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Swimming and swarming motility properties of peanut-nodulating rhizobia

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One sentence summary. Movement over surfaces allows rhizobia to colonize and survive on legume roots. Motility by peanut-nodulating rhizobia is clearly important during inoculation.

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ABSTRACT

Motility allows populations of bacteria to rapidly reach and colonize new microniches or microhabitats. The motility of rhizobia (symbiotic nitrogen-fixing bacteria that nodulate legume roots) is an important factor determining their competitive success. We evaluated the effects of temperature, incubation time, and seed exudates on swimming and swarming motility of five strains of *Bradyrhizobium* sp. (peanut-nodulating rhizobia). Swimming motility was increased by exudate exposure for all strains except native Pc34. In contrast, swarming motility was increased by exudate exposure for native 15A but unchanged for the other four strains. All five strains displayed the ability to differentiate into swarm cells. Morphological examination by scanning electron microscopy showed that the length of the swarm cells was variable, but generally greater than that of vegetative cells. Our findings suggest the importance of differential motility properties of peanut-nodulating rhizobial strains during agricultural inoculation and early steps of symbiotic interaction with the host.

Key words: rhizobia; peanut; swimming motility; swarming motility; seed exudates; scanning electron microscopy

INTRODUCTION

Inoculation of legume crop plants with effective rhizobial strains to increase productivity provides an ‘environmentally friendly’ alternative to chemical fertilization. Peanut (*Arachis hypogaea* L.) is cultivated worldwide under a variety of agronomic systems, with an estimated total annual production of 33.1 million tons. China is the leading peanut producer (~37.5% of total production), followed by India, USA, Argentina and Vietnam (Fabra et al., 2010). In Argentina, ~90% of peanut production occurs in Córdoba province.

Cultivated peanut plants are able to fix atmospheric nitrogen through their symbiotic association with specialized bacteria (rhizobia), reducing the need for expensive and environmentally harmful nitrogen fertilizers. The process of symbiosis involves hosting of rhizobia in nodule-forming plant root cells and a ‘molecular dialogue’ between the two partners to coordi-

nate the steps of symbiosis and to avoid host defense responses. Few studies to date have focused on the early interactions between the two partners.

Viable rhizobia can be introduced into peanut agricultural ecosystems by application either onto the seed (‘on-seed inoculation’) or into the soil (‘in-furrow inoculation’) (Bogino et al., 2011). On-seed inoculation typically results in a low rate of nodule occupancy because of (i) low bacterial motility and (ii) the ability of native rhizobial species to form nodules on the entire root system because of their wide distribution in the soil. The behavior of different rhizobial species in the rhizosphere determines their relative nodulation ability and fixation effectiveness (Thies, Singleton and Bohloul 1991). In-furrow inoculation prior to planting has several advantages over on-seed inoculation, e.g. (i) the plant seed coat is not damaged, (ii) the rhizobia are not affected by fungicides or pesticides applied to seeds and (iii)

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the rhizobia have a better opportunity to infect the legume root system because of their wide distribution in the soil (Fouilleux, Revellin and Catroux 1994).

In a study of soil temperature effect on seedling emergence and early growth of six peanut cultivars in natural field soils in temperature-gradient greenhouses in Gainesville, FL, USA, germination rates for all cultivars were lowest at the earliest sowing date, when soil temperature was lowest (Prasad, Boote and Allen 2006). In Córdoba province, peanuts are sown in the spring, preferably during three consecutive days at a soil depth of 10 cm and soil temperature $\geq 18^{\circ}\text{C}$. These conditions typically occur in late October and early November. During this period, the Córdoba region is often subject to cold fronts from the south that reduce soil temperature below normal levels for several days. Peanut crops are therefore frequently sown under suboptimal temperature conditions, resulting in slow seedling emergence, exposure of the plants to unfavorable growing conditions and consequent difficulty for inoculated rhizobia to establish symbiosis.

Various types of surface motility enable bacteria to establish symbiotic or pathogenic relationships with plants and animals. Depending on surface conditions and nutrient availability, bacteria may remain localized, move out to colonize larger areas, invade host tissues, or generate fruiting (spore-producing) bodies and wait for a more favorable season (Harshey 2003).

Motility is an essential and impressive feature of bacterial physiology. Movement of bacteria in aqueous environments by swimming along surfaces using various mechanisms has been classified into several distinct forms. Swimming along a surface occurs when the fluid film is sufficiently thick and the morphological pattern of the bacteria is unorganized. When the fluid layer on a surface is thin, or when cells are inoculated on the surface of agar medium, vegetative cells begin a process of differentiation into multinucleated cells and elongated, hyperflagellated cells termed 'swarm cells' (Braeken et al., 2008). The rapid movement of swarm cells at the edge of a colony is accompanied by cell growth within the colony, resulting in rapid colonization of the available area.

Swarming is a process requiring high energy consumption and synthesis of numerous flagellae (Harshey 2003; Verstraeten et al., 2008). Swarming is neither a starvation response nor an obligatory development stage, but rather a coordinated and reversible change in behavior in response to environmental factors (Senesi et al., 2010). This collective activity has several advantages over individual cell activity: it allows bacteria to move toward sources of nutrition (Wei et al., 2011), to move away from sources of potential damage (Macfarlane, Hopkins and Macfarlane 2001), to protect themselves from the action of antibiotics (Overhage et al., 2008; Butler, Wang and Harshey 2010), to avoid competition (Taylor and Buckling 2010) and to more efficiently engage in cooperative processes such as biofilm formation (Blair et al., 2008) and colonization of host plants (Ghelardi et al., 2007).

Swarming behavior has been documented in many genera of bacteria, including *Aeromonas*, *Azospirillum*, *Bacillus*, *Proteus*, *Pseudomonas* and *Escherichia* (Daniels, Vanderleyden and Michiels 2004). The rhizobial species reported (to date) to display swarming are *Ensifer meliloti* (Soto et al., 2002; Nogales et al., 2010, 2012), *Rhizobium etli* (Daniels et al., 2006; Braeken et al., 2008) and *R. leguminosarum* bv. *viciae* (Tambalo, Yost and Hynes 2010).

Swarming behavior of peanut-nodulating rhizobia has not been studied previously. We describe here the optimal conditions for swimming and swarming of five peanut-nodulating

Bradyrhizobium sp. strains, the morphology of swarm cells and the effects of various agents on swarming.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Five rhizobial strains were used in this study: three strains recommended for use as inoculants (C145 [Niftal, USA], SEMIA6144 [IPAGRO, Brazil], USDA4438 [USDA, ARS, USA]) and two native strains (15A, Pc34) (Bogino, Banchio and Giordano 2010). Bacterial cells were isolated from peanut root nodules and grown in yeast extract mannitol (YEM) medium (Vincent 1970) for 8 days at 30°C . Strains were selected based on their agricultural importance according to Bogino et al. (2010).

Swimming and swarming motility assays

Cells of the above strains were grown for 8 days in separate petri plates with 1.5% YEM medium at 30°C . An individual colony of each strain was then selected and inoculated (to establish further colonies) in the middle of a new petri plate, with 20 ml of 0.3% water-agar/10% YEM for swimming assays or 0.5% water-agar/10% YEM for swarming assays. Motility diameter was measured 8 days after this inoculation.

Modifications of motility testing procedures

Testing procedures as described previously were modified to facilitate studies of the effects of suboptimal temperature, incubation time, seed exudates and addition of various carbon sources on bacterial motility. For the suboptimal temperature test, plates were incubated at 23°C . For the incubation time test, plates were incubated for 16, 24 and 32 days at 30°C . The effect of seed exudates on motility diameter was evaluated by adding 8 μl of 10x concentrated exudates to culture medium. For evaluation of the attraction effect of seed exudates, 8 μl of 10x concentrated exudates was placed in a filter located 0.5 cm outside the motility radius of the strain. For the carbon source test, four monosaccharides (glucose, fructose, mannose, arabinose) and one polyol (mannitol) were added separately to the motility medium, each at a final concentration of 0.1%.

Preparation of seed exudates

Twenty peanut seeds were placed in a disinfected, autoclaved beaker, added with 20 ml sterile distilled water and incubated for 16 h in the dark. The resulting liquid (containing seed exudates) was lyophilized to dryness, resuspended in 2 ml physiological saline, concentrated 10x, sterilized by passage through cellulose nitrate filters (pore size $0.45\text{ }\mu\text{m}$), aliquoted into sterile Eppendorf tubes and stored at -20°C .

Scanning electron microscopy (SEM)

For cell size determination and morphological analysis, vegetative cells (obtained from the center of the plate) and swarm cells (obtained from the motility halo) were fixed in 2.5% glutaraldehyde, stored at 48°C for 24 h, and sequentially dehydrated as described by Kockro et al. (2000). After critical point drying, each sample was fixed on double-layer copper foil, placed on aluminium substrates coated with a thin gold layer on SCD 050 sputter (Bal-Tec; Canonsburg, PA, USA) at 40 mA/150 s, and examined by SEM (model JMST300, JEOL Ltd, Tokyo, Japan).

Statistical analysis

All experiments were performed using a randomized design. The values shown are mean \pm SD of three independent pairs of duplicate experiments. The data were subjected to analysis of variance (ANOVA) with multiple comparison variables by Fisher's least significant difference (LSD) test. Differences between means were considered to be significant at $P \leq 0.05$. The software program used was Infostat 1.0 (Department of Statistics and Biometry, Faculty of Agricultural Sciences, National University of Córdoba).

RESULTS AND DISCUSSION

Experimental conditions under which swimming and swarming were observed

Swimming and swarming motility of the five strains of peanut-nodulating rhizobia were evaluated for agar concentrations ranging from 0.3 to 1%. The agar concentration for which the largest motility diameters were observed was 0.3%, followed by 0.5%. Similarly, Albareda et al. (2006) reported maximal swimming and swarming motility at agar concentrations of 0.3 and 0.5%, respectively, for soybean-nodulating rhizobia. Butler et al. (2010) demonstrated that defined agar concentration ranges were optimal for swarming behavior by various groups of microorganisms; peanut-nodulating rhizobia showed swarming in the 0.4–1.0% range, with 0.5% as the optimal value. In view of the above findings, we used agar concentrations of 0.3 and 0.5% for subsequent studies of swimming and swarming behavior.

The five rhizobial strains used in our experiments are characterized by relatively slow growth. To ensure survival and growth of cells during the testing period, we supplemented the motility medium with 10% YEM to provide essential nutrients. Some of the values obtained under this condition were slightly higher than those obtained with water-agar medium (data not shown). The baseline conditions used for studying the effects of various factors were 0.3% agar + 10% YEM for swimming and 0.5% agar + 10% YEM for swarming.

Effects of temperature, incubation time and seed exudates on motility

Effects of temperature

Peanut crops are generally planted at temperatures ranging from 16 to 23°C, and rhizobia are introduced by inoculation. We examined the effect of temperature on swimming and swarming behavior of rhizobia.

Swimming and swarming motility were measured as described in Section 'Swimming and swarming motility assays', using incubation temperatures of 23 and 30°C. Results for swimming motility in 0.3% water-agar medium + 10% YEM are shown in Fig. 1A. The values obtained at 23°C were ~50% lower than those at 30°C. Reference strain USDA4438 showed the highest swimming motility value at 23°C, followed by native strain Pc34. Results for swarming motility in 0.5% water-agar medium + 10% YEM are shown in Fig. 1B. The values at 23°C were lower than those at 30°C, except for reference strain C145. In contrast to the results shown in Fig. 1A, the highest swarming motility value was observed for SEMIA6144, followed by C145.

Results contrary to ours were obtained in a study of *R. leguminosarum* swarming on plates incubated at 30 and 22°C; i.e. swarming motility was higher at the lower temperature (Tambalo et al., 2010). Enhancement of swarming motility at

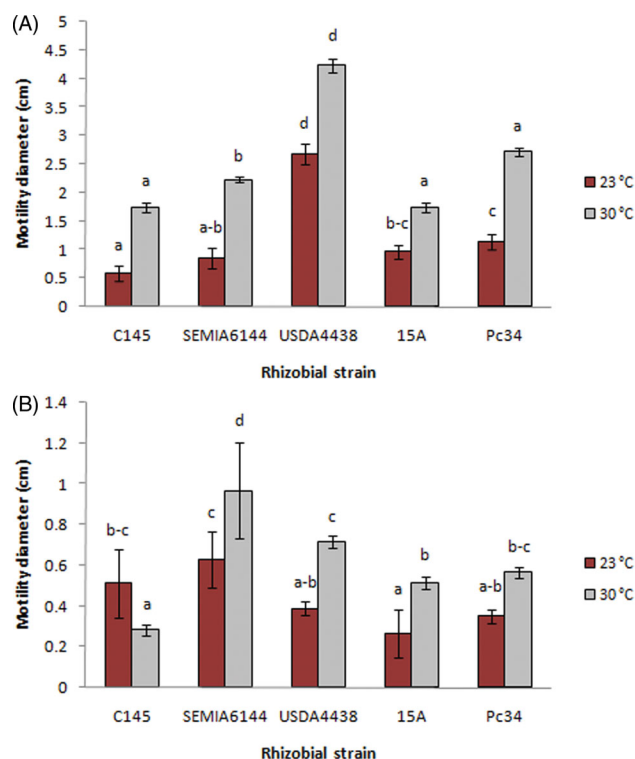


Figure 1. Swimming (A) and swarming (B) motility diameters of rhizobia grown in 0.3% (A) or 0.5% (B) water-agar medium + 10% YEM at two incubation temperatures. The values shown are mean \pm SD of three independent pairs of duplicate experiments. Differing letters above the bars indicate statistically significant (ANOVA, Fisher's LSD test, $P < 0.05$) differences between means.

lower temperature was also observed in *Pseudomonas* and *Serratia* (Lai, Soo and Wei 2005; Matilla et al., 2007). Reductions in temperature affect cell physiological state, wetness of the agar surface and production of biosurfactants for translocation (Lai et al., 2005). Further studies on the effects of temperatures below 23°C and production of motility-related molecules in our system are required to clarify the points of apparent contradiction as above.

Effects of incubation time

The strains used in this study display slow growth rates ($\mu = 0.079 \text{ CFU ml}^{-1} \text{ h}^{-1}$) and long generation times (up to 8.7 h) under laboratory conditions (data not shown). These characteristics presumably impact swimming and swarming behaviors in the field as well as the laboratory. To determine the effect of incubation time on swimming and swarming, we performed motility assays after 8, 16, 24 and 32 days. The choice of 8 days as the shortest interval was based on the slow growth rates of the strains and on our previous findings (Bogino et al., 2008). The choice of 32 days as the maximal interval was based on the dehydration effects observed at longer intervals.

Swimming and swarming motility assays were performed as described in Section 'Swimming and swarming motility assays', with an incubation temperature of 30°C. Swimming motility results are shown in Fig. 2A. The motility diameter increased as a function of incubation time for all five strains, except that the value for USDA4438 was slightly higher at 24 than at 32 days. The results for swarming motility (Fig. 2B) differed from those for swimming motility. The increase in motility diameter for reference strain C145 at greater incubation times was minimal. The

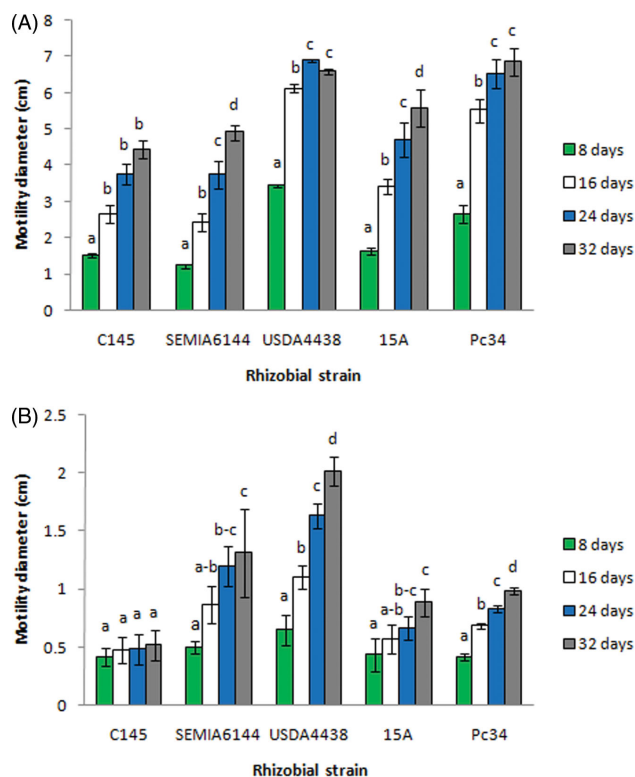


Figure 2. Swimming (A) and swarming (B) motility diameters of rhizobia grown in 0.3% (A) or 0.5% (B) water-agar medium + 10% YEM for four incubation times. Statistical details as in Fig. 1.

magnitudes of the diameters in all cases were smaller. Diameters increased as a function of incubation time, and were maximal at 32 days.

Similarly, Covell et al. (2013) observed maximal motility after 15 days incubation in soybean-nodulating rhizobia, which are also slow growing.

Effects of seed exudates

Values of swimming motility in the presence of concentrated seed exudates in the medium (as described in Section 'Modifications of motility testing procedures') are shown in Fig. 3A. USDA4438 and the two native strains showed the highest motility values. Values of swarming motility in the presence of exudates in the medium (Fig. 3B) were similar to those for swimming motility. All experimental values for both types of motility were lower than control values (without exudates). Possible explanations for this finding are that (i) we did not use an optimal exudate dose that would have increased motility or (ii) one or more molecular components in the exudates exert a motility-inhibitory effect.

The effect of adding exudates in a filter on swimming and swarming motility, in terms of percentage change of the motility radius, is summarized in Table 1. For swimming motility, the radius was increased by exudate exposure for all strains except native Pc34. In contrast, for swarming motility, the radius was unaffected by exudate exposure for all strains except native 15A, which showed a 25% increase.

Borisov et al. (2009) reported that swarming motility of various *Azospirillum* strains was increased by exposure to wheat exudates. The results were irregular, possibly because of the

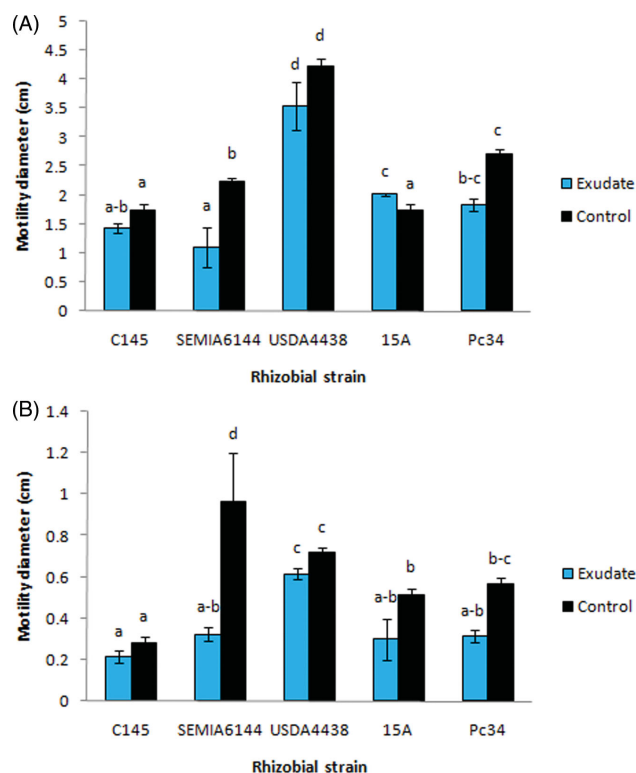


Figure 3. Swimming (A) and swarming (B) motility diameters of rhizobia grown in 0.3% (A) or 0.5% (B) water-agar medium + 10% YEM with or without addition of 8 μ l of 10x concentrated seed exudates. Statistical details as in Fig. 1.

Table 1. Percentage change of swimming and swarming motility radius resulting from exposure to peanut seed exudates.

Swimming motility (in 0.3% water-agar medium + 10% YEM)			
Strain	Control	+ Seed exudates	% increase
Motility radius (cm)			
C145	0.65 \pm 0.09	0.78 \pm 0.03	20.5
SEMIA6144	0.36 \pm 0.21	0.40 \pm 0.14	9.09
USDA4438	1.66 \pm 0.11	1.86 \pm 0.30	12
15A	0.91 \pm 0.03	1.11 \pm 0.03	21.81
Pc34	0.93 \pm 0.06	0.93 \pm 0.06	0
Swarming motility (in 0.5% water-agar medium + 10% YEM)			
C145	0.11 \pm 0.03	0.11 \pm 0.03	0
SEMIA6144	0.10 \pm 0.04	0.10 \pm 0.03	0
USDA4438	0.15 \pm 0.03	0.15 \pm 0.03	0
15A	0.13 \pm 0.06	0.16 \pm 0.06	25
Pc34	0.16 \pm 0.03	0.16 \pm 0.03	0

The values shown are mean \pm SD of three independent pairs of duplicate experiments.

presence of molecules in the exudates that had differential regulatory effects on the bacterial strains.

Rhizobacteria typically display positive chemotaxis toward root or seed exudates, and utilize chemical compounds present in the exudates as growth factors and/or nutrient sources (Yaryura et al., 2008). In-furrow inoculation of peanut-nodulating *Bradyrhizobium* results in variable positioning, and the bacteria may require a sufficient degree of motility to contact plant seeds or roots. It is important to elucidate the effects of seed and root exudates on such motility.

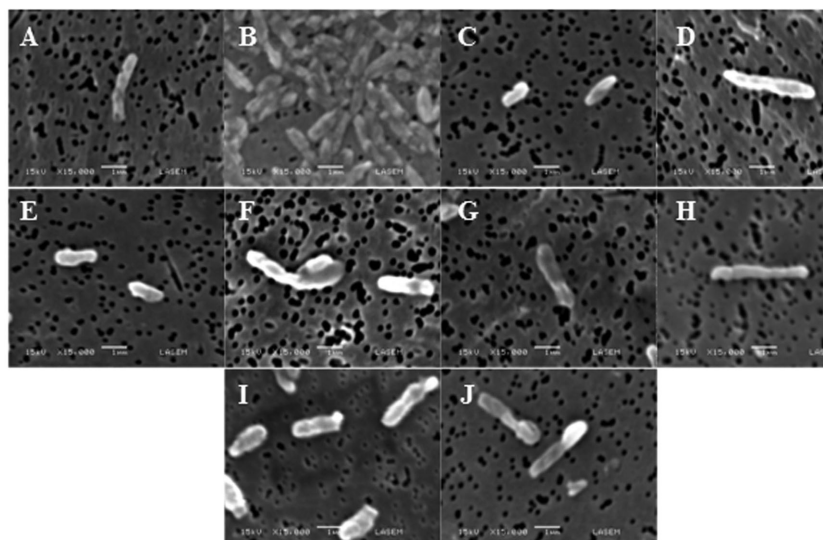


Figure 4. SEM images illustrating comparative morphology of vegetative cells (first panel) vs swarm cells taken from the edge of the swarm front (second panel). (A, B) strain C145. (C, D) strain SEMIA6144. (E, F) strain USDA4438. (G, H) strain 15A. (I, J) strain Pc34. Scale bars: 1 μ m.

Differences in motility halos of the various strains tested are presumably not due to variations in growth rate, since the strains have similar generation times (Bogino et al., 2010). Variability of motility among the strains could result from genetic differences, even though the strains all have peanut-nodulating ability.

Comparative morphology of vegetative vs swarm cells

Differentiation into swarm cells typically involves striking changes in cell morphology, particularly hyperflagellation and cell elongation (Fraser and Hughes 1999). Swarm cells also secrete surfactants and wetting agents that facilitate their movement along surfaces (Harshey 2003). We examined the morphology of vegetative and swarm cells by SEM. The five strains studied all displayed the ability to differentiate into swarm cells. The length of swarm cells was variable, but generally greater than that of vegetative cells (Fig. 4). Mean lengths (\pm SD) of vegetative and swarm cells for the five strains studied are summarized in Table 2. Similarly, Harshey (1994), Fraser and Hughes (1999) and Verstraeten et al. (2008) reported that swarm cells of various rhizobial strains were significantly longer than vegetative cells. In this study, C145 did not show a significant difference in length of swarm cells vs vegetative cells, in contrast to the other four strains (Fig. 4B vs A). Tambalo et al. (2010) reported that elongation is not essential for differentiation into swarm cells in *R. leguminosarum*.

Table 2. Lengths of vegetative and swarm cells of five rhizobial strains assessed by SEM.

	Vegetative cells	Swarm cells
	Cell length (μ m)	
C145	1.50 \pm 0.25	1.49 \pm 0.30
SEMIA6144	1.18 \pm 0.19	4.01 \pm 0.72
USDA4438	1.89 \pm 0.32	3.52 \pm 0.63
15A	2.09 \pm 0.36	3.40 \pm 0.64
Pc34	1.79 \pm 0.19	3.06 \pm 0.51

In most motility studies, it is not clear that whether elongated cells are required for swarming or whether they simply accumulate at the swarm edge. Although the ‘dogma’ of swarming motility emphasizes the importance of elongation, no study to date has demonstrated a mechanistic or regulatory connection at the molecular level for control of cell division during swarming. Studies of many bacterial species have not shown that substantial cell elongation is required for swarming motility, nor that these two processes are co-regulated (Kearns 2010).

CONCLUSIONS

When free-living bacteria colonize biotic or abiotic surfaces, the associated changes in physiology and morphology have important consequences for cell growth, development and survival. The manifestations of functional responses to surface colonization include surface motility, biofilm formation and host invasion (Harshey 2003). Movement over surfaces allows symbiotic bacterial species to migrate, adhere to host tissues or disperse from sites of infection.

The present findings demonstrate that symbiotic peanut-nodulating rhizobia are capable of swimming and swarming motility, and that the motility behavior is affected by temperature, incubation time and the presence of peanut seed exudates. Swimming and swarming motility by rhizobia are clearly important during in-furrow inoculation and the related early steps of symbiotic interaction; e.g. adhesion of the bacteria to peanut seeds. The motility capacity of peanut-nodulating rhizobial strains is largely unknown. Future studies by our group and others will clarify the roles of swimming motility, swarming motility and chemotaxis in rhizosphere colonization by inoculated strains, competition with native strain, adhesion to peanut seeds and roots, and nodulation of host plant roots.

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Conflict of interest statement. None declared.

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