Hanging by a thread: invasion of legume plants by rhizobia

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Nitrogen-fixing nodules on plants such as alfalfa, pea and vetch arise from the root inner cortex and grow via a persistent meristem. Thus, these nodules are defined as indeterminate. The formation of functional indeterminate nodules requires that symbiotic bacteria, collectively called rhizobia, gain access to the interior of roots and root nodules via infection threads. Recent work has begun to elucidate the important functions of the root cell cytoskeleton in infection thread formation. It has also recently become apparent that rhizobial Nod factors and rhizobial exopolysaccharides play key roles in the initiation and elongation of infection threads.

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Abbreviation
GFP green fluorescent protein
PIT pre-infection thread

Introduction

Bacteria belonging to the genera *Rhizobium*, *Sinorhizobium*, *Azorhizobium* and *Bradyrhizobium* (referred to here as rhizobia) can live inside root nodule cells of leguminous host plants and reduce atmospheric dinitrogen to ammonia, thereby providing the host with a ready source of nitrogen [1].

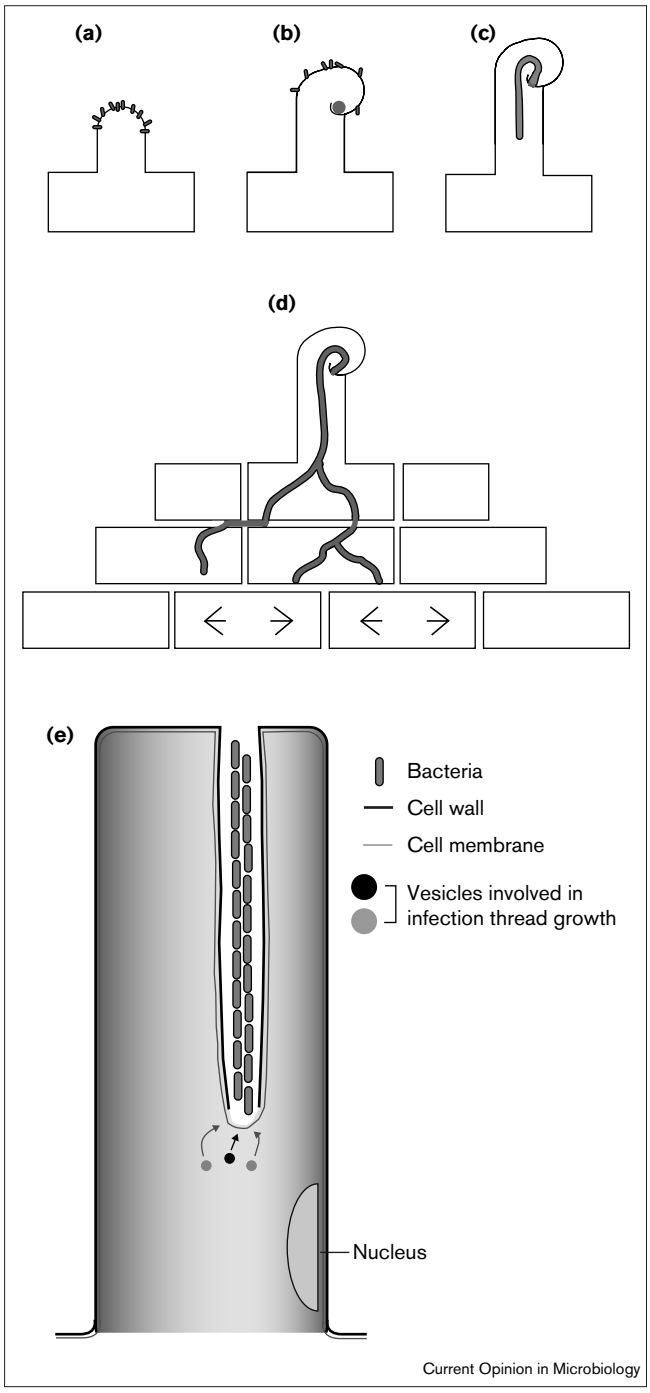
Nodulation requires the action of a lipo-oligosaccharide signaling molecule, Nod factor. Nod factors from all rhizobial species have the same basic chemical structure, but factors from different species differ with respect to the side groups bound to the oligosaccharide backbone and also in the length and degree of saturation of the lipid moiety [2,3•]. Plant responses mediated by Nod factor during nodulation of indeterminate hosts include: initiation of cell division in the inner cortex to form a nodule primordium, induction of plant genes, calcium oscillations and root hair distortion and curling [4–6].

Infection of the host begins when rhizobia become trapped between two root hair cell walls. This most often occurs when a deformed root hair forms a sharp bend or curl and bacteria bound to the root hair become trapped between appressed cell walls (Figure 1). Initiation of infection from such sites involves structural alterations of the root hair cell wall, followed by invagination of the root cell wall to form an incipient tubule and finally extension of the tubule by tip growth. This tubule, the infection thread, grows down the inside of the root hair and into the body of the root hair cell (epidermal cell). Rhizobia inside the infection thread grow and divide, keeping the tubule filled with bacteria. If the infection thread exits the epidermal cell, it does so by fusing with the distal cell wall, resulting in the release of bacteria into the intercellular space between the epidermal cell and the underlying cell layer. Invagination and tip growth, similar to that seen at the beginning of infection thread growth, occurs at the underlying cell wall and a bacteria-filled thread propagates further towards the inner root cortex [7]. The inward-growing infection thread network and the outward-growing nodule eventually meet. Branching of the thread as it approaches and then enters the nodule primordium ensures that a sufficient number of nodule cells are colonized. In indeterminate nodules, the infection threads do not penetrate the nodule meristem cells that constitute the growth zone, but rather grow and ramify in a zone behind the meristem called the infection zone or zone II. Bacteria eventually exit the infection thread network, thereby entering the cytoplasm of nodule cells; they then differentiate into bacteroids and fix atmospheric nitrogen [8•,9•]. In this review, we summarize the infection process in indeterminate hosts, and discuss recent advances in understanding the behavior of rhizobia in infection threads and the rhizobial genes and extracellular factors needed for the initiation and growth of these structures.

Redirection of root hair tip growth

Once rhizobia attach to root hairs and secrete Nod factor, the stage is set for their invasion of the plant. Successful invasion necessitates the reorientation of plant cell wall growth, in order to allow the root hairs to deform and the infection thread to initiate by invagination of the root hair cell wall. These major changes in cell shape and growth direction correlate with, and are probably caused by, significant alterations in the plant cytoskeleton. Actin depolymerization is one of the earliest effects seen in root hairs following exposure to Nod factor. Cárdenas *et al.* [10] followed the dynamics of filamentous actin (microfilaments) in live root hair cells of *Phaseolus vulgaris* (bean) by microinjecting FITC-phalloidin and monitoring its fluorescence after addition of *Rhizobium etli* Nod factor. Control root hairs displayed the normal complement of long microfilaments parallel to the long axis of the root hair. In contrast, 5–10 minutes after exposure to Nod factor, most of the fluorescence migrated to the root hair tip and changed from fibrous to diffuse. Because phalloidin binds polymerized actin and not actin monomers, it is likely that the diffuse fluorescence at the tip represents short actin polymers. Another study of actin dynamics during root hair growth also provided evidence for Nod factor-stimulated reorganization of the actin cytoskeleton. These authors hypothesize that this reorganization redirects vesicle

Figure 1



Schematic diagram of infection thread formation in alfalfa. **(a)** *S. meliloti* binding to a growing root hair. **(b)** Under the influence of Nod factors, the root hair curls and traps a microcolony of bacteria. **(c)** The root hair cell wall invaginates at the site of the microcolony and extends via tip growth down the root hair. **(d)** Infection threads ramify and grow toward inner cortex cells, which are dividing and forming the meristem of the nodule primordium. Mitotic figures are drawn in two of the bottom cells to represent the initiation of mitosis in these cells. **(e)** An enlarged view of the root hair shown in (c). The curl has been unrolled to show that topologically, the bacteria in the infection thread are still outside the root hair. Bacteria remain topologically outside the root until they bud from the tip of the thread and enter nodule cells as membrane-enclosed bacteria.

traffic from the root hair tip to the site of new root hair outgrowths, which eventually result in the typical root hair deformation and curling observed during infection [11•].

The microtubule cytoskeleton also displays significant alterations during the course of infection; for example, microtubule reorganization in the plant cortical cells is a prerequisite for formation of the nodule primordium [12]. Microtubules are probably also involved in regulating infection thread growth. They surround the nucleus and appear to connect it to the tip of the infection thread as they both migrate down the root hair [13••]. The general involvement of microtubules in tip growth [14] is consistent with the idea that infection thread extension may be a specialized form of tip growth.

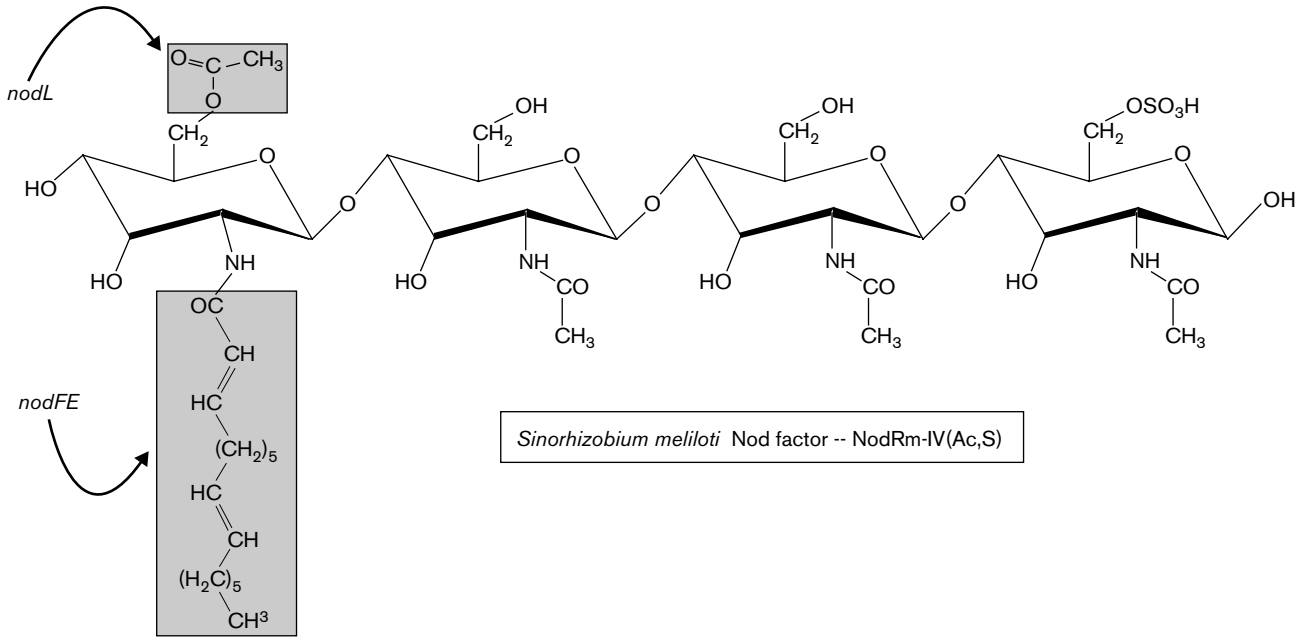
Plant cell division and growth of the thread

During the initiation of indeterminate nodules, root cells of the inner cortex respond to Nod factor by re-entering the cell cycle and dividing anticlinally, thereby giving rise to the nodule primordium and meristem [15,16]. In the outer cortex, above the developing nodule, a column or two of root cells also begin to divide. The cytoplasm moves from the cell periphery to a central position, as it normally does during cell division, but the cells usually progress no further and apparently arrest in the G2 phase of the cell cycle [17]. The cells in these columns give rise to columns of aligned cytoplasmic bridges called PITs (pre-infection threads) through which the inwardly growing infection thread can move. Thus the path of the growing infection thread through the outer cortex is marked by the aligned PITs [16].

Timmers *et al.* [13••] have recently shown that a *nodF/nodL* double mutant of *Sinorhizobium meliloti*, which synthesizes a Nod factor with an altered lipid group and no acetyl modification at the nonreducing end (Figure 2), is unable to induce PITs on its host alfalfa. This laboratory earlier showed that the *nodF/nodL* double mutant also did not readily initiate infection threads [18]. These phenotypes are not the result of a completely inactive Nod factor, because the mutant can induce root hair deformation and activate cell division in the root inner cortex. This suggests that the nonreducing end of wild-type *S. meliloti* Nod factor contains information essential for triggering formation of infection threads and PITs in alfalfa.

Interestingly, purified *S. meliloti* Nod factor alone is not sufficient to trigger PIT formation in alfalfa, even though it can induce inner cortex cell division. This is in contrast to the action of purified Nod factor of *Rhizobium leguminosarum*, which is sufficient to trigger PITs on its host plant, vetch. Thus, in alfalfa, formation of PITs may be distinct from initiation of nodule primordia, even though PIT formation superficially appears to be an arrested response in outer cortex cells to the process that triggers cell division in the inner cortex. The fact that *nodF/nodL* double mutants fail to initiate infection threads and the formation of PITs implies that these processes are somehow related [13••].

Figure 2



Nod factor structure. One form of Nod factor synthesized by *S. meliloti* is shown. The upper arrow indicates the acetyl group added by NodL (top gray box) and the lower arrow indicates the lipid moiety, the length and degree of saturation of which is modified by NodF and NodE (bottom gray box). Other species of rhizobia, such as *R. leguminosarum,* have NodL, NodE and NodF enzymes that perform similar functions.

Bacterial genes required for infection thread growth

nod genes

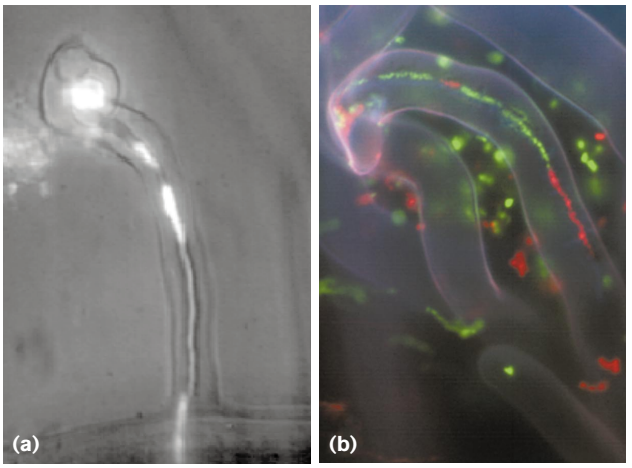
Recently, Walker and Downie [19••] published experiments in which they inoculated a *nodFEMNTLO* deletion mutant of *R. leguminosarum* biovar *vicae* onto vetch seedlings. The mutant synthesized Nod factor molecules that contained no host-specific decorations and it was unable to induce nodules on the seedlings. Microscopic observation of roots showed that the bacteria accumulated in large masses in the crooks of curled hairs and failed to initiate infection thread formation. Overexpression of *nodO* was able to rescue this phenotype to some extent: as the deletion mutant no longer accumulated in large masses, some root hairs initiated infection threads, and some nodules formed. This is a particularly intriguing result, because again it shows that Nod factor decorations are needed for the initiation of infection but not for all plant responses. NodO protein is known to be secreted from bacterial cells, has been shown to form pores in membranes, and can be a host-range determinant [20,21]. Perhaps NodO rescued the *nodFEMNTLO* phenotype by stimulating ion flow across a membrane, thereby amplifying a weaker-than-normal signal transmitted by the stripped-down version of Nod factor. Additional support for the importance of *nodO* and for modifications on the nonreducing end of Nod factor comes from experiments showing that a *nodE*/*nodO* double mutant induced aberrant infections, whereas *nodO* and *nodE* single mutants did not [19••]. The infection phenotype of the *nodO*/*nodE* mutant was similar to the phenotype of the *nodFEMNTLO* strain, except that a few infection threads formed. This was in spite of the presence of bacterial aggregates at infection sites. The infection threads were swollen, distorted and contained high numbers of infecting bacteria.

One striking phenotype of the *nodFEMNTLO* and *nodE*/*nodO* mutants is that the bacteria appear to be growing too much during their invasion of the host. Could Nod factor have a role in slowing the growth rate of rhizobia during the process of invasion and infection? Although Nod factor conceivably might act directly to influence the growth rate of the invading bacteria, it is more likely that it acts indirectly to trigger responses in the root cells, which in turn might down-regulate bacterial growth. Because the plant needs to control a rapidly proliferating population of bacteria within the infection thread, which itself has a limited expansion rate, it makes sense that the plant would exert some form of growth regulation on the invading rhizobia.

Exopolysaccharide synthesis genes

Nodules on alfalfa fail to develop properly when inoculated with mutants of *S. meliloti* that are unable to produce the exopolysaccharide succinoglycan [22,23]. Nodules induced by such mutants are small, lack a persistent meristem, and contain few or no bacteroids [24]. Work from several laboratories suggests that signaling is an important function of

Figure 3



The bacterial population inside alfalfa infection threads is derived from the clonal expansion of a few founder cells. **(a)** An alfalfa root hair infected with a mixed population of GFP-tagged and non-GFP-tagged *S. meliloti*. **(b)** An alfalfa root hair infected with a mixed population of *S. meliloti*. One strain expressed the red fluorescent protein DsRed (colored red) and the other expressed a variant of GFP (colored green). Both images show mixed infections that give rise to infection threads containing a small number of sectors, indicating that the entire population in the thread results from the clonal expansion of a few founder cells.

the exopolysaccharides [25,26]. It has been hypothesized that succinoglycan is important for symbiosis because it may suppress or delay host defense responses that would compromise the infection. Recently, it was shown that succinoglycan is important for initiating and extending infection threads, as *exo* mutants, which do not synthesize this exopolysaccharide, were inefficient at initiating infection threads, and threads that were initiated terminated early [24,27–29]. In certain cases, a second form of exopolysaccharide, EPSII, or lipopolysaccharide, KPS, can obviate the need for succinoglycan. Although these polysaccharides can replace the requirement for succinoglycan in terms of allowing the development of functional nodules, it has recently been shown that they are far less efficient than succinoglycan in promoting the initiation and extension of infection threads [30•].

Growth behavior of the bacterial population inside infection threads

Studies using green fluorescent protein (GFP)-tagged *S. meliloti* strains to infect alfalfa have shown that the population of bacteria inside the infection thread originates from the clonal expansion of a small number of founder cells which entered the thread early. This can be seen when a root hair is co-infected with two bacterial strains that can be differentiated on the basis of their fluorescence. When both strains enter the infection thread, they form alternating sectors with relatively sharp boundaries [31] (Figure 3). Each sector represents the clonal expansion of one or a few founding bacteria.

Microscopic observations show that when they nodulate alfalfa, *S. meliloti* cells usually form two or three columns inside the infection threads. The bacteria appear to be loosely associated with one another, and the columns have a braided appearance. Bacteria in the columns are aligned with their long axis more or less parallel to the long axis of the thread [29,31].

It is not currently known how rhizobia travel down infection threads. They do not have flagellae when inside the thread, and hence do not swim toward the growing tip. Because the bacteria remain in neatly ordered columns, and the sector boundaries remain sharp even in mixed infections, it is possible that the bacteria don't push past each other during growth, but rather move in unison down the thread. One form of movement compatible with these observations is sliding motility. In this rather obscure form of bacterial locomotion, loosely associated bacteria move in unison, and the bacterial cells themselves do not move with respect to each other during growth, division or translocation. This form of motility was first described in 1972 [32], and was recently shown to be operative in *Mycobacteria*, which were previously considered nonmotile [33•].

It is also possible that the bacterial columns inside the infection thread are cohesive because they are connected to a structure in the host cytoplasm such as the cytoskeleton and as such are under tension. This tension could provide the motive force for bacterial translocation down the thread, and could explain why sector boundaries remain sharp during infection thread growth.

Conclusions

The ability to monitor bacterial cells and cytoskeletal structures within plant cells with fluorescent probes has spurred recent progress in our understanding of the biogenesis of infection threads and the organization and movement of rhizobia within them. The completion of genome sequences of both bacterial and plant partners in the near future promises additional ways to gain a molecular understanding of the cellular reorganization that occurs after bacterial attachment to root hairs. It will also be necessary to learn more about how the host regulates invading microbes and how it distinguishes rhizobia from pathogens. It is likely that defense mechanisms used against pathogens are somehow modified, such that instead of sequestering the invading bacteria, the plant channels this response toward cell wall invagination, root hair distortion, and reactivation of cortical cell division. Ultimately, it will be crucial to begin to integrate the various events that occur early in infection and identify molecular mechanisms; for example, are asymmetric localization and spiking of Ca^{2+} prerequisites for actin filament reorganization, perhaps via Ca^{2+}-mediated actin depolymerization? Testing hypotheses such as these holds promise for revealing the molecular interactions driving early symbiotic events.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Hirsch AM: **Developmental biology of legume nodulation.** *New Phytol* 1992, **122**:211-237.

2. Pueppke SG: **The genetic and biochemical basis for nodulation of legumes by rhizobia.** *Crit Rev Biotechnol* 1996, **16**:1-51.

3. • Perret X, Staehelin C, Broughton WJ: **Molecular basis of symbiotic promiscuity**. *Micro Mol Biol Rev* 2000, **64**:180-201.
A comprehensive review of host range determinants in a variety of rhizobial–legume symbioses.

4. Cárdenas L, Feijo JA, Kunkel JG, Sánchez F, Holdaway-Clarke T, Hepler PK, Quinto C: ***Rhizobium* nod factors induce increases in intracellular free calcium and extracellular calcium influxes in bean root hairs.** *Plant J* 1999, **19**:347-352.

5. Denarié J, Debellé F, Promé JC: ***Rhizobium* lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis.** *Annu Rev Biochem* 1996, **65**:503-535.

6. Ehrhardt DW, Wais R, Long SR: **Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals.** *Cell* 1996, **85**:673-681.

7. van Spronsen PC, Bakhuizen R, van Brussel AAN, Kijne JW: **Cell wall degradation during infection thread formation by the root nodule bacteria *Rhizobium leguminosarum* is a two-step process.** *Eur J Cell Biol* 1994, **64**:88-94.

8. • Margolin W: **Differentiation of free-living rhizobia into nitrogen-fixing bacteroids.** In *Prokaryotic Development*. Edited by Brun YV, Shimkets LJ. Washington: ASM Press; 2000:441-466.
See annotation to [9•].

9. • Oke V, Long SR: **Bacteroid formation in the *Rhizobium*-legume symbiosis.** *Curr Opin Microbiol* 1999, **2**:641-646.
Two in-depth reviews [8•,9•] of the stages of the symbiosis from the bacterial point of view that focus on the infrequently discussed problem of bacteroid differentiation.

10. Cárdenas L, Vidali L, Dominguez J, Perez H, Sánchez F, Hepler PK, Quinto C: **Rearrangement of actin microfilaments in plant root hairs responding to *Rhizobium etli* nodulation signals.** *Plant Physiol* 1998, **116**:871-877.

11. • Miller DD, de Ruijter NCA, Bisseling T, Emons AMC**. The role of actin in root hair morphogenesis: studies with lipochito-oligosaccharide as a growth stimulator and cytochalasin as an actin perturbing drug.** *Plant J* 1999, **17**:141-154.
Actin dynamics during the course of root hair growth and rhizobial infection are examined, using Nod factors and an actin inhibitor as probes. A model is proposed in which actin localization guides vesicle trafficking to growing areas of the root hair; upon addition of Nod factor and reorganization of actin, vesicle traffic is redirected to new sites, ultimately resulting in root hair deformation.

12. Timmers ACJ, Auriac MC, De Billy F, Truchet G: **Nod factor internalization and microtubular cytoskeleton changes occur concomitantly during nodule differentiation in alfalfa.** *Development.* 1998, **125**:339-349.

13. •• Timmers AC, Auriac MC, Truchet G: **Refined analysis of early symbiotic steps of the *Rhizobium–Medicago* interaction in relationship with microtubular cytoskeleton rearrangements.** *Development* 1999, **126**:3617-3628.
A detailed histological description of microtubule cytoskeletal changes in the plant cells at all stages of infection, taking advantage of rhizobial mutants that synthesize altered Nod factor. The authors present a useful model that correlates known cytoskeletal changes with the infection time course.

14. Geitmann A, Emons AMC: **The cytoskeleton in plant and fungal cell tip growth.** *J Microsc* 2000, **198**:218-245.

15. Dudley ME, Jacobs TW, Long SR: **Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti*.** *Planta* 1987, **171**:289-301.

16. van Brussel AAN, Bakhuizen R, van Spronsen PC, Spaink HP, Tak T, Lugtenberg BJJ, Kijne JW: **Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*.** *Science* 1992, **257**:70-72.

17. Yang WC, De Blank C, Meskiene I, Hirt H, Bakker J, Van Kammen A, Franssen H, Bisseling T: ***Rhizobium* Nod factors reactivate the cell cycle during infection and nodule primordium formation, but the cycle is only completed in primordium formation.** *Plant Cell* 1994, **6**:1415-1426.

18. Ardourel M, Demont N, Debellé F, Maillet F, de Billy F, Promé J, Denarié J, Truchet G: ***Rhizobium meliloti* lipooligosaccharide nodulation factors: different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses.** *Plant Cell* 1994, **6**:1357-1374.

19. •• Walker SA, Downie JA: **Entry of *Rhizobium leguminosarum* bv. *viciae* into root hairs requires minimal nod factor specificity, but subsequent infection thread growth requires *nodO* or *nodE*.** *Mol Plant Microbe Interact* 2000, **13**:754-762.
This report shows that the nonreducing end of Nod factor is critical for the formation of functional infection threads. An especially important finding described here shows that expression of NodO, a putative pore-forming protein, can rescue the infection defects caused by *nod* mutations that result in Nod factors that lack host-specific decorations.

20. Sutton JM, Lea EJA, Downie JA: **The nodulation signaling protein NodO from *Rhizobium leguminosarum* bv *viciae* forms ion channels in membranes.** *Proc Natl Acad Sci USA* 1994, **91**:9990-9994.

21. Vlassak KM, Luyten E, Verreth C, van Rhijn P, Bisseling T, Vanderleyden J: **The *Rhizobium* sp. BR816 can function as a determinant for nodulation of *Leucaena leucocephala*, *Phaseolus vulgaris* and *Trifolium repens* by a diversity of *Rhizobium* spp.** *Mol Plant Microbe Interact* 1998, **11**:383-392.

22. Leigh JA, Signer ER, Walker GC: **Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules.** *Proc Natl Acad Sci USA* 1985, **82**:6231-6235.

23. Finan TM, Hirsch AM, Leigh JA, Johansen E, Kuldau GA, Deegan S, Walker GC, Signer ER: **Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation.** *Cell* 1985, **40**:869-877.

24. Yang C, Signer ER, Hirsch AM: **Nodules initiated by *Rhizobium meliloti* exopolysaccharide mutants lack a discrete, persistent nodule meristem.** *Plant Physiol* 1992, **98**:143-151.

25. Djordjevic SP, Chen H, Batley M, Redmond JW, Rolfe BG: **Nitrogen fixation ability of exopolysaccharide synthesis mutants of *Rhizobium* sp strain NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides.** *J Bacteriol* 1987, **169**:53-60.

26. González JE, Semino CE, Wang LX, Castellano-Torres LE, Walker GC: **Biosynthetic control of molecular weight in the polymerization of the octasaccharide subunits of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*.** *Proc Natl Acad Sci USA* 1998, **95**:13477-13482.

27. Leigh JA, Walker GC: **Exopolysaccharides of *Rhizobium*: synthesis, regulation and symbiotic function.** *Trends Genet* 1994, **10**:63-67.

28. Cheng HP, Walker GC: **Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*.** *J Bacteriol* 1998, **180**:5183-5191.

29. van Workum WAT, van Slageren S, van Brussel AAN, Kijne JW: **Role of exopolysaccharides of *Rhizobium leguminosarum* bv *viciae* as host plant-specific molecules required during nodulation of *Vicia sativa*.** *Mol Plant Microbe Interact* 1998, **11**:1233-1241.

30. • Pellock BJ, Cheng H-P, Walker GC: **Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides.** *J Bacteriol* 2000, **182**:4310-4318.
References [28,29,30•] demonstrate that succinoglycan is crucial for the initiation and extension of infection threads in alfalfa and vetch. Other polysaccharides, including EPSII and KPS, are less efficient at mediating invasion.

31. Gage DJ, Bobo T, Long SR: **Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa, *Medicago sativa*.** *J Bacteriol* 1996, **178**:7159-7164.

32. Henrichsen J: **Bacterial surface translocation: a survey and a classification.** *Bacteriol Rev* 1972, **36**:478-503.

33. • Martínez A, Torello S, Kolter R: **Sliding motility in mycobacteria.** *J Bacteriol* 2000, **181**:7331-7338.
This paper shows that *Mycobacteria*, previously thought to be nonmotile, can translocate across semisolid media by sliding. This form of motility has several features in common with *S. meliloti* translocation down infection threads.