Entropy-driven motility of *Sinorhizobium meliloti* on a semi-solid surface

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*Sinorhizobium meliloti* growing on soft agar can exhibit an unusual surface spreading behaviour that differs from other bacterial surface motilities. Bacteria in the colony secrete an exopolysaccharide-rich mucoid fluid that expands outward on the surface, carrying within it a suspension of actively dividing cells. The moving slime disperses the cells in complex and dynamic patterns indicative of simultaneous bacterial growth, swimming and aggregation. We find that while flagellar swimming is required to maintain the cells in suspension, the spreading and the associated pattern formation are primarily driven by the secreted exopolysaccharide EPS II, which creates two entropy-increasing effects: an osmotic flow of water from the agar to the mucoid fluid and a crowding or depletion attraction between the cells. Activation of these physical/chemical phenomena may be a useful function for the high molecular weight EPS II, a galactoglucan whose biosynthesis is tightly regulated by the ExpR/SinI/SinR quorum-sensing system: unlike bacterial colonies that spread via bacterium-generated, physical propulsive forces, *S. meliloti* under quorum conditions may use EPS II to activate purely entropic forces within its environment, so that it can disperse by passively ‘surfing’ on those forces.

1. Introduction

The ability to spread across moist surfaces provides a survival advantage to bacteria. It can allow a colony to expand into new territory or colonize a host and seek or migrate towards sources of nutrient or moisture. Bacteria achieve surface motility through a diversity of mechanisms, many of which have been studied in detail from both microbiological and physical perspectives. These include swimming, swarming, sliding, gliding and twitching [1–4]. They generally involve active propulsion of the bacterial cells by the physical work of flagellar rotation, or by other mechanical forces acting between the cells and the surface or between cells within a densely packed cell monolayer or biofilm. Given the variety of different mechanisms observed to date, it is highly likely that additional means of locomotion exist that have not yet been described.

*Sinorhizobium meliloti* is a soil α-proteobacterium that participates in an important nitrogen-fixing symbiosis with plants of the agriculturally important genus *Medicago*. *Sinorhizobium meliloti* is capable of at least two forms of motility on semi-solid agar surfaces [5–9]. One is a surface-swarming motion of hyperflagellated cells, described in detail by Soto *et al.* [6]. The other is an unusual type of surface spreading or translocation that has been observed by several groups [5,6,9] and was recently described in detail by Gao *et al.* [8] and Nogales *et al.* [7]. A newly inoculated colony that spreads by this mechanism first acquires a wet appearance as it secretes a nearly transparent mucoid fluid or slime. The mucoid layer then expands radially outward across the agar surface, at speeds of $\simeq 0.04–0.4\,\mu\text{m}\,\text{s}^{-1}$, over a period of 2–3 days [7,8]. The cells travel outward with the mucoid layer until the colony is dispersed over a final area as large as approximately 1 cm². During this spreading, the colony typically develops a feathered or curdled, patterned appearance as shown in figure 1 and in the images in references [7–9].

The mechanism of this spreading has been unclear. Although some authors have described it as a form of swarming or sliding motility, it is uncharacteristic...
of those behaviours in several respects. First, while swarming cells are normally hyperflagellated, the spreading *S. meliloti* possess only two to four flagella per cell [7,8]. In fact, this spreading does not absolutely require flagella [7]; flagellum-deficient strains form spreading mucoid layers like wild-type, although they do not disperse as efficiently throughout those layers and their colonies lack the distinctive feathered or curdled patterns [8].

Second, the spreading *Sinorhizobium* do not form the closely packed monolayer or biofilm that is typical of swarming or sliding colonies; rather the spreading *S. meliloti* colonies are heterogeneous in density, containing both high and very low cell density regions, giving rise to the feathered patterns [8]. We have collected time-lapse images (see the electronic supplementary material, movies SM1–SM7) that suggest that these patterns arise from complex and intriguing processes of cell division, migration, swimming, aggregation and settling in the spreading colony.

Regulation of microbial translocation over semi-solid surfaces often requires population-wide changes in gene expression. Quorum sensing is one of the best characterized mechanisms of bacterial population density-dependent gene regulation: by producing and responding to small diffusible molecules known as autoinducers, quorum-sensing bacteria can change patterns of gene expression in a community-wide fashion as their population changes [10]. *Sinorhizobium meliloti* spreading depends on the sinorhizobial SinI/SinR/ExpR quorum-sensing system and its acyl homoserine lactone (AHL) autoinducers [7,8]. This system includes the AHL synthase SinI, the intracellular AHL receptor ExpR and SinR, which regulates *sinI* [11]. ExpR interacts with the SinI-AHLS to effect transcription of wggR, whose product in turn activates production of the galactoglucofuran exopolysaccharide designated EPS II [12]. EPS II consists of low molecular weight (LMW, 15–20 disaccharide subunits) and high molecular weight (HMW, more than 25 disaccharide subunits) fractions [13], and it makes up the bulk of the mucoid that is generated by *S. meliloti* under the spreading conditions described here. The spreading requires biosynthesis of EPS II: strains that are deficient in EPS II biosynthesis, e.g. lacking *wggR, wgeB* (which encodes a glucosyl transferase) or genes essential to the SinI/SinR/ExpR quorum-sensing system, form dry colonies on soft agar and fail to spread [7,8].

Consequently, sinorhizobial spreading on semi-solid media requires EPS II biosynthesis under a functional SinI/SinR/ExpR quorum-sensing system and depends on the SinI-produced AHLSs. However, the addition of AHLSs to strains lacking *expR* or any EPS II biosynthesis genes did not restore the spreading behaviour. This finding suggests that EPS II production, rather than any surfactant action of the AHLSs, enables the spreading [8].

These intriguing properties of the quorum-sensing-controlled *S. meliloti* spreading and the richness of the colony patterns and their dynamics raise the question of what physical mechanisms underly this form of translocation. Here, we have combined physical/chemical studies of the organism and mutant strains with quantitative modelling to investigate the surface spreading. We find that two purely entropy-driven phenomena—osmotic flow [14] and the depletion (crowding) attraction [15–17]—attributable to the exopolysaccharide EPS II drive the physical dispersion of the colony. An osmotic model captures the observed kinetics and parameter-dependence of colony spreading, whereas flagellar swimming plays a more secondary role of maintaining the mucoid suspension of cells. Therefore, our data show that, through quorum-sensing controlled EPS II synthesis, the sinorhizobia achieve motility by collectively activating thermophysical forces in the surrounding medium, rather than by individually generating propulsive forces.

2. Material and methods

*Sinorhizobium meliloti* strains Rm8530 [18], Rm1021 (*expR−*) [19], MG32 (*sinI−*) [20] and MG320 (*fliP−*) [8] all harbouring the constitutive *gfp*-reporting plasmid pDG71 [21] were grown to an OD₆₀₀ = 0.5–0.8 in TY [22] at 30°C. *Escherichia coli* strain MT102 pJB132 [23] was grown to OD₆₀₀ = 0.2–0.3 in LB medium at 30°C. Media were supplemented with antibiotics as needed at final concentrations of streptomycin 400 µg ml⁻¹; tetracycline 5 µg ml⁻¹; kanamycin 50 µg ml⁻¹; 3-oxo-C₁₀-HSL (catalogue no. 10011238, Cayman Chemical, Ann Arbor, MI, USA) was prepared as a 10 mM stock solution in ethyl acetate.

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**Figure 1.** Spreading of *S. meliloti* Rm8530 on 0.4% (w/v) agar/5% (w/v) TY: (a) GFP fluorescence images (false colour) from a time-lapse study (see also the electronic supplementary material, movie SM1). The spreading colony deposits aggregates of cells onto the agar surface in a feathered pattern; (b) growth of the colony area *A*(t), following inoculation at time *t* = 0. Data are shown as a solid blue curve, and the dashed red curve shows a fit to equation (3.2) (*k* = 0.034 and *t₀* = 14). Arrows mark the times shown in (a); (c) *A*-time-derivative of the colony area *dA*(t)/dt (solid blue), and fit to exponential decay (dashed red); (d) *dA*(t)/dt versus *A*(t) during spreading (solid blue). The dashed red line indicates the linear relationship of equation (3.1) for *t* ≥ 14 h. (Online version in colour.)
We studied the spreading of bacteria on nutrient-poor, semi-solid agar by placing 3 μl of liquid culture onto the surface of a 0.4% (w/v) agar/5% (v/v) TY culture plate and then imaging the spreading colony in either white light or in green fluorescence. Plates were then incubated at room temperature and imaged in time lapse under blue light excitation or white light. The fluorescence imaging configuration is described in [24] and also in the electronic supplementary material.

To measure the effect of AHL concentration and agar concentration on colony spreading, we used a six-well plate with 5% TY agar and different AHL or agar concentrations in each well. Three microlitres of culture was loaded at the centre of each well and imaged at 0.001 Hz for 70 h. For agar concentration studies, the wells contained 5% (w/v) TY and agar concentrations in the range 0.4–0.8% (w/v). Five microlitres of EPS II concentrate was placed at the centre of each well and imaged in white light as described in the electronic supplementary material.

To obtain a washed concentrate of the HMW fraction of EPS II, we used a micropipette to collect mucoid secretion from spreading colonies of S. meliloti Rm8530, transferred the secretion to microcentrifuge tubes, and then spun for 20 min to remove cells. The supernatant was then filter-sterilized using a 0.22 μm syringe filter and then the HMW component was concentrated (approx. 10-fold) by spinning in a Centricon 10,000 MWCO centrifugal filter (YM-10, Millipore Corporation, Bedford, MA, USA) as per the manufacturer’s protocol. The EPS II concentrate was further washed twice with sterile deionized water and reconstituted (YM-10 filter) to ensure the removal of AHLs and other small molecule components. From weighing the dried concentrate, we estimate that, prior to spreading, the mucoid secretion contains 1–5% (w/v) EPS II.

To study EPS II-driven aggregation of sinorhizobia, we mixed 15 μl of either concentrated EPS II, water-diluted EPS II, or water with 15 μl of Rm1021 or Rm8530 culture. We deposited 1.5 μl of the mixture onto a glass slide in a sealed plate and incubated 12 h at 25°C. Droplets were imaged in phase contrast and fluorescence using a Nikon TE2000U microscope.

3. Results

(a) Kinetics of spreading

Although the colonies and their mucoid are visible in white light, cell swimming and aggregation are much more visible in GFP fluorescence images, in which the mucoid fluid is not visible. The fluorescence images of figure 1 show the spreading of an S. meliloti strain that carries a constitutive gfp reporter in a wild-type background (Rm8530 + pDG71). Before it begins radial spreading, the colony appears as a circular zone of radius \( R \approx 1 \text{ mm} \). At a later time (designated as \( t_0 \approx 14 \text{ h} \)), this disc begins to expand as the colony spreads, with its circumference or boundary coincident with the boundary of the mucoid layer that is generated by the colony: the cells and the fluid spread outward together. (Strains lacking flagella behave differently, as discussed below.) The spreading typically continues until the outer radius \( R \) of the mucoid (and colony) disc reaches at least 3–5 mm, i.e. until the colony covers an area of 30–80 mm².

While the total area \( A \) of the colony grows very rapidly when growth first begins, its rate of expansion \( dA/dt \) slows with time. In fact, figure 1d shows an intriguingly simple time-dependence in the kinetics of \( A \): once growth is underway (i.e. \( t > t_0 \)) the rate of area growth \( dA/dt \) decreases almost linearly with the area \( A \). That is, during active spreading the data are described by

\[
\frac{dA}{dt} = k(A_\infty - A),
\]

where \( k \) is a constant slope and \( A_\infty \) represents the final (\( dA/dt \to 0, t \to \infty \)) area of the fully spread colony. Integrating equation (3.1) implies that the kinetics of the growth of \( A(t) \) should resemble an exponential relaxation (for \( t \geq t_0 \))

\[
A(t) = A_\infty + (A_0 - A_\infty)e^{-k(C_0/C)}(t/t_0),
\]

where \( A_0 \) represents the initial (\( t = t_0 \)) area of the colony, \( A_\infty \) represents the final (\( t \to \infty \)) area and the constant \( k \) is a relaxation rate. Figure 1b shows that equation (3.2) gives a virtually perfect description of the colony spreading kinetics.

Fitting our data, we typically find values of \( k \approx 0.03\to0.10 \text{ h}^{-1} \).

Although complex biological or biochemical processes such as growth, swimming, nutrient diffusion and depletion, etc., are certainly under way in the colony, very simple exponential kinetics are typically the consequence of a simple physical or chemical equilibration. A plausible candidate mechanism is the osmotic equilibration between the agar medium and the EPS II-rich colony: the mucoid slime synthesized by sinorhizobia contains a high concentration of HMW EPS II, initially approximately 1–5% (w/v), which should act as an osmolyte—especially if it does not readily diffuse into the agar. If the osmotic gradient draws water from the agar into the mucoid layer, the layer may spread outward until it has become sufficiently diluted that it shares the same osmotic pressure as the agar. Such an osmotic mechanism was recently described in the spreading of Bacillus subtilis biofilms on semi-solid agar [14], although those colonies form a dense monolayer on the agar. This raises the possibility that cell–cell packing forces may contribute to B. subtilis spreading (‘sliding’ motility).

We observe in the electronic supplementary material that, if the colony has produced a quantity of osmolyte (such as EPS II) by \( t_0 \), then the osmotic disequilibrium between the colony and agar should pull water from the agar into the mucoid layer in such a manner that the colony area \( A \) (although not its radius \( R \)) exhibits exponential relaxation to equilibrium, exactly as in equations (3.1) and (3.2). The osmotic model also generates a rough prediction for the rate \( k \) (based on the hydraulic permeability [25] of agar) that is consistent with the data: an osmotic expansion of the mucoid layer is expected, regardless of any surfactant production, Marangoni effects, cell–cell forces, swimming motility, etc.

If area spreading is due to the osmotic effect of EPS II, we expect that a droplet of concentrated, purified EPS II on semi-solid agar (although not its radius \( R \)) exhibits exponential relaxation to equilibrium, exactly as in equations (3.1) and (3.2). The osmotic model also generates a rough prediction for the rate \( k \) (based on the hydraulic permeability [25] of agar) that is consistent with the data: an osmotic expansion of the mucoid layer is expected, regardless of any surfactant production, Marangoni effects, cell–cell forces, swimming motility, etc.

We expect that osmotically driven spreading should only occur on plates where the agar concentration is low enough that it cannot match the osmotic pressure of the EPS II in the colony; indeed the sinorhizobial spreading is well established as occurring only on soft agar (less than 1%) and is suppressed by surface dryness [9]. Quantitatively, the osmotic model (electronic supplementary material) predicts that the spreading rate \( k \) should scale in proportion to the agar concentration, and the
final equilibrated area $A_\infty$ of the mucoid layer should scale inversely with the agar concentration. Figure 2a–c confirms this expectation: when the washed, HMW EPS II concentrate is deposited onto plates containing agar concentrations in the range 0.4–0.8% (w/v), the spreading rate $k$ increases at high agar concentrations, while the amount of area gained decreases. Consequently, figure 2 demonstrates the physical mechanism whereby a concentrate of EPS II on a semi-solid agar surface spreads through osmotic action, with exponential relaxation kinetics.

If *S. meliloti* spread by the osmotic mechanism, then we expect that EPS II harvested from a mature (fully spread) colony will already be at osmotic equilibrium with the agar and should not be able to induce spreading behaviour when it is added to *S. meliloti* colonies that fail to spread. This expectation is consistent with data from Gao *et al.* [8], who found that harvested EPS II did not rescue spreading in mutants that were deficient in EPS II biosynthesis.

However, we also expect that reconcentrating the harvested EPS II should reestablish the osmotic gradient and allow the EPS II to induce spreading in a non-spreading strain. Therefore, we mixed washed, concentrated EPS II with Rm1021 (expR−, non-spreading) and with *E. coli* immediately before depositing these strains onto semi-solid agar. Electronic supplementary material, figure S1 and movies SM2 and SM3 show that spreading was initiated in both colonies, and that the colonies also exhibited dynamic streaming patterns very similar to those seen in the Rm8530 (expR+) strain.

We also investigated whether the cells’ tendency to migrate towards the outer perimeter of the mucoid layer is due to the EPS II or to chemotaxis towards nutrients available outside the colony. In general, the introduction of a chemottractant near a spreading colony did not notably affect the spreading kinetics or aggregation pattern in the colony (data not shown). To rule out a role for chemotaxis, we studied the behaviour of *S. meliloti* cultures in 1.5 μl droplets on a glass surface, where it is highly unlikely that nutrient or other gradients exist sufficient to induce chemotaxis: in figure 2a–j, each culture was first mixed with either concentrated EPS II, dilute EPS II, or water (no EPS II, as control). The addition of dilute or concentrated washed EPS II to droplets of Rm1021 (expR−) induced significant aggregation (compared to Rm1021 without exogenous EPS II), with large clusters of cells migrating to the outer perimeter of the droplet. Rm8530 (expR+) aggregates without addition of exogenous EPS II. These data show that the aggregation of cells and the migration of aggregate clusters to the outer perimeter of a droplet can be triggered simply by the presence of concentrated EPS II in the colony environment.

We also tested the effect of the autoinducer signal on the kinetics of spreading, as EPS II biosynthesis requires a functional SinI/SinR/ExpR quorum-sensing system. In figure 2, the sinI mutant (non-AHL producing) was inoculated onto semi-solid agar plates containing varying concentrations of exogenous 3-oxo-C16:1-HSL, which is the AHL autoinducer that was found to be most active in stimulating EPS II synthesis [8]. As expected, no spreading occurred on plates that lacked added AHL. The spreading is strongly activated when more than 200 nM AHL is present in the agar, consistent with induction of the quorum-sensing circuit for EPS II production. Interestingly, all AHL concentrations induced fairly similar spreading kinetics (i.e. similar rate $k$ in equation (3.2)), even though the final equilibrated area was sensitive to (AHL), as shown in figure 2e. This unintuitive result is predicted by the osmotic model in the electronic supplementary material: the rate $k$ (but not the amplitude $A_\infty$) of spreading is independent of the mass of EPS II that is present in the

Figure 2. Effect of agar concentration, AHL and EPS II on spreading and aggregation: (a) area of spreading EPS II pool (no cells) versus time after EPS II is deposited onto plates of 0.4–0.8% (w/v) agar; (b) the initial/initial ratio of areas ($A_0/A_\infty$) of the mucoid layer and (c) the rate $k$ of the spreading equilibration (from fitting data (a)) to equation (3.2)) both vary linearly with the agar concentration; (d) area-spreading kinetics of sin− strain (MG32 + pDG71) on 0.4% (w/v) agar/5% (w/v) TY containing different concentrations of exogenous autoinducer 3-oxo-C16:1-HSL; (e) the final/initial area ratio ($A_\infty/A_0$) obtained from (d) shows a typical quorum-sensing induction, increasing sharply for (AHL) $\geq$ 200 nM, while (f) the lag-time $t_\ell$ is insensitive to (AHL) ($\ell$–$j$) overlaid fluorescence + phase contrast images showing the outer perimeter of a droplet of culture on a glass surface, 12 h after addition of dilute or concentrated EPS II. EPS II causes Rm1021 (expR−) to form aggregates that migrate towards the edge of the droplet. Rm8530 (expR+) aggregates without addition of EPS II. (Online version in colour.)
mucoid layer: higher AHL concentrations can stimulate more EPS II production and yield a larger final colony area, but they do not affect the time required for equilibration.

(b) Formation of patterns
A striking feature of the electronic supplementary material, movie SM1 (as well as SM4, SM5 and SM6, which also show strain Rm8530) is the complex cellular motion and aggregation behaviour seen in the mucoid disc during spreading. Early in the spreading (by \( t \approx 10 \) h in the electronic supplementary material, movie SM1) most of the gfp-expressing cells are seen to migrate from the interior towards the perimeter of the mucoid layer. Subsequently, as the layer spreads, new cells are generated and shed rapidly from a very small number (as few as 20) of discrete sources located around the circumference. The electronic supplementary material presents a closer analysis of the fluorescence images, confirming that most new GFP is synthesized (i.e. most growth occurs) in these discrete sources, which appear to be clusters of cells that ride outward with the advancing edge of the mucoid disc. Daughter cells shed from the clusters travel away from the perimeter and eventually settle to the agar surface leaving the radial, feathered pattern. Close inspection of the time-lapse images further reveals—and phase contrast microscopy confirms—that only the younger cells near the moving perimeter are motile: older cells, closer to the centre of the disc, accumulate in aggregates on the agar surface. We interpret the eventual cessation of growth and motility in daughter cells as a consequence of the poor nutrient supply in the interior of the colony disc.

Previous authors have noted that motility-deficient \( S. \) meliloti do form a spreading mucoid layer, although lacking the complex patterns [8]. To study the relationship between motility and pattern formation, we imaged the spreading of the immotile \( fliP \) mutant. Like the wild-type, its area grows with exponential kinetics (see the electronic supplementary material, figure S2). However, the \( fliP \) cells disperse to a more limited extent within the fluid pool, so that the perimeter of the fluid layer spreads more rapidly than the cells. The \( fliP \) colony lacks the actively dividing clusters of cells on the perimeter of the mucoid layer; rather the cells remain closer to the initial droplet location and eventually settle onto the agar surface. Therefore, although flagella are not absolutely required for spreading of the mucoid layer, motility is important to maintaining the actively dividing cell clusters at the moving perimeter of the mucoid layer. The discussion addresses the physical mechanism by which EPS II and flagellar motility maintain the clusters at the perimeter.

In order to determine whether active swimming or simple Brownian diffusion (+ advection) of the cells is more important in generating the observed aggregation patterns, we performed computer simulations of spreading in a colony that contains clusters at the perimeter. (Details in the electronic supplementary material.) Figure 3 compares experimental data (a) with simulated patterns (b,c) generated by two different simulations. Simulations (b,c) both employ identical parameters for the physical dimension of the mucoid layer and its rate of spreading. They also both assume that clusters located on the moving perimeter shed new daughter cells into the mucoid layer. However, they make different assumptions about the motility of the daughter cells. In figure 3b, each daughter cell swims actively in a random direction, while in figure 3c each daughter cell is passively carried by the moving fluid (advection) while diffusing randomly within that fluid. Although both simulations generate a radial dispersion pattern of the cells, the swimming model (figure 3b) more closely resembles the experimental data. As discussed in the electronic supplementary material, the active swimming model captures the conical streaming motion of the daughter cells away from the source clusters that are located on the boundary. Cells moving by diffusion/advection deposit only short, narrow trails on the agar as the colony spreads.

Taken together, our data indicate that EPS II (rather than flagellar swimming) enables the spreading of the mucoid disc, the migration of the actively dividing clusters to the disc perimeter and the aggregation of the cells. However, the data and simulations also show that active swimming of the daughter cells (rather than passive diffusion) is important.

![Figure 3](http://rsbp.royalsocietypublishing.org/) Observed and simulated colony patterns: (a) green fluorescence image (false colour) of strain Rm8530 spreading on semi-solid agar; (b,c) cell location maps from two simulations of spreading. Simulations (electronic supplementary material) assume that new daughter cells move in the fluid either by (b) active swimming or (c) diffusion and advection in the spreading fluid and (d) entropic phenomena at work during \( S. \) meliloti spreading. Cells retaining quorum synthesize the osmolyte and crowding agent EPS II, causing them to aggregate and to cluster at the air/fluid/agar interface [26] at the perimeter of the colony. The osmolyte also draws water from the agar, causing the mucoid layer to swell and spread outward until it reaches osmotic equilibrium. (Online version in colour.)
in maintaining clusters at the perimeter of the colony and shaping the trails that are deposited by the moving boundary.

4. Discussion

Nogales et al. [7] showed that *S. meliloti* exhibits at least two different modes of motility on semi-solid agar. The first mode is a typical swarming behaviour that requires flagella and rhizobactin 1021 but does not require the quorum-sensing regulator ExpR. Here, we study the second mode, which is an ExpR-dependent, flagellum-independent motility. It requires the ExpR-regulated production of the galactoglucom exopolysaccharide II (EPS II) and it leads to the complex colony spreading patterns shown in figure 1 and the movies in the electronic supplementary material.

As its physical mechanism has been unclear, this behaviour was initially described as a form of swarming [5,9]. However, Nogales et al. argued that the behaviour was not necessarily true swarming, which is generally a coordinated motion of hyperflagellated cells that populate a surface at high density, as a biofilm or monolayer, and propel themselves by a complex, coordinated swimming motion [2–4]. Nogales et al. proposed that *S. meliloti* spreading is a form of sliding, a passive bacterial translocation in which expansive forces generated by growing cells push the colony outward on a low friction surface [2–4]. As sliding is often facilitated by the surfactant action of amphiphilic secretions such as lipopolysaccharides and glycolipids [2–4], Nogales et al. [7] suggested that the spreading of *S. meliloti* may be aided by a secreted surfactant, for example siderophore.

While the ExpR-dependent (and flagellum-independent) translocation of *S. meliloti* is not a form of swarming, our data indicate that it is not sliding either. A sliding colony typically appears as a compact, densely packed monolayer of cells that spreads virtually as a unit across the surface, without apparent motion of the individual cells. Yet, we find that the spreading *S. meliloti* do not form a dense layer on the agar, their spreading is driven by an osmotic equilibration (rather than by inter-cellular forces), and no surfactant needs be present for this spreading to occur: spreading was restored to non-spreading (e.g. expR-deficient) strains, and even induced in *E. coli*, by addition of the washed, concentrated HMW EPS II. Osmotic equilibration as a driving force for *S. meliloti* spreading explains the importance of EPS II production (via expR) in this behaviour, as well as the ability of succinoglycan (EPS I) overproduction to enable surface spreading in expR− strains [7] by acting as an alternative osmolyte.

Osmotic equilibration may contribute to spreading in other bacterial species. Osmotic flow was recently proposed to drive the spreading of *B. subtilis* biofilms on soft agar [14,27]. A recently described, mucin-driven spreading motility in *Pseudomonas aeruginosa* on soft agar also shares many features of sinorhizobial spreading [28]. However, those systems maintain a high cell density and do not form the complex patterns of cell aggregation that are seen in *S. meliloti*. Yeung et al. [28] proposed that the *P. aeruginosa* spreading be named ‘surfing’, a term that aptly describes the movement seen in the electronic supplementary material, movie SM1: clusters of actively dividing *S. meliloti* ride or surf on the advancing edge of the mucoid disc.

A key question is why the aggregates form and migrate to the colony edge. Poon and co-workers [15–17] showed that the depletion attraction causes cell aggregation in *S. meliloti* cultures that contain high concentrations of exopolysaccharide. Briefly, the depletion attraction is a crowding phenomenon whereby a high concentration of an inert, high molecular weight solute can induce larger particles suspended in the solution to aggregate [29,30]. The effect is entropic in origin, in that it does not arise from a particular interaction between the particles and the solute, but rather results from the full system’s (solution + suspended particles) tendency to increase its entropy: when the larger particles aggregate, or are pushed against the bounding surfaces of the solution, the solute molecules can access greater volume and therefore increase their entropy. Depletion plays an important role in intramolecular interactions within living cells, although its role at the extracellular level is less understood [30]. Osmotic flow is likewise an entropic phenomenon.

Overproduction of succinoglycan EPS I by *S. meliloti* generated sufficient depletion attraction to induce aggregation and sedimentation of a live culture, so that a liquid culture of *S. meliloti* separated into a clear supernatant phase and a settled (crystal-like) aggregate [15,17]. This phase separation is a crowding effect not attributable to sticking interactions between the cells or between the cells and the EPS. In fact, EPS I (MW ≈ 6 × 10⁶ g mol⁻¹ [16]) also induced aggregation of *E. coli* as well as heat-killed *S. meliloti*. Notably, cell motility inhibits the sedimentation: flagellar swimming shifted the sedimentation equilibrium, allowing cell aggregates to remain suspended at higher solute concentrations [16].

Those studies were performed on variants of the *S. meliloti* strain Rm1021 (which does not produce EPS II) and at polymer concentrations up to 1–2% (w/v). The Rm8530-based strains studied here appear to produce HMW EPS II at 1–5% (w/v), which should be ample to induce similar crowding effects. Sorroche et al. [31] have demonstrated that EPS II produced by the Rm8530 strain induces aggregation in planktonic cultures over 24 h and that EPS I has no role in this aggregation: mutants deficient in expR do not aggregate, while mutants deficient in EPS I production aggregated like the parental strains. These data indicate that EPS II production generates a depletion attraction between suspended sinorhizobia, sufficient to induce aggregation.

Further, depletion attractions drive larger particles out of a bulk volume and towards the boundaries and (especially) the corners of the container [26]; the entropic force pushing a particle towards a boundary surface is roughly twice as large as the force between two particles. Therefore, we expect the depletion attraction to push suspended, actively dividing cell clusters towards the fluid/air/agar interface at the outer perimeter of the colony and to keep them at this interface while the mucoid disc expands. This allows the clusters to ride at the expanding boundary where they have access to fresh nutrient, while they shed daughter cells into the interior of the mucoid disc. We found no evidence that chemotaxis is responsible for migration of the clusters towards the outer perimeter.

Our experiments indicate that flagellar swimming does not physically drive spreading of the colony; motility-deficient strains can generate mucoid pools that spread with the same osmotically driven kinetics as the wild-type. However, as the immotile cells aggregate and sediment more quickly [16], they are not carried outward with the spreading mucoid layer and the characteristic feathered patterns do not develop. Consequently, the significance of
flagellar motion is that it weakens the depletion attraction and delays the settling of the cells [16]; this facilitates spreading and pattern formation by preventing the actively dividing clusters from settling onto the agar surface. Our simulations also show that flagellar swimming of the daughter cells away from the actively growing clusters is responsible for the characteristic ‘streaming’ behaviour seen in the images and movies.

We see no physical reason why the ‘surfing’ motility should be limited to the soft agar surfaces. Rather, we expect it should also be possible in the natural habitat of S. meliloti. The ability of EPS II to drive colony spreading may then provide a biological rationale for EPS II production; although the LMW fraction of EPS II has a role in nodule invasion, the function of the HMW fraction of EPS II has been elusive [8,13]. In this regard, it is interesting that EPS II is regulated by quorum sensing. Two SinI-produced AHLs in particular, the C16:1 and 3-oxo-C16:1 homoserine lactones, are most effective in stimulating spreading [8]. If the purpose of EPS II production is to activate osmotic forces that disperse the colony, then quorum-sensing regulation ensures that the energetically costly EPS II is only produced when there is the potential for useful, macroscopic amounts of the exopolysaccharide to accumulate. In fact, only a small fraction (10–30%) of cells express wggR [8], indicating that the colony’s investment in biofilm synthesis of EPS II is tightly regulated in a community-wide fashion.

In summary, the efficient dispersion of S. meliloti across semi-solid agar is driven not by physical propulsive forces (such as flagellar rotation) generated within the cells, but rather by two physical chemical effects associated with the high concentration of polysaccharide. The osmotic pressure of the polysaccharide pulls water from the agar into the mucoid layer, while the depletion effect pushes the growing clusters to the moving perimeter of the colony. Both of these forces are ultimately driven by entropy increase in the EPS II-rich fluid. This does not mean that the colony expends no energy in dispersing itself through the environment: by performing the chemical work of synthesizing and secreting macroscopic amounts of exopolysaccharide EPS II into its environment—an energetically costly process that is accordingly controlled by a quorum-sensing network—the bacteria build up the free energy of their mucoid slime. They can then ride passively with the mucoid layer as it moves downhill in free energy (i.e. rising entropy), spreading outward until it reaches equilibrium with the larger environment.

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Data accessibility. No large datasets are involved in this work. However, data and software will be provided upon request.

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