogales et al. 2010; Amaya-Gómez et al. 2015).

Except for the above mentioned information, genetic determinants and regulatory mechanisms controlling surface motility in *S. meliloti* as well as the role played by the different modes of surface translocation in the establishment of symbiosis are largely unknown. To gain insights into these issues, we have characterized the surface translocation shown by the two ExpR$^-$ deficient *S. meliloti* reference strains Rm1021 and GR4 by analyzing the behavior of a set of genetically defined mutants under new and more permissive conditions for surface spreading. Our results unveil the complexity of surface motility in *S. meliloti* and suggest the existence of flagella-independent modes of surface translocation which seem to be controlled in a co-ordinated manner with the stage of flagellar assembly. In addition, the availability of isogenic flagella-less *S. meliloti* mutants with opposite surface motility phenotypes, allowed us to investigate the impact on rhizobial symbiotic characteristics of both flagellar-mediated motility as well as the flagella-independent motility exhibited by an *flgK* mutant.

2 Materials and methods

2.1 Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al. 1989) at 37ºC; *S. meliloti* strains were grown at 30ºC in complex tryptone yeast (TY) medium (Beringer 1974), Bromfield medium (BM) (0.04% tryptone, 0.01% yeast extract, and 0.01% $CaCl_2 \cdot 2H_2O$) or in minimal medium (MM) containing glutamate (6.5 mM), mannitol (55 mM), mineral salts (1.3 mM K_2HPO_4, 2.2 mM $KH_2PO_4 \cdot 3H_2O$, 0.6 mM $MgSO_4 \cdot 7H2O$, 0.34 mM $CaCl_2 \cdot 2H_2O$, 0.022 mM $FeCl_3 \cdot 6H_2O$, 0.86 mM NaCl), and vitamins (0.2 mg/liter biotin, 0.1 mg/liter calcium pantothenate). When required, antibiotics were added at the following final concentrations: 50 µg ml^{-1} streptomycin (Sm), 100 µg ml^{-1} spectinomycin (Sp), 50 µg ml^{-1} kanamycin (Km) and 200 µg ml^{-1} ampicillin (Ap) for *E. coli*; 200 µg ml^{-1} streptomycin, 100 µg ml^{-1} spectinomycin, 75 µg ml^{-1} hygromycin (Hyg), 120 µg ml^{-1} neomycin (Nm) and 200 µg ml^{-1} kanamycin for *S. meliloti*. To improve reproducibility, all liquid cultures of *S. meliloti* were routinely initiated from glycerol stocks.

2.2 Construction of *S.meliloti* strains.

Phage ΦM12 transduction (Finan et al. 1984) was employed to transfer mutations among Rm1021-derivative strains in the following manners (i) The *flaA flaB* mutant 1021fadDflaAB was obtained by transferring the Δ*flaA flaB*::Hyg mutation from the strain 1021F to 1021FDCSS. (ii) The 1021fadDrhbD mutant was obtained by transferring the *fadD*::SmSp mutation from 1021FDCSS strain to 1021rhbD. (iii) The 1021flgE mutant was obtained by transferring the *flgE*::mini-Tn*5* mutation from strain Sm2B5005 to Rm1021. For the construction of *flaA flaB* mutants in GR4 derivative strains, a 3.300-bp *Bgl*II fragment containing the *flaA flaB*::Hyg mutation from strain Rm11601 was first cloned into pUC18 to yield plasmid pUCflaAB::Hyg, and then subcloned as a *Hind*III fragment into vector pk18*mobsacB* to yield plasmid pK18flaAB::Hyg. This plasmid was introduced into GR4 and GR4FDCSS via conjugation with *E. coli* strain S17-1, and allele replacement events were selected as described previously (Schäfer et al. 1994). For the construction of the GR4flgk mutant, the *Eco*RI fragment from plasmid pUCGNS5 containing an *flgK*::Tn*5* insertion was subcloned into pSUP202 to yield plasmid pSUP202flgK. This plasmid was introduced into GR4 via conjugation with *E. coli* strain S17-1 and kamamycin resistant mutants were selected. All mutants constructed in this work were checked by PCR and Southern hybridation with specific probes.

2.3 Motility assays.

Surface motility of *S. meliloti* strains was assayed essentially as described in Soto et al. (2002) with the only difference of using 0.6% Noble Agar Difco (BD) instead of purified agar (Pronadisa). Briefly, *S. meliloti* cells grown in TY broth to late logarithmic phase ($O.D._{600nm}$ = 1-1.2) were pelleted, washed twice in MM and resuspended in 0.1 volume of the latter medium. Two µl aliquots of this bacterial suspension (ca. 2 x 10^7cells) were dispensed and allowed to dry for 10 minutes onto the surface of plates containing 20 ml semisolid MM previously air dried at room temperature for 15 min. Plates were incubated at 30ºC for 24 h or 48 h. Surface migration was determined as the average length of the two sides of a rectangle able to exactly frame each colony. Swimming motility was examined on plates prepared with BM containing 0.3% Bacto agar and inoculated with 3-µl droplets of rhizobial cultures grown in TY (optical density at 600 nm [$OD_{600\ nm}$]=1) and incubated at 30ºC for 2 days. For swimming tests, migration zone was determined as the colony diameter in millimeters after 48 hours incubation.

2.4 Plant assays.

Surface sterilization and germination of alfalfa (*Medicago sativa L. cv. Aragón*) seeds were performed as described previously (Olivares et al. 1980). To test the infectivity and competitive ability of each strain, seedlings were grown in hydroponic cultures under axenic conditions either in glass tubes or in Leonard jars containing nitrogen-free nutrient solution (Olivares et al. 1980). When glass tubes were used, 10 day-old plants (one plant per tube) were inoculated with 1 ml of a rhizobial suspension containing approximately 10^6 cells. When Leonard jars were used, 10 seedlings per jar were placed equidistantly from the center and immediately inoculated by applying 5 ml of a rhizobial suspension containing $5x10^2$ cells to the center of the jar. The rhizobial suspension used in each case contained either a single rhizobial strain (for infectivity assays) or a 1:1 mixture of the strain to be tested and GR4(pGUS3) (for competitiveness assays). Prior to inoculation, bacteria were grown to exponential phase (OD_{600} = 0.5-0.6) in TY broth and diluted accordingly in sterile water to reach the desired concentration of cells. After inoculation, the nodule formation efficiency or infectivity of each rhizobial strain was determined by counting the number of nodules developed by a total of 20 alfalfa plants. These data were recorded either daily (in the case of plants grown in glass tubes) or 14 and 28 days after inoculation (in the case of plants grown in Leonard jars). To determine nodule occupancy by competing strains, roots were collected either 15 or 21 days after inoculation depending on the system used to grow the plants (glass tubes or Leonard jars, respectively), briefly washed with water and incubated overnight in the dark at 37ºC in 1mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide; Apollo Scientific) in 50 mM sodium phosphate buffer (pH

7.5) with 1% SDS. Those nodules occupied by the strain harboring the pGUS3 plasmid stained blue, so nodule occupancy could be determined by counting blue and white nodules. Alfalfa shoot dry weight was determined after drying the shoots of 28 day-old plants at 70ºC for 2 days.

2.5 Transmission electron microscopy (TEM).

Cells for TEM observations were obtained from the edge of swarming colonies. Carbon-coated Formvar grids were placed for 5 minutes on top of a drop of water previously applied to the colony border. The grids were then washed twice in water for 1 min and stained with 2% (w/v) uranyl acetate for 3 min. Grids were allowed to air dry for at least 1 hour and visualized using a JEOL JEM-1011 transmission electron microscope with a 100 kV beam at the Microscopy Service of the Estación Experimental del Zaidín, Granada, Spain. Images were captured using an Orius Gatan charge-coupled-device (CCD) camera.

3 Results

3.1 Noble agar as gelling agent in semisolid minimal medium facilitates surface migration in *S. meliloti*

As described in the Introduction section, surface motility of ExpR deficient *S. meliloti* strains has been observed on surfaces of semisolid minimal medium prepared with Purified Agar (Pronadisa) (hereafter MM_{PA}). However, migration under these conditions is rather limiting and for some strains like GR4, conditions are so restrictive that they prevent bacterial translocation (Soto et al. 2002; Nogales et al. 2010, 2012). With the aim of identifying more permissive conditions that allow surface motility in *S. meliloti* GR4 and based on the knowledge that the concentration and type of agar are crucial factors influencing this surface behavior, standard surface motility tests (Soto et al. 2002) were performed using MM containing different gelling agents at varying concentrations. It was found that semisolid MM prepared with Noble Agar (Difco, BD) (hereafter MM_{NA}) at 0.6% and 0.8% permitted surface spreading of strain GR4 (Fig. 1a). On MM_{NA} (0.6%) GR4 was able to colonize most of the surface of the plate after 48 h of incubation. Under the new conditions, surface translocation of strain Rm1021 also improved as revealed by the profuse surface colonization exhibited on MM_{NA} 0.6, 0.8% and even some on MM_{NA} 1% (Fig. 1b). In agreement with our previous reports (Soto et al. 2002; Nogales et al. 2010) strain Rm1021 exhibited better surface motility than strain GR4. The magnitude of surface propagation exhibited by Rm1021 on

MM_{NA} 1% and the fact that the migration zone did not increase with time, together with the non-motile phenotype shown by strain GR4 in this medium resembled previous observations made on MM_{PA} 0.6%. Given that MM_{NA} 0.6% permitted surface translocation of strain GR4 while maintaining differences in surface translocation with Rm1021, we decided to use these new experimental conditions to characterize the motilities exhibited by these two reference strains.

3.2 GR4 moves on surfaces using swarming but inactivation of the *flgK* gene triggers a flagella-independent translocation

To characterize the surface translocation exhibited by strain GR4 under the new conditions, the behavior on semisolid surfaces of two mutants GR4flaAB and GR4flgK was tested. GR4flaAB is unable to produce the principal flagellin subunit FlaA as well as FlaB, one of the other three accessory subunits which are required to form the complex *S. meliloti* flagellar filament (Scharf et al. 2001). GR4flgK contains a Tn*5* insertion that interrupts the *flgK* gene annotated as potentially coding for a hook-filament junction protein. As expected, both mutants lacked flagella (Fig. 2) and did not show swimming motility (Fig. 3a). However, when surface motility tests were performed, a different behavior was observed. GR4flaAB did not disperse beyond the inoculation drop even after 48 h of incubation, indicating the inability of the mutant to move (Fig. 4a). This result demonstrates that the translocation exhibited by GR4 is dependent on flagellar action, i. e. GR4 moves on surfaces using swarming motility. Although lack of surface motility was the phenotype observed for GR4flaAB in most of the experiments, occasionally, colonies of this mutant exhibited some degree of surface translocation although the surface area colonized was smaller than that of GR4 (spreading mutant colonies exhibited migration zones of 13.7 ± 0.5 mm and 55.2 ± 1.8 mm after 24 h and 48 h incubation, respectively). Cells present in these spreading colonies of GR4flaAB lacked flagella and when re-inoculated in new motility tests they showed the same heterogeneous behavior as the initial inoculum: with the usual exceptions, most of the colonies were non-motile (data not shown). This suggests that the motility occasionally associated with GR4flaAB is not the result of genetic changes. At present, we do not know the reasons for this reversion of phenotype. Interestingly, and in contrast to GR4flaAB, the flagella-less GR4flgK mutant always exhibited surface motility on semisolid MM plates with distinctive characteristics from the motility exhibited by the wild type such as the macroscopic appearance and smaller extension of the migration zone (Fig. 4a).

3.3 Inactivation of *fadD* in *S. meliloti* GR4 stimulates surface spreading by promoting a flagella-independent translocation

In line with our previous published data, inactivation of *fadD* promoted GR4 surface translocation also under the new and more permissive conditions for surface motility. Twenty four hours after incubation, GR4FDCSS colonized a surface area 35% bigger than the wild-type strain (Fig. 4b). No differences in surface area colonization were appreciated after 48 hours of incubation most likely due to limitations of the set up (the wt strain colonizes the entire surface plate area after 48 h). As in the case of GR4, to characterize the surface translocation exhibited by the *fadD* mutant, the behavior on semisolid MM plates of GR4fadD *flaAflaB* and *flgK* mutants was analyzed. In agreement with being flagella-less strains, GR4fadDflaAB and GR4fadDflgK were defective in swimming motility (Fig. 3b). In this case, the extensive surface spreading shown by the flagella-less GR4fadDflaAB strain on semisolid MM indicated that the absence of a functional *fadD* gene in GR4 favors mostly a flagella-independent motility. Only 14% of the displacement shown by GR4FDCSS strain can be attributed to flagellar action. Surprisingly, once more, the effect on surface translocation caused by inactivating the *flgK* gene in a *S. meliloti* strain (in this case GR4FDCSS) was different from that caused by a mutation that prevents from building the flagellar filament (*flaAflaB* mutation). The reduced surface spreading shown by the GR4fadDflgK strain compared to that shown by the GR4fadDflaAB (Fig. 4b) could be interpreted as the *flgK* mutation negatively interfering with the flagella-independent movement characteristic of a GR4 *fadD* mutant. On the other hand, by comparing the surface migration exhibited by GR4flgK (Fig. 4a) and GR4fadDflgK (Fig. 4b), one can conclude that inactivation of *fadD* facilitates the flagella-independent translocation that takes place in the GR4flgK strain.

3.4 *S. meliloti* Rm1021 translocates on semisolid MM using mostly a flagella-independent type of motility which is highly dependent on siderophore production.

In our previous studies in which surface motility tests were conducted on semisolid MM_{PA}, we concluded that the ExpR deficient strain Rm1021 could move over semisolid surfaces using at least two different types of motility: swarming and a flagella-independent translocation, both of which were highly dependent on siderophore rhizobactin 1021 production (Nogales et al. 2012). The limited surface spreading shown by the different strains under the experimental conditions used in those studies hampered the analysis of the contribution of each type of motility to the overall Rm1021 motility. With

the availability of more permissive conditions for *S. meliloti* surface motility identified in this work, we decided to re-evaluate the surface translocation exhibited by Rm1021 by characterizing the behavior on semisolid MM surfaces of different Rm1021 derivative mutants: two flagella-less mutants, 1021F lacking flagellin FlaA and FlaB subunits and 1021flgE affected in hook protein synthesis, and the mutant 1021rhbD, unable to produce siderophore. As expected for strains devoid of flagella, 1021F and 1021flgE behaved as non-motile in swimming motility assays, while clear swimming halos of similar size could be observed for Rm1021 and the siderophore 1021rhbD mutant (Fig. 5a). The wide surface spreading shown by the flaAflaB mutant (1021F) on semisolid MM indicated that, in contrast to GR4 that moves using swarming motility, Rm1021 translocates on semisolid surfaces using mostly a flagella-independent motility (Fig. 6a). By comparing the surface area colonized by Rm1021 and 1021F, it is deduced that swarming motility represents only 13% of the translocation exhibited by Rm1021. On the other hand, the non-motile phenotype shown by the siderophore 1021rhbD mutant on semisolid MM confirms our previous finding about the crucial role that rhizobactin 1021, a particular iron chelator with surfactant properties, plays on Rm1021 motility (Nogales et al. 2010, 2012). Surprisingly, and as occurred in the GR4 genetic background, a different behavior on semisolid surfaces was observed between the two flagella-less Rm1021 derivative mutants. While 1021F colonized the surface of the semisolid MM plate almost as efficiently as the wild-type strain Rm1021, 1021flgE was highly impaired in surface translocation. This result indicates that the stage in which flagellar assembly is blocked influences the surface spreading behavior of Rm1021.

3.5 Inactivation of *fadD* hampers surface spreading of Rm1021 *flaAflaB* mutant while promoting a flagella-independent translocation in a siderophore mutant

Based on motility tests performed on semisolid MM_{PA}, we reported that a mutation in *fadD* promotes surface motility not only in the GR4 strain but also in Rm1021 (Nogales et al. 2010). Results obtained in this study using MM_{NA} did not show differences concerning the magnitude of surface translocation exhibited by Rm1021 or its *fadD* mutant (Fig. 6a and b). However, striking differences were found between the corresponding *flaA flaB* mutants (1021F and 1021fadDflaAB, respectively). The significant decrease in surface motility and surface area colonization shown by 1021fadDflaAB compared to 1021F and 1021FDCSS indicates that: i) FadD loss-of-function negatively interferes with surface spreading of a Rm1021 *flaA flaB* mutant and ii) in contrast to Rm1021, which translocates on semisolid surfaces using mostly a flagella-independent motility, flagellar action is a major contributor of the surface motility

exhibited by 1021FDCSS. This is in agreement with the observed up-regulation of flagellar gene expression in 1021FDCSS in response to growth on semisolid MM surfaces (Nogales et al. 2010). Contrasting with the effects caused by *fadD* inactivation in 1021F, the same mutation had no significant effects on the limited surface translocation exhibited by mutant 1021flgE, indicating that in Rm1021 the *flgE* mutation is epistatic.

On the other hand and in agreement with our published data, motility tests performed on the new semisolid MM demonstrate that the lack of a functional *fadD* gene partially restores surface motility in the Rm1021 siderophore mutant (1021rhbD) (Fig. 6b). Additionally, we show that the surface translocation promoted by inactivating the *fadD* gene is mediated by a flagella-independent mechanism as revealed by the similar surface colonization shown by 1021fadDrhbD and its flagella-less derivative mutant 1021fadDrhbDflaAB. Furthermore, by comparing the phenotypes exhibited by 1021fadDflaAB and 1021fadDrhbDflaAB we can suggest that although rhizobactin 1021 is not crucial for surface translocation of Rm1021 *fadD* mutant, the siderophore impacts this phenotype by both increasing the distance covered by cells of the colony and altering its morphology to a characteristic dendritic pattern.

3.6 Symbiotic characteristics of flagella-less GR4 mutants with opposite surface motility behavior

The role of flagella and flagella-mediated motility in the symbiotic performance of rhizobial strains has not been seriously investigated. Less information is available about the role played by the different types of bacterial surface translocation on the establishment of symbiosis with the plant host. As a first approach to investigate these issues, in this study we analyzed the symbiotic phenotypes shown by two isogenic flagella-less mutants with opposite surface motility behavior: GR4flaAB which is nonmotile on surfaces and GR4flgK that spreads on semisolid surfaces by an as yet unknown mechanism. Differences in the symbiotic phenotypes of the two mutant strains relative to the wild-type strain would be attributed to the lack of flagella, whereas differences observed between the two mutant strains could be the result of the ability/inability to move on surfaces. GR4flaAB and GR4flgK were able to induce nitrogen-fixing root nodules on alfalfa plants. When symbiotic tests were performed in glass tubes, no significant differences were detected either in infectivity or competitive ability between the mutants and the wild-type strain GR4 (data not shown). In these assays, a high density of inoculum is applied next to the roots, which could make bacterial motility an unnecessary trait for optimal symbiotic performance. However, when we performed the symbiotic assays using Leonard jars, in which an inoculum of lower cell density

was applied at certain distance from the plant roots, we found that GR4flaAB exhibited a less efficient nodulation phenotype at the end of the experiment than the wild-type strain (Fig. 7a) whereas GR4flgK behaved as infective as GR4 throughout the trial (Fig. 7b). The reduction in nodule number observed in plants inoculated with GR4flaAB compared to that of plant inoculated with GR4 correlated with reductions in alfalfa shoot dry weight (34 mg and 48 mg per plant, respectively). This result could suggest that, as generally accepted, flagella contribute to nodule formation efficiency of the rhizobial strain but optimal infectivity can also be achieved by flagella-less mutants that exhibit some kind of surface translocation as is the case for GR4flgK strain. Interestingly, the two flagella-less mutants showed a significant reduction in competitive ability of about 33% compared to the wild-type strain GR4 (Fig.7b) indicating that flagella and/or flagella-dependent motility positively influences competitiveness of *S. meliloti* strains.

4 Discussion

The ability to move across surfaces is a strictly regulated bacterial trait that can impact the interaction with a host. Different modes of surface translocation have been reported for different *S. meliloti* strains, but the mechanisms involved, their regulation as well as their role in the establishment of symbiosis have not yet been deeply investigated. To gain insights into these issues, in this study we have characterized the behavior on surfaces exhibited by the reference strains GR4 and Rm1021 and their *fadD*-derivative mutants under more permissive conditions for surface translocation. Both strains lack a functional ExpR, the quorum sensing transcriptional regulator required for exopolysaccharide EPS II production, and therefore cannot exhibit the EPS II-dependent surfing motility described for *expR*$^+$ strains (Gao et al. 2012; Dilanji et al. 2014). At the initiation of this study, surface motility assays performed on semisolid minimal medium (MM_{PA}) demonstrated that whereas GR4 behaved as non-motile, Rm1021 could translocate over surfaces using two types of motility which were highly dependent on siderophore rhizobactin 1021 production: swarming that requires flagellar action, and a flagellum-independent surface spreading or sliding (Nogales et al. 2010, 2012). It was also reported that inactivation of *fadD*, a gene involved in lipid metabolism, promoted surface translocation in the two *S. meliloti* strains by a yet unknown mechanism (Soto et al. 2002; Nogales et al. 2010). In the present study, we found that motility assays performed on semisolid MM (0.6%) in which purified agar (Pronadisa) was replaced with Noble Agar (Difco, BD) permitted surface spreading of GR4 and improved the surface propagation of Rm1021. Rm1021 colonized a larger surface area than GR4, which was in agreement with our previous

observations indicating that Rm1021 is more efficient in surface translocation than GR4. Moreover, under the new experimental conditions to test surface motility, we found that the inactivation of *fadD* promoted surface translocation in GR4 but not in Rm1021. Perhaps, the promoting effect of the *fadD* mutation on Rm1021 surface translocation observed earlier on MM_{PA} might be masked under the new experimental conditions due to the overall increased motility of this strain.

The phenotype exhibited by mutants unable to produce a functional flagellar filament (*flaA flaB* mutants) was sufficient to unveil the different mechanisms used by GR4, Rm1021 and their corresponding *fadD* mutants to propagate over surfaces. It was found that whereas GR4 moves using swarming motility, Rm1021 translocates on semisolid surfaces using mostly a flagella-independent motility that is facilitated by the siderophore rhizobactin 1021. These results clearly point at GR4 rather than Rm1021 as the *S. meliloti* strain of choice for characterizing swarming motility. Our experiments also revealed that whereas the inactivation of *fadD* in GR4 increases surface translocation by promoting mostly a flagella-independent motility, in Rm1021 FadD loss-of-function has the opposite effect, negatively interfering with the flagella-independent translocation exhibited by a *flaAflaB* mutant. These results clearly demonstrate that although wild-type reference *S. meliloti* strains rely on different components to move over surfaces, i.e. flagella in GR4 and rhizobactin 1021 in Rm1021, the lack of FadD activity impacts surface behavior in both strains. At present, we do not know the exact mechanism by which these effects are exerted and we can only speculate. In *S. meliloti*, FadD codes for a fatty acyl-CoA ligase that allows the utilization of exogenous and endogenous long chain fatty acids via their activation with CoA (Pech-Canul et al. 2011). In culture, *S. meliloti fadD* mutants accumulate free fatty acids during stationary phase (Pech-Canul et al. 2011), and fatty acids and fatty acid-related signals are known to influence motility in different bacteria (Ryan and Dow 2011; Winans 2011). Inactivation of *fadD* in GR4 could lead to changes in lipid-related compounds that could facilitate the spreading of cells over the surface without the need for flagellar action. In Rm1021, the crucial role played by rhizobactin 1021 (a hydroxamate-type siderophore containing a lipid moiety) in the surface motility exhibited by this strain, suggests the possibility that the *fadD* mutation could have some effect on the siderophore structure, altering perhaps its surfactant properties. On the other hand and in agreement with our previous results, we have shown here that the inactivation of *fadD* partially restores the inability to move over surfaces exhibited by the Rm1021 siderophore mutant. In addition, in this work we demonstrate that this effect is exerted by promoting a flagella-independent mode of translocation which is less effective in covering distances but more

effective in surface area colonization than that shown by the siderophore producing but flagella-less *fadD* mutant 1021fadDflaAB. Thus, there is a close link between the effect that the *fadD* mutation generates on the surface motility exhibited by flaAflaB Rm1021 mutants and their ability to produce rhizobactin 1021 that awaits further investigation.

One of the most unexpected findings in our study was highlighted with the opposite surface behaviors exhibited by different flagella-less mutants which were derived from the same genetic background. Thus, in contrast to the non-motile phenotype exhibited by the GR4 *flaA flaB* mutant on semisolid surfaces, the flagella-less GR4 *flgK* mutant, under the same conditions, displayed surface translocation. Likewise, whereas the Rm1021 *flaA flaB* mutant showed extensive surface translocation, the flagella-less Rm1021 *flgE* mutant was highly impaired in surface motility. Theoretically, *flaA flaB* and *flgK/E* mutants are blocked in different stages of the flagellar assembly (flagellar filament and hook, respectively). Our results suggest that, regardless of the mechanism involved, flagella-independent translocation in *S. meliloti* seems to be regulated in a co-ordinated manner with the stage of flagellar assembly. There are examples in literature in which bacterial phenotypes have been shown to be under coordinated control with the state of flagellar assembly. For example, the expression of late flagellar genes in enterobacteria requires the completion of the flagellar hook-basal body (Smith and Hoover 2009), and the expression of some virulence factors in bacteria such as *Salmonella*, *Proteus* or *Yersinia*, has been linked to the flagellar apparatus (Allison et al. 1992; Young et al. 1999; Iyoda et al. 2001; Duan et al. 2013). Interestingly, the synthesis of a swarming-enabling surfactant in *Pseudomonas syringae* pv syringae (Pss) is coordinated with flagellar assembly: mutations in genes involved in early flagellar assembly abolish or reduce surfactant production, while mutations in flagellin genes increase its production (Burch et al. 2012). Coordinating surfactant production and flagellar assembly was suggested to help swarming by lubricating flagella and/or the surface. The behavior of Rm1021 flagella-less mutants resemble in some ways the phenotypes described for Pss mutants. Rhizobactin 1021 is crucial for Rm1021 surface motility and its amphipatic structure confers surfactant activity to the siderophore. Could Rhizobactin 1021 production be under coordinated control with flagellar assembly? We could not detect differences in the CAS activity present in Rm1021, 1021flaAB and 1021flgE cultures. However, this analysis does not rule out the possibility of changes in siderophore structure that could impact its surfactant activity. Therefore, coordinated control of flagellar assembly with the production of Rhizobactin 1021 or any other surfactant in Rm1021 warrants further investigation.

Finally, to gain insights about the role that *S. meliloti* flagella and/or flagellar mediated motility as well as flagella-independent surface translocation play in the interaction with alfalfa plants, the symbiotic phenotype of two GR4 flagella-less mutants which display opposite surface motility behaviors was assessed and compared to that of the wild-type strain. Our results confirm that motility is a dispensable trait for nodulation and nitrogen fixation. On the other hand, the lower competitive ability exhibited by the two flagella-less mutants compared to the wild-type strain, demonstrate that flagella-dependent motility positively influences the competitiveness for nodule occupation most likely by enabling bacteria to rapidly reach proper infection sites. However, the fact that the flagella-less GR4 *flgK* mutant behaves as effective as the wild-type strain in nodule formation, indicates that flagella and/or flagellar mediated motility are not essential for infectivity and suggest a role for some kind of flagellar-independent motility in this symbiotic trait that might be relevant under field conditions.

In summary, identification of more permissive conditions for surface tranlocation and the use of a set of isogenic mutants unveiled the complexity of *S. meliloti* surface motility and suggests the existence of flagella-independent modes of surface translocation which seem to be controlled in a co-ordinated manner with the stage of flagellar assembly. In addition, the symbiotic phenotypes exhibited by two non-flagellated GR4-derivative mutants with different capacities to spread over surfaces indicate that flagella positively influence the competitiveness for nodule occupation but they are not essential for optimal bacterial infectivity. Flagella-independent modes of translocation that remain to be characterized at the molecular level could play important roles at certain stages of the symbiotic interaction.

REFERENCES

Allison C, Lai HC, Hughes C (1992) Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. Mol Microbiol 6: 1583-1591

Amaya-Gómez CV (2013) Transcriptomic approach for the identification of genes and signals palying a role in swarming motility of *Sinorhizobium meliloti*: connection with biofilm formation and symbiosis. Doctoral Thesis, University of Granada

Amaya-Gómez CV, Hirsch AM, Soto MJ (2015) Biofilm formation assessment in *Sinorhizobium meliloti* reveals interlinked control with surface motility. BMC Microbiol 15: 8. doi: 10.1186/s12866-015-0390-z

Ames P. and Bergman K (1981) Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. J Bacteriol 148: 728-908

Bahlawane C, McIntosh M, Krol E, Becker A (2008) *Sinorhizobium meliloti* regulator MucR couples exopolysaccharide synthesis and motility. Mol Plant Microbe Interact 21:1498-1509. doi: 10.1094/MPMI-21-11-1498

Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84: 188-198

Bernabéu-Roda L (2014) Bases moleculares que gobiernan la movilidad *swarming* en *Sinorhizobium meliloti:* conexión con formación de biopelículas y establecimiento de simbiosis. Tesis Doctoral, Universidad de Granada

Burch AY, Shimada BK, Mullin SW, Dunlap CA, Bowman MJ, Lindow SE (2012) *Pseudomonas syringae* coordinates production of a motility-enabling surfactant with flagellar assembly. J Bacteriol 194: 1287-1298. doi: 10.1128/JB.06058-11

Caetano-Anollés G, Crist-Estes DK, Bauer WD (1988) Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J Bacteriol 170: 3164-3169

Caetano-Anollés G, Wrobel-Boerner E, Bauer WD (1992) Growth and movement of spot inoculated *Rhizobium meliloti* on the root surface of alfalfa. Plant Physiol 98: 1181-1189

Casadesús J, Olivares J (1979) Rough and fine linkage mapping of the *Rhizobium meliloti* chromosome. Mol Gen Genet 174: 203-209

Dilanji GE, Teplitski M, Hagen SJ (2014) Entropy-driven motility of *Sinorhizobium meliloti* on a semi-solid surface. Proc Biol Sci 281: 20132575. doi: 10.1098/rspb.2013.2575

Duan Q, Zhou M, Zhu L, Zhu G (2013) Flagella and bacterial pathogenicity. J Basic Microbiol 53: 1-8. doi:10.1002/jobm.201100335

Finan TM, Hartweig E, LeMieux K, Bergman K, Walker GC, Signer ER (1984) General transduction in *Rhizobium meliloti*. J Bacteriol 159: 120-124

Fujishige NA, Kapadia NN, De Hoff PL, Hirsch AM (2006) Investigations of Rhizobium biofilm formation. FEMS Microbiol Ecol. 56:195-206

Gao M, Coggin A, Yagnik K, Teplitski M. (2012) Role of specific quorum-sensing signals in the regulation of exopolysaccharide II production within *Sinorhizobium meliloti* spreading colonies. PLoS One 7: e42611. doi: 10.1371/journal.pone.0042611

García-Rodríguez FM, Toro N (2000) *Sinorhizobium meliloti* nfe (nodulation formation efficiency) genes exhibit temporal and spatial expression patterns similar to those of genes involved in symbiotic nitrogen fixation. Mol Plant Microbe Interact 13: 583-591

Gurich N, González JE (2009) Role of quorum sensing in *Sinorhizobium meliloti*-alfalfa symbiosis. J Bacteriol 191: 4372-4382. doi: 10.1128/JB.00376-09

Harshey RM (2003) Bacterial motility on a surface: many ways to a common goal. Annu Rev Microbiol 57: 249-273. doi: 10.1128/JB.00376-09

Henrichsen J (1972) Bacterial surface translocation: a survey and a classification. Bacteriol. Rev 36: 478-503

Iyoda S, Kamidoi T, Hirose K, Kutsukake K, Watanabe H (2001) A flagellar gene *fliZ* regulates the expression of invasion genes and virulence phenotype in *Salmonella enterica* serovar Typhimurium. Microb Pathog 30: 81-90

Kearns DB (2010) A field guide to bacterial swarming motility. Nat Rev Microbiol 8: 634-644. doi: 10.1038/nrmicro2405

Meade HM, Signer ER (1977) Genetic mapping of *Rhizobium meliloti*. Proc Natl Acad Sci U S A 74: 2076-2078

Mellor HY, Glenn A R, Arwas R (1987) Symbiotic and competitive properties of motility mutants of Rhizobium trifolii TA1. Arch Microbiol 148: 34-39

Miller LD, Yost CK, Hynes MF, Alexandre G (2007) The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. viciae is essential for competitive nodulation. Mol Microbiol 63: 348-362

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. doi: 10.1186/1471-2164-11-157

Nogales J, Bernabéu-Roda L, Cuéllar V, Soto MJ (2012) ExpR is not required for swarming but promotes sliding in *Sinorhizobium meliloti*. J Bacteriol 194:2027-35. doi: 10.1128/JB.06524-11

Oldroyd GE and Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59:519-46. doi: 10.1146/annurev.arplant.59.032607.092839

Olivares J, Casadesús J, Bedmar EJ (1980) Method for testing degree of infectivity of *Rhizobium meliloti* strains. Appl Environ Microbiol 39:967-970

Pech-Canul A, Nogales J, Miranda-Molina A, Alvarez L, Geiger O, Soto MJ, Lopez-Lara IM (2011) *FadD* is required for utilization of endogenous fatty acids released from membrane lipids. J Bacteriol 193:6295-304. doi: 10.1128/JB.05450-11.

Ryan RP and Dow JM (2011) Communication with a growing family: diffusible signal factor (DSF) signaling in bacteria. Trends Microbiol 19:145-52. doi: 10.1016/j.tim.2010.12.003

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press

Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69-73

Scharf B, Schuster-Wolff-Buhring H, Rachel R, Schmitt R (2001). Mutational analysis of the *Rhizobium lupini* H13-3 and *Sinorhizobium meliloti* flagellin genes: importance of flagellin A for flagellar filament structure and transcriptional regulation. J Bacteriol 183:5334-5342

Scharf B and Schmitt R (2002) Sensory transduction to the flagellar motor of *Sinorhizobium meliloti.* J Mol Microbiol Biotechnol 4:183-6

Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram- negative bacteria. Nat Biotechnol 1:784-791. doi:10.1038/nbt1183-784.

Smith TG and Hoover TR (2009) Deciphering bacterial flagellar gene regulatory networks in the genomic era. Adv Appl Microbiol 67: 257-295. doi: 10.1016/S0065-2164(08)01008-3.

Soby S and Bergman K (1983) Motility and chemotaxis of *Rhizobium meliloti* in soil. Appl Environ Microbiol 46:995-998

Soto MJ, Fernández-Pascual M, Sanjuán J, Olivares J (2002) A *fadD* mutant of *Sinorhizobium meliloti* shows multicellular swarming migration and is impaired in nodulation efficiency on alfalfa roots. Mol Microbiol 43: 371-382

Winans SC (2011) A new family of quorum sensing pheromones synthesized using S-adenosylmethionine and Acyl-CoAs. Mol Microbiol 79:1403-6. doi: 10.1111/j.1365-2958.2011.07551.x

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119

Yost CK, Rochepeau P, Hynes MF (1998) *Rhizobium leguminosarum* contains a group of genes that appear to code for methyl-accepting chemotaxis proteins. Microbiol 144: 1945-1956

Young GM, Schmiel DH, Miller VL (1999) A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. Proc Natl Acad Sci USA 96: 6456-6461

Figure Captions

Fig. 1 Surface motility of *S. meliloti* strains **a** GR4 and **b** Rm1021 on minimal medium (MM) containing different concentrations of Noble agar. Graphs represent the average of surface migration (given in millimeters and determined as the average length of the two sides of a rectangle able to exactly frame each colony) obtained in each medium after 24 and 48 hours of incubation at 30ºC. Means and standard deviations were obtained from at least 3 replicates in three independent experiments (n=9). Below each graph, representative pictures of the motilities exhibited by each strain on MM containing different concentrations of agar are shown

Fig. 2 Transmission electron microscope images of *S. meliloti* **a** GR4 (wild-type), and its flagella-less derivative mutants **b** GR4flaAB and **c** GR4flgK. Cells were isolated from the edge of colonies grown on semisolid MM 0.6% Noble agar after 20 hours of incubation at 30ºC and stained with 2% uranyl acetate

Fig. 3 Swimming motility in BM 0.3% agar of *S. meliloti* **a** GR4 and **b** GR4 *fadD* mutant (GR4FDCSS) and their corresponding flagella-less *flaAflaB* and *flgK* derivative mutants. Pictures were taken after 48 hours of incubation at 30ºC

Fig. 4 Surface motility on semisolid MM (0.6% Noble agar) of *S. meliloti* **a** GR4 and **b** GR4 *fadD* mutant (GR4FDCSS) and their corresponding flagella-less *flaAflaB* and *flgK* derivative mutants. Representative pictures of the motility exhibited by each strain after 24 and 48 hours of incubation at 30ºC are shown. Values represent the mean and the standard error of the surface migration (given in millimeters and determined as the average length of the two sides of a rectangle able to exactly frame each colony) obtained from at least three independent experiments with at least three technical replicates

Fig. 5 Swimming motility in BM 0.3% agar of **a** Rm1021 and **b** Rm1021 *fadD* mutant (1021FDCSS), and their flagella-less and siderophore defective derivative mutants. Pictures were taken after 48 hours of incubation at 30ºC

Fig. 6 Surface motility on semisolid MM (0.6% Noble agar) of *S. meliloti* **a** Rm1021 and **b** Rm1021 *fadD* mutant (1021FDCSS) and their corresponding flagella-less (*flaAflaB* and *flgE*) and siderophore defective derivative mutants. Representative pictures of the motility exhibited by each strain after 24 and 48 hours of incubation at 30ºC are shown. Values represent the mean and the standard error of the migration zone (given in millimeters and determined as the average length of the two sides of a rectangle able to exactly

frame each colony) obtained from at least three independent experiments with at least three technical replicates

Fig. 7 Symbiotic phenotype of non-flagellated GR4 derivative mutants. **a** Infectivity test. Nodule formation efficiency of GR4 (wt) and flagella-less mutants GR4flaAB and GR4flgK expressed as the average number of nodules present in alfalfa roots 14 and 28 days after inoculation. **b** Competitive nodulation assays. Each strain was mixed with marked GR4(pGUS3) at a ratio of 1:1. After inoculating alfalfa roots with these mixtures, the number of nodules occupied by each strain was estimated (see experimental procedures for details). Values are the mean and standard errors obtained in at least three independent experiments. Same letter indicates no significant differences according to ANOVA test ($p \leq 0.05$)

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
***S. meliloti* strains**		
GR4	Wild type	Casadesús and Olivares 1979
GR4flaAB	GR4 *flaA flaB;*Hyg^r	This work
GR4flgK	GR4 *flgK*::Tn*5*; Km^r	This work
GR4FDCSS	GR4 Δ*fadD*::SmSp; Sm^r Sp^r	Amaya-Gómez et al. 2015
GR4fadDflaAB	GR4FDCSS *flaA flaB;*Sm^r Sp^r Hyg^r	This work
GR4fadDflgK	GR4FDCSS *flgK*::Tn*5*; Sm^r Sp^r Km^r	Bernabéu-Roda 2014
Rm1021	SU47 *expR*102::IS*Rm*2011-1; Sm^r.	Meade and Signer 1977
1021F	Rm1021 *flaA flaB::*Hyg;Sm^r Hyg^r	Nogales et al. 2012
1021flgE	Rm1021 *flgE*::mini-Tn*5*; Sm^r Neo^r	This work
1021rhbD	Rm1021 Δ*rhbD;* Sm^r	Amaya-Gómez 2013
1021FDCSS	Rm1021 Δ*fadD*::SmSp; Sm^r Sp^r	Nogales et al. 2010
1021fadDflaAB	1021FDCSS *flaA flaB;*Sm^r Sp^r Hyg^r	This work
1021fadDflgG	1021FDCSS *flgG*::Tn*5*; Sm^r Sp^r Km^r	Bernabéu-Roda 2014
1021fadDrhbD	1021FDCSS Δ*rhbD;* Sm^r Sp^r	This work
1021fadDrhbDflaAB	1021fadDrhbD *flaA flaB::*Hyg;Sm^r Sp^r Hyg^r	This work
Rm11601	Rm8530 *flaA flaB*; Sm^r, Hyg^r	Gurich and González 2009
Sm2B5005	Sm2B3001 *flgE*::mini-Tn*5*; Sm^r Neo^r	Bahlawane et al. 2008
***E. coli* strains**		
DH5α	*sup*E44 *Δlac*U169Φ80 *LacZ*ΔM1 *rec*A1 *end*A1 *gyr*A96 *thi*1 *rel*A1 *5hsd*R171	Bethesda Research Lab
S17-1	*thi pro rec*A *hsd*R, *hsd;* Rp4Tc::Mu, Km::Tn*7*; Tmp^r, Sm^r, Spc^r	Simon et al. 1983
Plasmids		
pUC18	Cloning vector; Ap^r	Yanisch-Perron et al. 1985
pUCGNS5	pUC18 derivative carrying the genomic *Eco*RI fragment containing the *flgK*::Tn*5* insertion from GR4fadDflgK. Ap^r, Km^r	Bernabéu-Roda 2014
pSUP202	Suicide plasmid; Ap^r, Tc^r, Cm^r	Simon et al. 1983
pSUP202flgK	pSUP202 derivative carrying the *Eco*RI fragment from pUCGNS5.Ap^r, Tc^r, Km^r	This work
pUCflaAB::Hyg	pUC18 derivative carrying a *Bgl*II fragment containing the *flaA flaB*::Hyg mutation from Rm11601. Ap^r, Hyg^r	This work
pK18*mobsacB*	Suicide plasmid; Km^r	Schäfer et al. 1994
pK18flaAB::Hyg	pK18*mobsacB* derivative carrying the insert from pUCflaAB::Hyg.	This work
pGUS3	Plasmid which contains an *nfeD::gusA* fusion. Km^r.	García-Rodríguez and Toro 2000

[a] Hyg^r, Km^r, Sm^r, Sp^r, Neo^r, Tmp^r, Ap^r, Tc^r and Cm^r indicate hygromycin, kanamycin, streptomycin, spectinomycin, neomycin, trimethoprim, ampicylin, tetracyclin and chloramphenychol resistance, respectively.

Abstract *Sinorhizobium meliloti* can exhibit diverse modes of surface translocation whose manifestation depends on the strain. The mechanisms involved and the role played by the different modes of surface motility in the establishment of symbiosis are largely unknown. In this work, we have characterized the surface motility shown by two *S. meliloti* reference strains (Rm1021 and GR4) under more permissive conditions for surface spreading and analyzed the symbiotic properties of two flagella-less *S. meliloti* mutants with different behavior on surfaces. The use of Noble agar in semisolid minimal medium induces surface motility in GR4, a strain described so far as non-motile on surfaces. The motility exhibited by GR4 is swarming as revealed by the non-motile phenotype of the flagella-less *flaAB* mutant. Intriguingly, a *flgK* mutation which also abolishes flagella production, triggers surface translocation in GR4 through an as yet unknown mechanism. In contrast to GR4, Rm1021 moves over surfaces using mostly a flagella-independent motility which is highly reliant on siderophore rhizobactin 1021 production. Surprisingly, this motility is absent in a flagella-less *flgE* mutant. In addition, we found that *fadD* loss-of-function, known to promote surface motility in *S. meliloti*, exerts different effects on the two reference strains: while *fadD* inactivation promotes a flagella-independent type of motility in GR4, the same mutation interferes with the surface translocation exhibited by the Rm1021 *flaAB* mutant. The symbiotic phenotypes shown by GR4flaAB and GR4flgK, non-flagellated mutants with opposite surface motility behavior, demonstrate that flagella-dependent motility positively influences competitiveness for nodule occupation, but is not crucial for optimal infectivity.

Keywords: Rhizobium**,** swarming, flagella, nodulation, infectivity, competitiveness

1 Introduction

The ability to move provides bacteria with a competitive advantage allowing them to occupy environmental niches that are optimal for growth and survival. Bacteria can develop different types of motility, but only two require the presence of bacterial flagella: swimming and swarming. The first occurs as an individual movement in liquid media or environments with low agar concentrations (0.2-0.4%), while swarming takes place over semisolid surfaces (0.4-1.2%) as a social spreading phenomenon. Besides swarming, bacteria can also translocate over surfaces by twitching and sliding. While twitching motility is mediated by the extension and retraction of type IV pili, sliding is a passive surface translocation promoted by bacterial growth and facilitated by compounds that reduce friction between cells and surfaces (Henrichsen 1972; Harshey 2003; Kearns 2010).

Rhizobia are motile bacteria that can exist either as free-living organisms in the soil or in symbiotic association with their host legume plants in which they induce the formation of nitrogen-fixing root nodules, a process that is the result of a complex and continuous molecular dialogue between bacteria and the host plant that co-ordinates bacterial infection with nodule organogenesis (Oldroyd and Downie 2008). It is generally accepted that motility is not essential for nodulation or nitrogen fixation although it is thought to be important in the initial stages of the symbiosis by directing bacteria to the proper infection sites which could impact bacterial infectivity and competitiveness. In agreement with this, different studies performed with non-flagellated, non-motile or non-chemotactic rhizobial strains, showed that these bacteria were less competitive and less efficient in nodulation (Ames and Bergman 1981; Soby and Bergman 1983; Mellor et al. 1987; Caetano-Anollés et al. 1988, 1992; Yost et al. 1998; Fujishige et al. 2006; Miller et al. 2007). However, most of these results were obtained using genetically undefined mutants defective in swimming motility.

Sinorhizobium meliloti, the alfalfa endosymbiont, has been used as a model organism for the study of several aspects of swimming motility and chemotaxis since they substantially differ from the *Escherichia coli* paradigm, such as the mode of flagellar rotation, signal processing, and gene regulation (Scharf and Schmitt 2002). Recently, it has been shown that *S. meliloti* can translocate over semisolid surfaces by employing different mechanisms depending on the strain. Strains carrying a functional *expR* gene (encoding the transcriptional regulator of the ExpR/Sin quorum sensing system) exhibit an unusual quorum-sensing-controlled spreading (or surfing) which is mostly driven by physical/chemical effects

created by the secreted exopolysaccharide EPS II (Gao et al. 2012; Dilanji et al. 2014). ExpR-deficient strains like Rm1021 can also translocate over surfaces. Rm1021 moves over surfaces of semisolid minimal medium (0.6% purified Pronadisa agar) using at least two different types of motility. One type is flagellum-independent surface spreading or sliding, which is dependent on the siderophore rhizobactin 1021 (Nogales et al. 2010, 2012). The second type of surface translocation shown by *S. meliloti* Rm1021 is swarming, dependent on flagellar action and positively influenced by exopolysaccharides (Nogales et al. 2012). Under the same experimental conditions used to test the surface motility of Rm1021, strain GR4 which is also ExpR deficient but unable to produce rhizobactin 1021, behaves as non-motile (Soto et al. 2002). Nevertheless, inactivation of the *fadD* gene, which codes for a long-chain fatty acyl Coenzyme A ligase, promotes swarming motility on semisolid minimal medium in GR4 and increases the surface translocation exhibited by Rm1021 (Soto et al. 2002; Nogales et al. 2010). The molecular mechanism by which the lack of FadD induces surface motility is still unknown. Among environmental signals, iron has been identified as a cue playing an important role in controlling surface-associated phenotypes in *S. meliloti*. High iron conditions inhibit surface motility while promoting biofilm formation (Nogales et al. 2010; Amaya-Gómez et al. 2015).

Except for the above mentioned information, genetic determinants and regulatory mechanisms controlling surface motility in *S. meliloti* as well as the role played by the different modes of surface translocation in the establishment of symbiosis are largely unknown. To gain insights into these issues, we have characterized the surface translocation shown by the two ExpR$^-$ deficient *S. meliloti* reference strains Rm1021 and GR4 by analyzing the behavior of a set of genetically defined mutants under new and more permissive conditions for surface spreading. Our results unveil the complexity of surface motility in *S. meliloti* and suggest the existence of flagella-independent modes of surface translocation which seem to be controlled in a co-ordinated manner with the stage of flagellar assembly. In addition, the availability of isogenic flagella-less *S. meliloti* mutants with opposite surface motility phenotypes, allowed us to investigate the impact on rhizobial symbiotic characteristics of both flagellar-mediated motility as well as the flagella-independent motility exhibited by an *flgK* mutant.

2 Materials and methods

2.1 Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al. 1989) at 37ºC; *S. meliloti* strains were grown at 30ºC in complex tryptone yeast (TY) medium (Beringer 1974), Bromfield medium (BM) (0.04% tryptone, 0.01% yeast extract, and 0.01% $CaCl_2 \cdot 2H_2O$) or in minimal medium (MM) containing glutamate (6.5 mM), mannitol (55 mM), mineral salts (1.3 mM K_2HPO_4, 2.2 mM $KH_2PO_4 \cdot 3H_2O$, 0.6 mM $MgSO_4 \cdot 7H2O$, 0.34 mM $CaCl_2 \cdot 2H_2O$, 0.022 mM $FeCl_3 \cdot 6H_2O$, 0.86 mM NaCl), and vitamins (0.2 mg/liter biotin, 0.1 mg/liter calcium pantothenate). When required, antibiotics were added at the following final concentrations: 50 µg ml^{-1} streptomycin (Sm), 100 µg ml^{-1} spectinomycin (Sp), 50 µg ml^{-1} kanamycin (Km) and 200 µg ml^{-1} ampicillin (Ap) for *E. coli*; 200 µg ml^{-1} streptomycin, 100 µg ml^{-1} spectinomycin, 75 µg ml^{-1} hygromycin (Hyg), 120 µg ml^{-1} neomycin (Nm) and 200 µg ml^{-1} kanamycin for *S. meliloti*. To improve reproducibility, all liquid cultures of *S. meliloti* were routinely initiated from glycerol stocks.

2.2 Construction of *S.meliloti* strains.

Phage ΦM12 transduction (Finan et al. 1984) was employed to transfer mutations among Rm1021-derivative strains in the following manners (i) The *flaA flaB* mutant 1021fadDflaAB was obtained by transferring the Δ*flaA flaB*::Hyg mutation from the strain 1021F to 1021FDCSS. (ii) The 1021fadDrhbD mutant was obtained by transferring the *fadD*::SmSp mutation from 1021FDCSS strain to 1021rhbD. (iii) The 1021flgE mutant was obtained by transferring the *flgE*::mini-Tn*5* mutation from strain Sm2B5005 to Rm1021. For the construction of *flaA flaB* mutants in GR4 derivative strains, a 3.300-bp *Bgl*II fragment containing the *flaA flaB*::Hyg mutation from strain Rm11601 was first cloned into pUC18 to yield plasmid pUCflaAB::Hyg, and then subcloned as a *Hind*III fragment into vector pk18*mobsacB* to yield plasmid pK18flaAB::Hyg. This plasmid was introduced into GR4 and GR4FDCSS via conjugation with *E. coli* strain S17-1, and allele replacement events were selected as described previously (Schäfer et al. 1994). For the construction of the GR4flgk mutant, the *Eco*RI fragment from plasmid pUCGNS5 containing an *flgK*::Tn*5* insertion was subcloned into pSUP202 to yield plasmid pSUP202flgK. This plasmid was introduced into GR4 via conjugation with *E. coli* strain S17-1 and kamamycin resistant mutants were selected. All mutants constructed in this work were checked by PCR and Southern hybridation with specific probes.

2.3 Motility assays.

Surface motility of *S. meliloti* strains was assayed essentially as described in Soto et al. (2002) with the only difference of using 0.6% Noble Agar Difco (BD) instead of purified agar (Pronadisa). Briefly, *S. meliloti* cells grown in TY broth to late logarithmic phase ($O.D._{600nm}$ = 1-1.2) were pelleted, washed twice in MM and resuspended in 0.1 volume of the latter medium. Two µl aliquots of this bacterial suspension (ca. 2 x 10^7cells) were dispensed and allowed to dry for 10 minutes onto the surface of plates containing 20 ml semisolid MM previously air dried at room temperature for 15 min. Plates were incubated at 30ºC for 24 h or 48 h. Surface migration was determined as the average length of the two sides of a rectangle able to exactly frame each colony. Swimming motility was examined on plates prepared with BM containing 0.3% Bacto agar and inoculated with 3-µl droplets of rhizobial cultures grown in TY (optical density at 600 nm [$OD_{600\ nm}$]=1) and incubated at 30ºC for 2 days. For swimming tests, migration zone was determined as the colony diameter in millimeters after 48 hours incubation.

2.4 Plant assays.

Surface sterilization and germination of alfalfa (*Medicago sativa L. cv. Aragón*) seeds were performed as described previously (Olivares et al. 1980). To test the infectivity and competitive ability of each strain, seedlings were grown in hydroponic cultures under axenic conditions either in glass tubes or in Leonard jars containing nitrogen-free nutrient solution (Olivares et al. 1980). When glass tubes were used, 10 day-old plants (one plant per tube) were inoculated with 1 ml of a rhizobial suspension containing approximately 10^6 cells. When Leonard jars were used, 10 seedlings per jar were placed equidistantly from the center and immediately inoculated by applying 5 ml of a rhizobial suspension containing $5x10^2$ cells to the center of the jar. The rhizobial suspension used in each case contained either a single rhizobial strain (for infectivity assays) or a 1:1 mixture of the strain to be tested and GR4(pGUS3) (for competitiveness assays). Prior to inoculation, bacteria were grown to exponential phase (OD_{600} = 0.5-0.6) in TY broth and diluted accordingly in sterile water to reach the desired concentration of cells. After inoculation, the nodule formation efficiency or infectivity of each rhizobial strain was determined by counting the number of nodules developed by a total of 20 alfalfa plants. These data were recorded either daily (in the case of plants grown in glass tubes) or 14 and 28 days after inoculation (in the case of plants grown in Leonard jars). To determine nodule occupancy by competing strains, roots were collected either 15 or 21 days after inoculation depending on the system used to grow the plants (glass tubes or Leonard jars, respectively), briefly washed with water and incubated overnight in the dark at 37ºC in 1mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide; Apollo Scientific) in 50 mM sodium phosphate buffer (pH

7.5) with 1% SDS. Those nodules occupied by the strain harboring the pGUS3 plasmid stained blue, so nodule occupancy could be determined by counting blue and white nodules. Alfalfa shoot dry weight was determined after drying the shoots of 28 day-old plants at 70ºC for 2 days.

2.5 Transmission electron microscopy (TEM).

Cells for TEM observations were obtained from the edge of swarming colonies. Carbon-coated Formvar grids were placed for 5 minutes on top of a drop of water previously applied to the colony border. The grids were then washed twice in water for 1 min and stained with 2% (w/v) uranyl acetate for 3 min. Grids were allowed to air dry for at least 1 hour and visualized using a JEOL JEM-1011 transmission electron microscope with a 100 kV beam at the Microscopy Service of the Estación Experimental del Zaidín, Granada, Spain. Images were captured using an Orius Gatan charge-coupled-device (CCD) camera.

3 Results

3.1 Noble agar as gelling agent in semisolid minimal medium facilitates surface migration in *S. meliloti*

As described in the Introduction section, surface motility of ExpR deficient *S. meliloti* strains has been observed on surfaces of semisolid minimal medium prepared with Purified Agar (Pronadisa) (hereafter MM_{PA}). However, migration under these conditions is rather limiting and for some strains like GR4, conditions are so restrictive that they prevent bacterial translocation (Soto et al. 2002; Nogales et al. 2010, 2012). With the aim of identifying more permissive conditions that allow surface motility in *S. meliloti* GR4 and based on the knowledge that the concentration and type of agar are crucial factors influencing this surface behavior, standard surface motility tests (Soto et al. 2002) were performed using MM containing different gelling agents at varying concentrations. It was found that semisolid MM prepared with Noble Agar (Difco, BD) (hereafter MM_{NA}) at 0.6% and 0.8% permitted surface spreading of strain GR4 (Fig. 1a). On MM_{NA} (0.6%) GR4 was able to colonize most of the surface of the plate after 48 h of incubation. Under the new conditions, surface translocation of strain Rm1021 also improved as revealed by the profuse surface colonization exhibited on MM_{NA} 0.6, 0.8% and even some on MM_{NA} 1% (Fig. 1b). In agreement with our previous reports (Soto et al. 2002; Nogales et al. 2010) strain Rm1021 exhibited better surface motility than strain GR4. The magnitude of surface propagation exhibited by Rm1021 on

MM_{NA} 1% and the fact that the migration zone did not increase with time, together with the non-motile phenotype shown by strain GR4 in this medium resembled previous observations made on MM_{PA} 0.6%. Given that MM_{NA} 0.6% permitted surface translocation of strain GR4 while maintaining differences in surface translocation with Rm1021, we decided to use these new experimental conditions to characterize the motilities exhibited by these two reference strains.

3.2 GR4 moves on surfaces using swarming but inactivation of the *flgK* gene triggers a flagella-independent translocation

To characterize the surface translocation exhibited by strain GR4 under the new conditions, the behavior on semisolid surfaces of two mutants GR4flaAB and GR4flgK was tested. GR4flaAB is unable to produce the principal flagellin subunit FlaA as well as FlaB, one of the other three accessory subunits which are required to form the complex *S. meliloti* flagellar filament (Scharf et al. 2001). GR4flgK contains a Tn*5* insertion that interrupts the *flgK* gene annotated as potentially coding for a hook-filament junction protein. As expected, both mutants lacked flagella (Fig. 2) and did not show swimming motility (Fig. 3a). However, when surface motility tests were performed, a different behavior was observed. GR4flaAB did not disperse beyond the inoculation drop even after 48 h of incubation, indicating the inability of the mutant to move (Fig. 4a). This result demonstrates that the translocation exhibited by GR4 is dependent on flagellar action, i. e. GR4 moves on surfaces using swarming motility. Although lack of surface motility was the phenotype observed for GR4flaAB in most of the experiments, occasionally, colonies of this mutant exhibited some degree of surface translocation although the surface area colonized was smaller than that of GR4 (spreading mutant colonies exhibited migration zones of 13.7 ± 0.5 mm and 55.2 ± 1.8 mm after 24 h and 48 h incubation, respectively). Cells present in these spreading colonies of GR4flaAB lacked flagella and when re-inoculated in new motility tests they showed the same heterogeneous behavior as the initial inoculum: with the usual exceptions, most of the colonies were non-motile (data not shown). This suggests that the motility occasionally associated with GR4flaAB is not the result of genetic changes. At present, we do not know the reasons for this reversion of phenotype. Interestingly, and in contrast to GR4flaAB, the flagella-less GR4flgK mutant always exhibited surface motility on semisolid MM plates with distinctive characteristics from the motility exhibited by the wild type such as the macroscopic appearance and smaller extension of the migration zone (Fig. 4a).

3.3 Inactivation of *fadD* in *S. meliloti* GR4 stimulates surface spreading by promoting a flagella-independent translocation

In line with our previous published data, inactivation of *fadD* promoted GR4 surface translocation also under the new and more permissive conditions for surface motility. Twenty four hours after incubation, GR4FDCSS colonized a surface area 35% bigger than the wild-type strain (Fig. 4b). No differences in surface area colonization were appreciated after 48 hours of incubation most likely due to limitations of the set up (the wt strain colonizes the entire surface plate area after 48 h). As in the case of GR4, to characterize the surface translocation exhibited by the *fadD* mutant, the behavior on semisolid MM plates of GR4fadD *flaAflaB* and *flgK* mutants was analyzed. In agreement with being flagella-less strains, GR4fadDflaAB and GR4fadDflgK were defective in swimming motility (Fig. 3b). In this case, the extensive surface spreading shown by the flagella-less GR4fadDflaAB strain on semisolid MM indicated that the absence of a functional *fadD* gene in GR4 favors mostly a flagella-independent motility. Only 14% of the displacement shown by GR4FDCSS strain can be attributed to flagellar action. Surprisingly, once more, the effect on surface translocation caused by inactivating the *flgK* gene in a *S. meliloti* strain (in this case GR4FDCSS) was different from that caused by a mutation that prevents from building the flagellar filament (*flaAflaB* mutation). The reduced surface spreading shown by the GR4fadDflgK strain compared to that shown by the GR4fadDflaAB (Fig. 4b) could be interpreted as the *flgK* mutation negatively interfering with the flagella-independent movement characteristic of a GR4 *fadD* mutant. On the other hand, by comparing the surface migration exhibited by GR4flgK (Fig. 4a) and GR4fadDflgK (Fig. 4b), one can conclude that inactivation of *fadD* facilitates the flagella-independent translocation that takes place in the GR4flgK strain.

3.4 *S. meliloti* Rm1021 translocates on semisolid MM using mostly a flagella-independent type of motility which is highly dependent on siderophore production.

In our previous studies in which surface motility tests were conducted on semisolid MM_{PA}, we concluded that the ExpR deficient strain Rm1021 could move over semisolid surfaces using at least two different types of motility: swarming and a flagella-independent translocation, both of which were highly dependent on siderophore rhizobactin 1021 production (Nogales et al. 2012). The limited surface spreading shown by the different strains under the experimental conditions used in those studies hampered the analysis of the contribution of each type of motility to the overall Rm1021 motility. With

the availability of more permissive conditions for *S. meliloti* surface motility identified in this work, we decided to re-evaluate the surface translocation exhibited by Rm1021 by characterizing the behavior on semisolid MM surfaces of different Rm1021 derivative mutants: two flagella-less mutants, 1021F lacking flagellin FlaA and FlaB subunits and 1021flgE affected in hook protein synthesis, and the mutant 1021rhbD, unable to produce siderophore. As expected for strains devoid of flagella, 1021F and 1021flgE behaved as non-motile in swimming motility assays, while clear swimming halos of similar size could be observed for Rm1021 and the siderophore 1021rhbD mutant (Fig. 5a). The wide surface spreading shown by the flaAflaB mutant (1021F) on semisolid MM indicated that, in contrast to GR4 that moves using swarming motility, Rm1021 translocates on semisolid surfaces using mostly a flagella-independent motility (Fig. 6a). By comparing the surface area colonized by Rm1021 and 1021F, it is deduced that swarming motility represents only 13% of the translocation exhibited by Rm1021. On the other hand, the non-motile phenotype shown by the siderophore 1021rhbD mutant on semisolid MM confirms our previous finding about the crucial role that rhizobactin 1021, a particular iron chelator with surfactant properties, plays on Rm1021 motility (Nogales et al. 2010, 2012). Surprisingly, and as occurred in the GR4 genetic background, a different behavior on semisolid surfaces was observed between the two flagella-less Rm1021 derivative mutants. While 1021F colonized the surface of the semisolid MM plate almost as efficiently as the wild-type strain Rm1021, 1021flgE was highly impaired in surface translocation. This result indicates that the stage in which flagellar assembly is blocked influences the surface spreading behavior of Rm1021.

3.5 Inactivation of *fadD* hampers surface spreading of Rm1021 *flaAflaB* mutant while promoting a flagella-independent translocation in a siderophore mutant

Based on motility tests performed on semisolid MM_{PA}, we reported that a mutation in *fadD* promotes surface motility not only in the GR4 strain but also in Rm1021 (Nogales et al. 2010). Results obtained in this study using MM_{NA} did not show differences concerning the magnitude of surface translocation exhibited by Rm1021 or its *fadD* mutant (Fig. 6a and b). However, striking differences were found between the corresponding *flaA flaB* mutants (1021F and 1021fadDflaAB, respectively). The significant decrease in surface motility and surface area colonization shown by 1021fadDflaAB compared to 1021F and 1021FDCSS indicates that: i) FadD loss-of-function negatively interferes with surface spreading of a Rm1021 *flaA flaB* mutant and ii) in contrast to Rm1021, which translocates on semisolid surfaces using mostly a flagella-independent motility, flagellar action is a major contributor of the surface motility

exhibited by 1021FDCSS. This is in agreement with the observed up-regulation of flagellar gene expression in 1021FDCSS in response to growth on semisolid MM surfaces (Nogales et al. 2010). Contrasting with the effects caused by *fadD* inactivation in 1021F, the same mutation had no significant effects on the limited surface translocation exhibited by mutant 1021flgE, indicating that in Rm1021 the *flgE* mutation is epistatic.

On the other hand and in agreement with our published data, motility tests performed on the new semisolid MM demonstrate that the lack of a functional *fadD* gene partially restores surface motility in the Rm1021 siderophore mutant (1021rhbD) (Fig. 6b). Additionally, we show that the surface translocation promoted by inactivating the *fadD* gene is mediated by a flagella-independent mechanism as revealed by the similar surface colonization shown by 1021fadDrhbD and its flagella-less derivative mutant 1021fadDrhbDflaAB. Furthermore, by comparing the phenotypes exhibited by 1021fadDflaAB and 1021fadDrhbDflaAB we can suggest that although rhizobactin 1021 is not crucial for surface translocation of Rm1021 *fadD* mutant, the siderophore impacts this phenotype by both increasing the distance covered by cells of the colony and altering its morphology to a characteristic dendritic pattern.

3.6 Symbiotic characteristics of flagella-less GR4 mutants with opposite surface motility behavior

The role of flagella and flagella-mediated motility in the symbiotic performance of rhizobial strains has not been seriously investigated. Less information is available about the role played by the different types of bacterial surface translocation on the establishment of symbiosis with the plant host. As a first approach to investigate these issues, in this study we analyzed the symbiotic phenotypes shown by two isogenic flagella-less mutants with opposite surface motility behavior: GR4flaAB which is nonmotile on surfaces and GR4flgK that spreads on semisolid surfaces by an as yet unknown mechanism. Differences in the symbiotic phenotypes of the two mutant strains relative to the wild-type strain would be attributed to the lack of flagella, whereas differences observed between the two mutant strains could be the result of the ability/inability to move on surfaces. GR4flaAB and GR4flgK were able to induce nitrogen-fixing root nodules on alfalfa plants. When symbiotic tests were performed in glass tubes, no significant differences were detected either in infectivity or competitive ability between the mutants and the wild-type strain GR4 (data not shown). In these assays, a high density of inoculum is applied next to the roots, which could make bacterial motility an unnecessary trait for optimal symbiotic performance. However, when we performed the symbiotic assays using Leonard jars, in which an inoculum of lower cell density

was applied at certain distance from the plant roots, we found that GR4flaAB exhibited a less efficient nodulation phenotype at the end of the experiment than the wild-type strain (Fig. 7a) whereas GR4flgK behaved as infective as GR4 throughout the trial (Fig. 7b). The reduction in nodule number observed in plants inoculated with GR4flaAB compared to that of plant inoculated with GR4 correlated with reductions in alfalfa shoot dry weight (34 mg and 48 mg per plant, respectively). This result could suggest that, as generally accepted, flagella contribute to nodule formation efficiency of the rhizobial strain but optimal infectivity can also be achieved by flagella-less mutants that exhibit some kind of surface translocation as is the case for GR4flgK strain. Interestingly, the two flagella-less mutants showed a significant reduction in competitive ability of about 33% compared to the wild-type strain GR4 (Fig.7b) indicating that flagella and/or flagella-dependent motility positively influences competitiveness of *S. meliloti* strains.

4 Discussion

The ability to move across surfaces is a strictly regulated bacterial trait that can impact the interaction with a host. Different modes of surface translocation have been reported for different *S. meliloti* strains, but the mechanisms involved, their regulation as well as their role in the establishment of symbiosis have not yet been deeply investigated. To gain insights into these issues, in this study we have characterized the behavior on surfaces exhibited by the reference strains GR4 and Rm1021 and their *fadD*-derivative mutants under more permissive conditions for surface translocation. Both strains lack a functional ExpR, the quorum sensing transcriptional regulator required for exopolysaccharide EPS II production, and therefore cannot exhibit the EPS II-dependent surfing motility described for *expR*$^{+}$ strains (Gao et al. 2012; Dilanji et al. 2014). At the initiation of this study, surface motility assays performed on semisolid minimal medium (MM_{PA}) demonstrated that whereas GR4 behaved as non-motile, Rm1021 could translocate over surfaces using two types of motility which were highly dependent on siderophore rhizobactin 1021 production: swarming that requires flagellar action, and a flagellum-independent surface spreading or sliding (Nogales et al. 2010, 2012). It was also reported that inactivation of *fadD*, a gene involved in lipid metabolism, promoted surface translocation in the two *S. meliloti* strains by a yet unknown mechanism (Soto et al. 2002; Nogales et al. 2010). In the present study, we found that motility assays performed on semisolid MM (0.6%) in which purified agar (Pronadisa) was replaced with Noble Agar (Difco, BD) permitted surface spreading of GR4 and improved the surface propagation of Rm1021. Rm1021 colonized a larger surface area than GR4, which was in agreement with our previous

observations indicating that Rm1021 is more efficient in surface translocation than GR4. Moreover, under the new experimental conditions to test surface motility, we found that the inactivation of *fadD* promoted surface translocation in GR4 but not in Rm1021. Perhaps, the promoting effect of the *fadD* mutation on Rm1021 surface translocation observed earlier on MM_{PA} might be masked under the new experimental conditions due to the overall increased motility of this strain.

The phenotype exhibited by mutants unable to produce a functional flagellar filament (*flaA flaB* mutants) was sufficient to unveil the different mechanisms used by GR4, Rm1021 and their corresponding *fadD* mutants to propagate over surfaces. It was found that whereas GR4 moves using swarming motility, Rm1021 translocates on semisolid surfaces using mostly a flagella-independent motility that is facilitated by the siderophore rhizobactin 1021. These results clearly point at GR4 rather than Rm1021 as the *S. meliloti* strain of choice for characterizing swarming motility. Our experiments also revealed that whereas the inactivation of *fadD* in GR4 increases surface translocation by promoting mostly a flagella-independent motility, in Rm1021 FadD loss-of-function has the opposite effect, negatively interfering with the flagella-independent translocation exhibited by a *flaAflaB* mutant. These results clearly demonstrate that although wild-type reference *S. meliloti* strains rely on different components to move over surfaces, i.e. flagella in GR4 and rhizobactin 1021 in Rm1021, the lack of FadD activity impacts surface behavior in both strains. At present, we do not know the exact mechanism by which these effects are exerted and we can only speculate. In *S. meliloti*, FadD codes for a fatty acyl-CoA ligase that allows the utilization of exogenous and endogenous long chain fatty acids via their activation with CoA (Pech-Canul et al. 2011). In culture, *S. meliloti fadD* mutants accumulate free fatty acids during stationary phase (Pech-Canul et al. 2011), and fatty acids and fatty acid-related signals are known to influence motility in different bacteria (Ryan and Dow 2011; Winans 2011). Inactivation of *fadD* in GR4 could lead to changes in lipid-related compounds that could facilitate the spreading of cells over the surface without the need for flagellar action. In Rm1021, the crucial role played by rhizobactin 1021 (a hydroxamate-type siderophore containing a lipid moiety) in the surface motility exhibited by this strain, suggests the possibility that the *fadD* mutation could have some effect on the siderophore structure, altering perhaps its surfactant properties. On the other hand and in agreement with our previous results, we have shown here that the inactivation of *fadD* partially restores the inability to move over surfaces exhibited by the Rm1021 siderophore mutant. In addition, in this work we demonstrate that this effect is exerted by promoting a flagella-independent mode of translocation which is less effective in covering distances but more

effective in surface area colonization than that shown by the siderophore producing but flagella-less *fadD* mutant 1021fadDflaAB. Thus, there is a close link between the effect that the *fadD* mutation generates on the surface motility exhibited by flaAflaB Rm1021 mutants and their ability to produce rhizobactin 1021 that awaits further investigation.

One of the most unexpected findings in our study was highlighted with the opposite surface behaviors exhibited by different flagella-less mutants which were derived from the same genetic background. Thus, in contrast to the non-motile phenotype exhibited by the GR4 *flaA flaB* mutant on semisolid surfaces, the flagella-less GR4 *flgK* mutant, under the same conditions, displayed surface translocation. Likewise, whereas the Rm1021 *flaA flaB* mutant showed extensive surface translocation, the flagella-less Rm1021 *flgE* mutant was highly impaired in surface motility. Theoretically, *flaA flaB* and *flgK/E* mutants are blocked in different stages of the flagellar assembly (flagellar filament and hook, respectively). Our results suggest that, regardless of the mechanism involved, flagella-independent translocation in *S. meliloti* seems to be regulated in a co-ordinated manner with the stage of flagellar assembly. There are examples in literature in which bacterial phenotypes have been shown to be under coordinated control with the state of flagellar assembly. For example, the expression of late flagellar genes in enterobacteria requires the completion of the flagellar hook-basal body (Smith and Hoover 2009), and the expression of some virulence factors in bacteria such as *Salmonella*, *Proteus* or *Yersinia*, has been linked to the flagellar apparatus (Allison et al. 1992; Young et al. 1999; Iyoda et al. 2001; Duan et al. 2013). Interestingly, the synthesis of a swarming-enabling surfactant in *Pseudomonas syringae* pv syringae (Pss) is coordinated with flagellar assembly: mutations in genes involved in early flagellar assembly abolish or reduce surfactant production, while mutations in flagellin genes increase its production (Burch et al. 2012). Coordinating surfactant production and flagellar assembly was suggested to help swarming by lubricating flagella and/or the surface. The behavior of Rm1021 flagella-less mutants resemble in some ways the phenotypes described for Pss mutants. Rhizobactin 1021 is crucial for Rm1021 surface motility and its amphipatic structure confers surfactant activity to the siderophore. Could Rhizobactin 1021 production be under coordinated control with flagellar assembly? We could not detect differences in the CAS activity present in Rm1021, 1021flaAB and 1021flgE cultures. However, this analysis does not rule out the possibility of changes in siderophore structure that could impact its surfactant activity. Therefore, coordinated control of flagellar assembly with the production of Rhizobactin 1021 or any other surfactant in Rm1021 warrants further investigation.

Finally, to gain insights about the role that *S. meliloti* flagella and/or flagellar mediated motility as well as flagella-independent surface translocation play in the interaction with alfalfa plants, the symbiotic phenotype of two GR4 flagella-less mutants which display opposite surface motility behaviors was assessed and compared to that of the wild-type strain. Our results confirm that motility is a dispensable trait for nodulation and nitrogen fixation. On the other hand, the lower competitive ability exhibited by the two flagella-less mutants compared to the wild-type strain, demonstrate that flagella-dependent motility positively influences the competitiveness for nodule occupation most likely by enabling bacteria to rapidly reach proper infection sites. However, the fact that the flagella-less GR4 *flgK* mutant behaves as effective as the wild-type strain in nodule formation, indicates that flagella and/or flagellar mediated motility are not essential for infectivity and suggest a role for some kind of flagellar-independent motility in this symbiotic trait that might be relevant under field conditions.

In summary, identification of more permissive conditions for surface tranlocation and the use of a set of isogenic mutants unveiled the complexity of *S. meliloti* surface motility and suggests the existence of flagella-independent modes of surface translocation which seem to be controlled in a co-ordinated manner with the stage of flagellar assembly. In addition, the symbiotic phenotypes exhibited by two non-flagellated GR4-derivative mutants with different capacities to spread over surfaces indicate that flagella positively influence the competitiveness for nodule occupation but they are not essential for optimal bacterial infectivity. Flagella-independent modes of translocation that remain to be characterized at the molecular level could play important roles at certain stages of the symbiotic interaction.

REFERENCES

Allison C, Lai HC, Hughes C (1992) Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. Mol Microbiol 6: 1583-1591

Amaya-Gómez CV (2013) Transcriptomic approach for the identification of genes and signals palying a role in swarming motility of *Sinorhizobium meliloti*: connection with biofilm formation and symbiosis. Doctoral Thesis, University of Granada

Amaya-Gómez CV, Hirsch AM, Soto MJ (2015) Biofilm formation assessment in *Sinorhizobium meliloti* reveals interlinked control with surface motility. BMC Microbiol 15: 8. doi: 10.1186/s12866-015-0390-z

Ames P. and Bergman K (1981) Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. J Bacteriol 148: 728-908

Bahlawane C, McIntosh M, Krol E, Becker A (2008) *Sinorhizobium meliloti* regulator MucR couples exopolysaccharide synthesis and motility. Mol Plant Microbe Interact 21:1498-1509. doi: 10.1094/MPMI-21-11-1498

Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84: 188-198

Bernabéu-Roda L (2014) Bases moleculares que gobiernan la movilidad *swarming* en *Sinorhizobium meliloti:* conexión con formación de biopelículas y establecimiento de simbiosis. Tesis Doctoral, Universidad de Granada

Burch AY, Shimada BK, Mullin SW, Dunlap CA, Bowman MJ, Lindow SE (2012) *Pseudomonas syringae* coordinates production of a motility-enabling surfactant with flagellar assembly. J Bacteriol 194: 1287-1298. doi: 10.1128/JB.06058-11

Caetano-Anollés G, Crist-Estes DK, Bauer WD (1988) Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J Bacteriol 170: 3164-3169

Caetano-Anollés G, Wrobel-Boerner E, Bauer WD (1992) Growth and movement of spot inoculated *Rhizobium meliloti* on the root surface of alfalfa. Plant Physiol 98: 1181-1189

Casadesús J, Olivares J (1979) Rough and fine linkage mapping of the *Rhizobium meliloti* chromosome. Mol Gen Genet 174: 203-209

Dilanji GE, Teplitski M, Hagen SJ (2014) Entropy-driven motility of *Sinorhizobium meliloti* on a semi-solid surface. Proc Biol Sci 281: 20132575. doi: 10.1098/rspb.2013.2575

Duan Q, Zhou M, Zhu L, Zhu G (2013) Flagella and bacterial pathogenicity. J Basic Microbiol 53: 1-8. doi:10.1002/jobm.201100335

Finan TM, Hartweig E, LeMieux K, Bergman K, Walker GC, Signer ER (1984) General transduction in *Rhizobium meliloti*. J Bacteriol 159: 120-124

Fujishige NA, Kapadia NN, De Hoff PL, Hirsch AM (2006) Investigations of Rhizobium biofilm formation. FEMS Microbiol Ecol. 56:195-206

Gao M, Coggin A, Yagnik K, Teplitski M. (2012) Role of specific quorum-sensing signals in the regulation of exopolysaccharide II production within *Sinorhizobium meliloti* spreading colonies. PLoS One 7: e42611. doi: 10.1371/journal.pone.0042611

García-Rodríguez FM, Toro N (2000) *Sinorhizobium meliloti* nfe (nodulation formation efficiency) genes exhibit temporal and spatial expression patterns similar to those of genes involved in symbiotic nitrogen fixation. Mol Plant Microbe Interact 13: 583-591

Gurich N, González JE (2009) Role of quorum sensing in *Sinorhizobium meliloti*-alfalfa symbiosis. J Bacteriol 191: 4372-4382. doi: 10.1128/JB.00376-09

Harshey RM (2003) Bacterial motility on a surface: many ways to a common goal. Annu Rev Microbiol 57: 249-273. doi: 10.1128/JB.00376-09

Henrichsen J (1972) Bacterial surface translocation: a survey and a classification. Bacteriol. Rev 36: 478-503

Iyoda S, Kamidoi T, Hirose K, Kutsukake K, Watanabe H (2001) A flagellar gene *fliZ* regulates the expression of invasion genes and virulence phenotype in *Salmonella enterica* serovar Typhimurium. Microb Pathog 30: 81-90

Kearns DB (2010) A field guide to bacterial swarming motility. Nat Rev Microbiol 8: 634-644. doi: 10.1038/nrmicro2405

Meade HM, Signer ER (1977) Genetic mapping of *Rhizobium meliloti*. Proc Natl Acad Sci U S A 74: 2076-2078

Mellor HY, Glenn A R, Arwas R (1987) Symbiotic and competitive properties of motility mutants of Rhizobium trifolii TA1. Arch Microbiol 148: 34-39

Miller LD, Yost CK, Hynes MF, Alexandre G (2007) The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. viciae is essential for competitive nodulation. Mol Microbiol 63: 348-362

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. doi: 10.1186/1471-2164-11-157

Nogales J, Bernabéu-Roda L, Cuéllar V, Soto MJ (2012) ExpR is not required for swarming but promotes sliding in *Sinorhizobium meliloti*. J Bacteriol 194:2027-35. doi: 10.1128/JB.06524-11

Oldroyd GE and Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59:519-46. doi: 10.1146/annurev.arplant.59.032607.092839

Olivares J, Casadesús J, Bedmar EJ (1980) Method for testing degree of infectivity of *Rhizobium meliloti* strains. Appl Environ Microbiol 39:967-970

Pech-Canul A, Nogales J, Miranda-Molina A, Alvarez L, Geiger O, Soto MJ, Lopez-Lara IM (2011) *FadD* is required for utilization of endogenous fatty acids released from membrane lipids. J Bacteriol 193:6295-304. doi: 10.1128/JB.05450-11.

Ryan RP and Dow JM (2011) Communication with a growing family: diffusible signal factor (DSF) signaling in bacteria. Trends Microbiol 19:145-52. doi: 10.1016/j.tim.2010.12.003

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press

Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69-73

Scharf B, Schuster-Wolff-Buhring H, Rachel R, Schmitt R (2001). Mutational analysis of the *Rhizobium lupini* H13-3 and *Sinorhizobium meliloti* flagellin genes: importance of flagellin A for flagellar filament structure and transcriptional regulation. J Bacteriol 183:5334-5342

Scharf B and Schmitt R (2002) Sensory transduction to the flagellar motor of *Sinorhizobium meliloti.* J Mol Microbiol Biotechnol 4:183-6

Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram- negative bacteria. Nat Biotechnol 1:784-791. doi:10.1038/nbt1183-784.

Smith TG and Hoover TR (2009) Deciphering bacterial flagellar gene regulatory networks in the genomic era. Adv Appl Microbiol 67: 257-295. doi: 10.1016/S0065-2164(08)01008-3.

Soby S and Bergman K (1983) Motility and chemotaxis of *Rhizobium meliloti* in soil. Appl Environ Microbiol 46:995-998

Soto MJ, Fernández-Pascual M, Sanjuán J, Olivares J (2002) A *fadD* mutant of *Sinorhizobium meliloti* shows multicellular swarming migration and is impaired in nodulation efficiency on alfalfa roots. Mol Microbiol 43: 371-382

Winans SC (2011) A new family of quorum sensing pheromones synthesized using S-adenosylmethionine and Acyl-CoAs. Mol Microbiol 79:1403-6. doi: 10.1111/j.1365-2958.2011.07551.x

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119

Yost CK, Rochepeau P, Hynes MF (1998) *Rhizobium leguminosarum* contains a group of genes that appear to code for methyl-accepting chemotaxis proteins. Microbiol 144: 1945-1956

Young GM, Schmiel DH, Miller VL (1999) A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. Proc Natl Acad Sci USA 96: 6456-6461

Figure Captions

Fig. 1 Surface motility of *S. meliloti* strains **a** GR4 and **b** Rm1021 on minimal medium (MM) containing different concentrations of Noble agar. Graphs represent the average of surface migration (given in millimeters and determined as the average length of the two sides of a rectangle able to exactly frame each colony) obtained in each medium after 24 and 48 hours of incubation at 30ºC. Means and standard deviations were obtained from at least 3 replicates in three independent experiments (n=9). Below each graph, representative pictures of the motilities exhibited by each strain on MM containing different concentrations of agar are shown

Fig. 2 Transmission electron microscope images of *S. meliloti* **a** GR4 (wild-type), and its flagella-less derivative mutants **b** GR4flaAB and **c** GR4flgK. Cells were isolated from the edge of colonies grown on semisolid MM 0.6% Noble agar after 20 hours of incubation at 30ºC and stained with 2% uranyl acetate

Fig. 3 Swimming motility in BM 0.3% agar of *S. meliloti* **a** GR4 and **b** GR4 *fadD* mutant (GR4FDCSS) and their corresponding flagella-less *flaAflaB* and *flgK* derivative mutants. Pictures were taken after 48 hours of incubation at 30ºC

Fig. 4 Surface motility on semisolid MM (0.6% Noble agar) of *S. meliloti* **a** GR4 and **b** GR4 *fadD* mutant (GR4FDCSS) and their corresponding flagella-less *flaAflaB* and *flgK* derivative mutants. Representative pictures of the motility exhibited by each strain after 24 and 48 hours of incubation at 30ºC are shown. Values represent the mean and the standard error of the surface migration (given in millimeters and determined as the average length of the two sides of a rectangle able to exactly frame each colony) obtained from at least three independent experiments with at least three technical replicates

Fig. 5 Swimming motility in BM 0.3% agar of **a** Rm1021 and **b** Rm1021 *fadD* mutant (1021FDCSS), and their flagella-less and siderophore defective derivative mutants. Pictures were taken after 48 hours of incubation at 30ºC

Fig. 6 Surface motility on semisolid MM (0.6% Noble agar) of *S. meliloti* **a** Rm1021 and **b** Rm1021 *fadD* mutant (1021FDCSS) and their corresponding flagella-less (*flaAflaB* and *flgE*) and siderophore defective derivative mutants. Representative pictures of the motility exhibited by each strain after 24 and 48 hours of incubation at 30ºC are shown. Values represent the mean and the standard error of the migration zone (given in millimeters and determined as the average length of the two sides of a rectangle able to exactly

frame each colony) obtained from at least three independent experiments with at least three technical replicates

Fig. 7 Symbiotic phenotype of non-flagellated GR4 derivative mutants. **a** Infectivity test. Nodule formation efficiency of GR4 (wt) and flagella-less mutants GR4flaAB and GR4flgK expressed as the average number of nodules present in alfalfa roots 14 and 28 days after inoculation. **b** Competitive nodulation assays. Each strain was mixed with marked GR4(pGUS3) at a ratio of 1:1. After inoculating alfalfa roots with these mixtures, the number of nodules occupied by each strain was estimated (see experimental procedures for details). Values are the mean and standard errors obtained in at least three independent experiments. Same letter indicates no significant differences according to ANOVA test ($p \leq 0.05$)

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
***S. meliloti* strains**		
GR4	Wild type	Casadesús and Olivares 1979
GR4flaAB	GR4 *flaA flaB;*Hyg^r	This work
GR4flgK	GR4 *flgK*::Tn*5*; Km^r	This work
GR4FDCSS	GR4 Δ*fadD*::SmSp; Sm^r Sp^r	Amaya-Gómez et al. 2015
GR4fadDflaAB	GR4FDCSS *flaA flaB;*Sm^r Sp^r Hyg^r	This work
GR4fadDflgK	GR4FDCSS *flgK*::Tn*5*; Sm^r Sp^r Km^r	Bernabéu-Roda 2014
Rm1021	SU47 *expR*102::IS*Rm*2011-1; Sm^r.	Meade and Signer 1977
1021F	Rm1021 *flaA flaB::*Hyg*;*Sm^r Hyg^r	Nogales et al. 2012
1021flgE	Rm1021 *flgE*::mini-Tn*5*; Sm^r Neo^r	This work
1021rhbD	Rm1021 Δ*rhbD;* Sm^r	Amaya-Gómez 2013
1021FDCSS	Rm1021 Δ*fadD*::SmSp; Sm^r Sp^r	Nogales et al. 2010
1021fadDflaAB	1021FDCSS *flaA flaB;*Sm^r Sp^r Hyg^r	This work
1021fadDflgG	1021FDCSS *flgG*::Tn*5*; Sm^r Sp^r Km^r	Bernabéu-Roda 2014
1021fadDrhbD	1021FDCSS Δ*rhbD;* Sm^r Sp^r	This work
1021fadDrhbDflaAB	1021fadDrhbD *flaA flaB::*Hyg*;*Sm^r Sp^r Hyg^r	This work
Rm11601	Rm8530 *flaA flaB*; Sm^r, Hyg^r	Gurich and González 2009
Sm2B5005	Sm2B3001 *flgE*::mini-Tn*5*; Sm^r Neo^r	Bahlawane et al. 2008
***E. coli* strains**		
DH5α	*sup*E44 *Δlac*U169Φ80 *LacZ*ΔM1 *rec*A1 *end*A1 *gyr*A96 *thi*1 *rel*A1 *5hsd*R171	Bethesda Research Lab
S17-1	*thi pro rec*A *hsd*R, *hsd;* Rp4Tc::Mu, Km::Tn*7*; Tmp^r, Sm^r, Spc^r	Simon et al. 1983
Plasmids		
pUC18	Cloning vector; Ap^r	Yanisch-Perron et al. 1985
pUCGNS5	pUC18 derivative carrying the genomic *Eco*RI fragment containing the *flgK*::Tn*5* insertion from GR4fadDflgK. Ap^r, Km^r	Bernabéu-Roda 2014
pSUP202	Suicide plasmid; Ap^r, Tc^r, Cm^r	Simon et al. 1983
pSUP202flgK	pSUP202 derivative carrying the *Eco*RI fragment from pUCGNS5.Ap^r, Tc^r, Km^r	This work
pUCflaAB::Hyg	pUC18 derivative carrying a *Bgl*II fragment containing the *flaA flaB*::Hyg mutation from Rm11601. Ap^r, Hyg^r	This work
pK18*mobsacB*	Suicide plasmid; Km^r	Schäfer et al. 1994
pK18flaAB::Hyg	pK18*mobsacB* derivative carrying the insert from pUCflaAB::Hyg.	This work
pGUS3	Plasmid which contains an *nfeD::gusA* fusion. Km^r.	García-Rodríguez and Toro 2000

[a] Hyg^r, Km^r, Sm^r, Sp^r, Neo^r, Tmp^r, Ap^r, Tc^r and Cm^r indicate hygromycin, kanamycin, streptomycin, spectinomycin, neomycin, trimethoprim, ampicylin, tetracyclin and chloramphenychol resistance, respectively.