region, with analytical errors (1σ) of 4% or less for both $^{14}N/^{15}N$ and $^{12}C/^{13}C$.
19. While the anomalous regions in C and N have slightly different shapes, the differences are at the limit of the lateral resolution of these smoothed images and are, therefore, of limited importance. However, all extracted C and N isotopic ratios (e.g., Fig. 1) refer to identical regions of interest and are, thus, spatially correlated.
20. Th. Fouchet *et al*., *Icarus* **143**, 223 (2000).
21. K. Hashizume, M. Chaussidon, B. Marty, F. Robert, *Science* **290**, 1142 (2000).
22. T. Owen, P. R. Mahaffy, H. B. Nieman, S. Atreya, M. Wong, *Astrophys. J.* **553**, L77 (2001).
23. K. Hashizume, M. Chaussidon, B. Marty, K. Terada, *Astrophys. J.* **600**, 480 (2004).
24. J. Aléon, F. Robert, M. Chaussidon, B. Marty, *Geochim. Cosmochim. Acta* **67**, 3773 (2003).
25. M. N. Fomenkova, S. Chang, L. M. Mukhin, *Geochim. Cosmochim. Acta* **58**, 4503 (1994).
26. C. Arpigny *et al*., *Science* **301**, 1522 (2003).
27. P. J. Heaney, E. P. Vicenzi, L. A. Giannuzzi, K. J. T. Livi, *Am. Mineral.* **86**, 1094 (2001).
28. J. P. Bradley, *Science* **265**, 925 (1994).
29. P. Hoppe, S. Amari, E. Zinner, R. S. Lewis, *Geochim. Cosmochim. Acta* **59**, 4029 (1995).
30. L. P. Keller, S. Messenger, J. P. Bradley, *J. Geophys. Res.* **105**, 10397 (2000).
31. S. J. Clemett, C. R. Maechling, R. N. Zare, P. D. Swan, R. M. Walker, *Science* **262**, 721 (1993).
32. G. J. Flynn, L. P. Keller, D. Joswiak, D. E. Brownlee, *Lunar Planet. Sci.* **33**, 1320 (2002).
33. D. E. Brownlee, in *Analysis of Interplanetary Dust*, M. E. Zolensky, T. L. Wilson, F. Y. M. Rietmeijer, G. J. Flynn, Eds. (AIP Conference Proceedings 310, American Institute of Physics, Woodbury, NY, 1994), pp. 5–8.
34. D. E. Brownlee, D. J. Joswik, M. E. Kress, S. Taylor, J. Bradley, *Lunar Planet. Sci.* **33**, 1786 (2002).
35. J. P. Bradley, D. E. Brownlee, *Science* **231**, 1542 (1986).
36. W. D. Langer, T. E. Graedel, M. A. Frerking, P. B. Armentrout, *Astrophys. J.* **277**, 581 (1984).
37. W. D. Langer, T. E. Graedel, *Astrophys. J.* **69**, 241 (1989).
38. S. A. Sandford, M. P. Bernstein, J. P. Dworkin, *Meteorit. Planet. Sci.* **36**, 1117 (2001).
39. This work was supported by NASA grants NAG5-13467 to C.F., and NAG5-10623 and NAG5-10696 to J.B.. The work by J.B., Z.D., S.B., and G.G. was performed under the auspices of the U.S. Department of Energy, National Nuclear Security Administration by the University of California, Lawrence Livermore National Laboratory (LLNL) under contract No. W-7405-Eng-48. Department of Energy (DOE) contract DE-AC03-76SF00098 supported electron microscopy performed at the National Center for Electron Microscopy at Lawrence Berkeley National Laboratory (LBNL) and infrared spectroscopy performed at the Advanced Light Source (ALS) at LBNL. We thank T. Smolar of Washington University for NanoSIMS maintenance and support, M. Martin and ALS personnel for providing the ALS beamline, and K. Moore at LLNL for assistance with energy-filtered imaging.

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Legionella Effectors That Promote Nonlytic Release from Protozoa

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***Legionella pneumophila*, the bacterial agent of legionnaires' disease, replicates intracellularly within a specialized vacuole of mammalian and protozoan host cells. Little is known about the specialized vacuole except that the Icm/Dot type IV secretion system is essential for its formation and maintenance. The *Legionella* genome database contains two open reading frames encoding polypeptides (LepA and LepB) with predicted coiled-coil regions and weak homology to SNAREs; these are delivered to host cells by an Icm/Dot–dependent mechanism. Analysis of mutant strains suggests that the Lep proteins may enable the *Legionella* to commandeer a protozoan exocytic pathway for dissemination of the pathogen.**

Several intracellular pathogens including *Legionella pneumophila*, *Mycobacterium avium* (*1*), *Chlamydia spp.* (*2*), and *Francisella tularensis* (*3*) are able to replicate within protozoan trophozoites. Thus, free-living amoebae may serve as a significant reservoir for pathogens in the environment, perhaps even as a "training environment" for the selection of virulence-related traits in these pathogens (*4*). *L. pneumophila*, the causative agent of legionnaires' disease, is frequently detected in association with *Hartmanella vermiformis* at the sources of infection during outbreaks (*5*). Under experimental conditions, *L. pneumophila* can multiply within and kill a variety of phylogenetically unrelated protozoa ranging from *Acanthamoeba castellani* to the genetically well characterized social amoeba *Dictyostelium discoideum* (*6*, *7*).

Intracellular pathogens have evolved three distinct strategies for surviving phagosome-lysosome fusion. Two of these mechanisms, tolerance of the toxic environment and escape from the phago-lysosome into the cytosol, are used by a variety of pathogens such as *Salmonella enterica* and *Listeria monocytogenes*, respectively. *L. pneumophila* and several other prokaryotic and eukaryotic intracellular pathogens use a third strategy—prevention of phagosome-lysosome fusion. After uptake of *L. pneumophila* by macrophages or protozoa, the bacteria are found within a specialized vacuole that does not fuse with lysosomes and does not acidify (*8*, *9*); this allows replication to proceed. The specialized vacuole associates with endoplasmic reticulum (ER)–derived secretory vesicles (*10*), mitochondria, and rough ER (*11*, *12*) and, near the end of the replicative cycle, acquires late endosomal markers (*13*). These observations strongly suggest that the bacteria play an active and continuous role in modulating organelle trafficking events from within the confines of their specialized vacuole.

A group of 24 genes called *icm* (intracellular multiplication) (*14*) or *dot* (defect in organelle trafficking) (*15*) are required for intracellular multiplication of *L. pneumophila*. Sequence similarity between several Icm/Dot proteins and those of the conjugative system of IncI plasmids colIb-9 and R64 (*16*) indicates that the Icm/Dot proteins form a type IV secretion system (TFSS) that delivers effectors to host cells. However, *L. pneumophila* mutants lacking the previously identified RalF or LidA effectors do not display obvious defects in organelle trafficking or intracellular replication (*17*, *18*). Because direct biochemical observation of infected cells has not led to the identification of additional effectors in *L. pneumophila*, we searched the genome sequence database for candidate genes.

As the modulation of organelle trafficking events appears to be important during intracellular multiplication, we looked for *L. pneumophila* effectors that resemble components of the SNARE system (*19*) and that might somehow disable or alter its function. Two such open reading frames (ORFs) were identified. Both exhibit limited sequence similarity to mammalian EEA1 and yeast USO1, proteins known to be involved in vesicle trafficking. Both *Legionella* ORFs are predicted to encode large regions of α-helical coiled-coils, structures present in EEA1 and USO1 and also commonly found in SNAREs (table S1).

As there are no functional assays for these putative effectors, we investigated whether *L.*

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pneumophila delivers the ORF products to host cells. We used a gene fusion approach that takes advantage of the calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*. Adenylate cyclase hybrid proteins have very low activity in the bacterial cells in which they are synthesized but are strongly activated if they are translocated to the cytosol of eukaryotes and interact with calmodulin (*20*). We constructed gene fusions that encoded chimeras of the *Legionella* effector proteins LepA and LepB with the catalytic domain encoded by *cyaA*. We then tested the ability of *L. pneumophila* to deliver the hybrid proteins to host cells by measuring the levels of cAMP in J774 macrophages infected by different *L. pneumophila* strains. When wild-type *L. pneumophila* expressing LepA-CyaA and LepB-CyaA under control of the Ptac promoter were used to infect J774 cells, large amounts of adenosine 3′,5′-monophosphate (cAMP) were produced within 30 min after infection (table S2). Controls showed that cAMP production depended on the presence of the LepA or LepB sequences. Translocation also required the DotA and IcmT proteins, which indicates that the proteins are delivered via the Icm/Dot TFSS.

To evaluate whether LepA or LepB plays any role during intracellular multiplication of *L. pneumophila*, we constructed strains lacking either or both genes. We used a standard assay for intracellular replication to examine the abilities of the strains to replicate in both human and protozoan hosts. This assay detects colony-forming units (CFUs) released into the supernatant at different times following infection. None of the three strains showed a detectable replication defect in several mammalian phagocytic cells, including human macrophages, murine J774 macrophages, and human macrophage-like U937cells (fig. S1). In contrast, all three mutant strains were recovered in smaller numbers than wild-type *L. pneumophila* during cocultivation with the protozoan host *A. castellani*. Much smaller numbers of CFUs were recovered for strain JC51 in which both genes were deleted (Fig. 1A). To verify that the defect is due to the absence of the LepA and LepB proteins, we used plasmids encoding either LepA or LepB. As expected, either plasmid could reverse the defect in JC51 (fig. S2). Similar results were obtained with another protozoan, the social amoeba *D. discoideum* (*21*). We conclude that although LepA and LepB are not required for replication in mammalian cells, they each play a partially redundant role in protozoan host cells.

To test whether the bacteria were capable of intracellular growth but were not released from the host cells, we performed single-step growth experiments in which the total bacterial contents of the host cells and the extracellular medium were counted after artificial lysis of the infected amoebae (Fig. 1B). These experiments revealed that the *lepA* and *lepB* mutations, either singly or together, had little effect on intracellular multiplication; even the JC51 double mutant replicated almost as well as the JR32 wild-type strain. Therefore, the lower number of CFUs recovered for the mutants in the standard assay depicted in Fig. 1A must be due to a defect in release from the protozoan cells, magnified over the course of several infection cycles.

To confirm that the bacteria are able to replicate inside protozoa and to observe their intracellular location, *D. discoideum* cells infected with either the wild-type JR32 or the JC51 mutant strain were examined by transmission electron microscopy (TEM) at 0.5, 5, and 24 hours after infection. After 0.5 hour and 5 hours, the samples contained similar numbers of bacteria in membrane-bound compartments (fig. S3). By 24 hours, there were few intact cells remaining for either sample. A rare intact *D. discoideum* cell infected with JC51 24 hours after infection is shown in Fig. 1C; there are

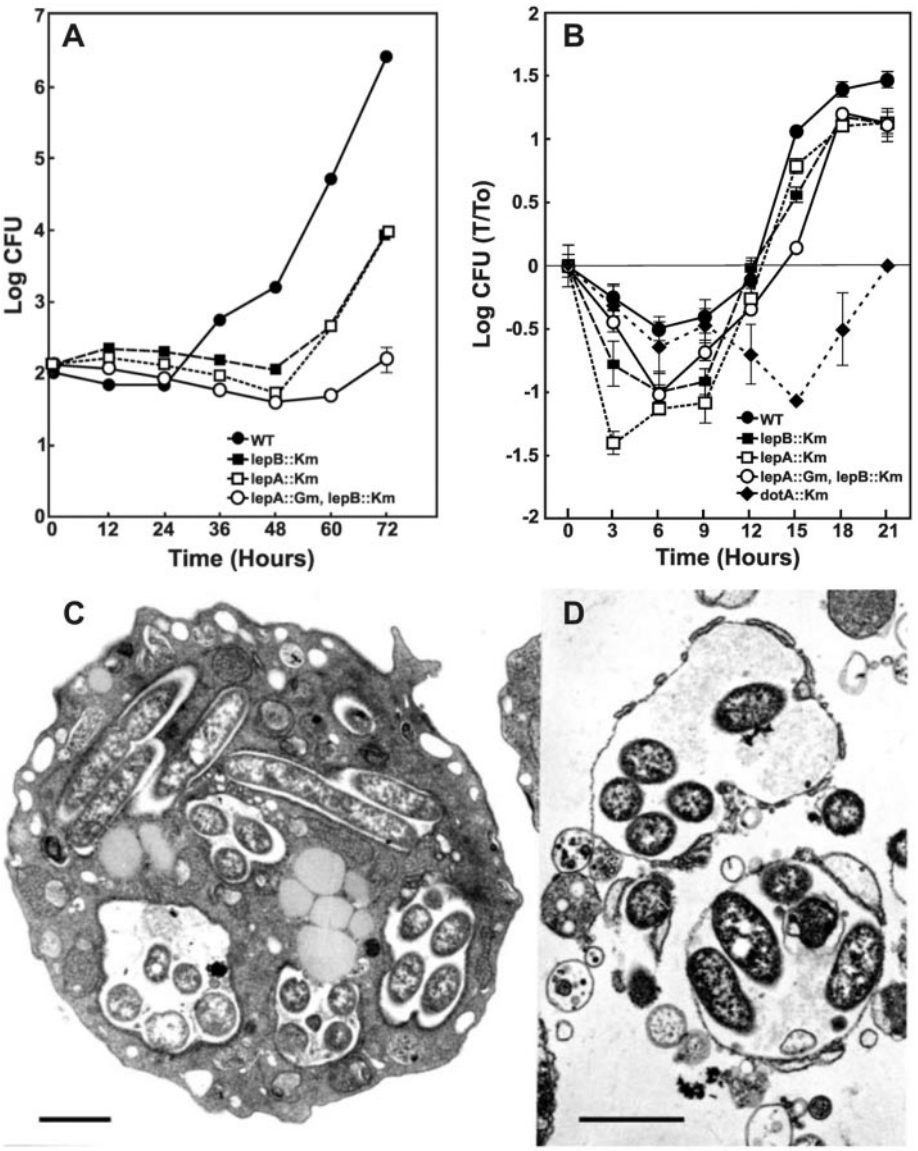

Fig. 1. *L. pneumophila lepA* and *lepB* mutants are defective for release from *A. castellanii*, but can multiply intracellularly. *A. castellanii* trophozooites were seeded into 24-well tissue culture plates, infected with *L. pneumophila* strains, and the numbers of CFUs released into the supernatants were measured. (**A**) The *lepA* or *lepB* single mutants are partially defective for release of *Legionella* when cocultured with *A. castellanii*. The *lepA, lepB* double mutant is severely defective for release of *Legionella*. (**B**) CFUs from a single step of growth for wild-type and the *lepA* and *lepB* mutants were measured in cell lysates. The single or double *lep* mutants exhibited levels of intracellular replication similar to wild type's in the first round of infection. Values represent the mean of at least three independent samples ± SD. (**C** and **D**) TEM images of *D. discoideum* infected with *L. pneumophila* strain JC51, showing examples of material remaining attached to the plastic surface of a tissue culture well 24 hours after infection. (C), An intact cell containing several *L. pneumophila* inside vacuoles; (D), remnants of a lysed cell including several membrane-bound *L. pneumophila*. See Materials and Methods for details. Scale bars, 10 μm.

several membrane-bound compartments containing bacteria. In the samples infected with JC51, numerous vesicles containing bacteria were observed with the attached remains of lysed cells (Fig. 1D). Such structures were not observed in samples infected with wild-type *L. pneumophila*, presumably because the vesicles had already been released into the medium. These results are entirely consistent with the view that the LepA and LepB proteins allow nonlytic release of *L. pneumophila*. Others have documented that *L. pneumophila* are released from protozoa enclosed within "faecal vesicles" (*6*) or "respirable vesicles" (*22*), which are believed to be highly infectious to humans. JC51 exhibits a defect in release from protozoan but not mammalian host cells; this suggests that the bacteria are normally released via an exocytic pathway in protozoa that is either absent or unused in mammalian cells.

Because release of *Legionella* from host cells has been suggested to involve pore-forming cytotoxicity (*23*), we evaluated the alternative possibility that the LepA and LepB products facilitate bacterial release by lysing host cells. We used a vital dye to measure the viability of amoebae after infection by wild-type and mutant strains of *L. pneumophila*. Infection of *A. castellani* with a *dotA* mutant had no effect on the ability of the amoebae to reduce the dye, because this strain is incapable of intracellular growth or host cell killing. In contrast, the wild-type and the *lep* mutant strains completely prevented dye reduction above an input multiplicity of infection of 1 bacterium per amoeba (fig. S4). Similar results were obtained with *D. discoideum* (*21*). We conclude that the Lep proteins play no role in cytotoxicity or host cell killing.

The ability of the JC51 strain to grow within and to kill protozoa suggests that it retains the ability to prevent phagosome-lysosome fusion. To verify this, we used *D. discoideum* cells that express a fusion of green fluorescent protein (GFP) to VatM, the 100-kD transmembrane subunit of the vacuolar H^+-translocating adenosine triphosphatase (V-ATPase). Earlier studies using VatM-GFP revealed that the V-ATPase is delivered to the membrane of new phagosomes by fusion with preexisting endo-lysosomes (*24*). Amoebae expressing VatM-GFP were infected with different *L. pneumophila* strains expressing DsRed-Express. Living infected cells were observed by confocal fluorescence microscopy. Representative images of the VatM-GFP expressing *D. discoideum* cells infected with wild-type (JR32), *icmT* (GS3011), or Δ*lepA, lepB* (JC51) are shown in Fig. 2. The red signal of the *icmT* mutant bacteria is clearly surrounded by the green VatM-GFP signal, indicating fusion of the phagosomes with endo-lysosomes (Fig. 2, C, D, and E). In contrast, neither the red signal of JR32 nor that of JC51 associates with the green signal of the VatM-GFP (Fig. 2, A and B). Thus, the V-ATPase is delivered to the membranes of phagosomes containing the *icmT* mutant but not to phagosomes containing either the wild-type or the *lepA, lepB* JC51 double mutant. We conclude that neither LepA nor LepB is required for preventing fusion of the *Legionella* phagosome with lysosomes.

Although we have no information about the mechanism used by the Lep proteins to promote release of *Legionella* from protozoa, the availability of *D. discoideum* mutants with defects in the late stages of endosomal transit including exocytosis (*25*) may provide a way to test whether a known exocytic pathway is involved. In addition, ectopic expression of the Lep proteins in amoebae may provide additional information about their function at the molecular level.

The ability of *L. pneumophila* and other bacteria to prey on protozoa is becoming more widely recognized. Several bacterial species, some of which may be phylogenetically related to *L. pneumophila* (*26*), have been found to exist as symbionts within protozoa (*27*). The genetic and physiologic factors that contribute to the development of host-pathogen versus host-symbiont relations can best be studied using both hosts and bacteria that are genetically tractable, like those described here.

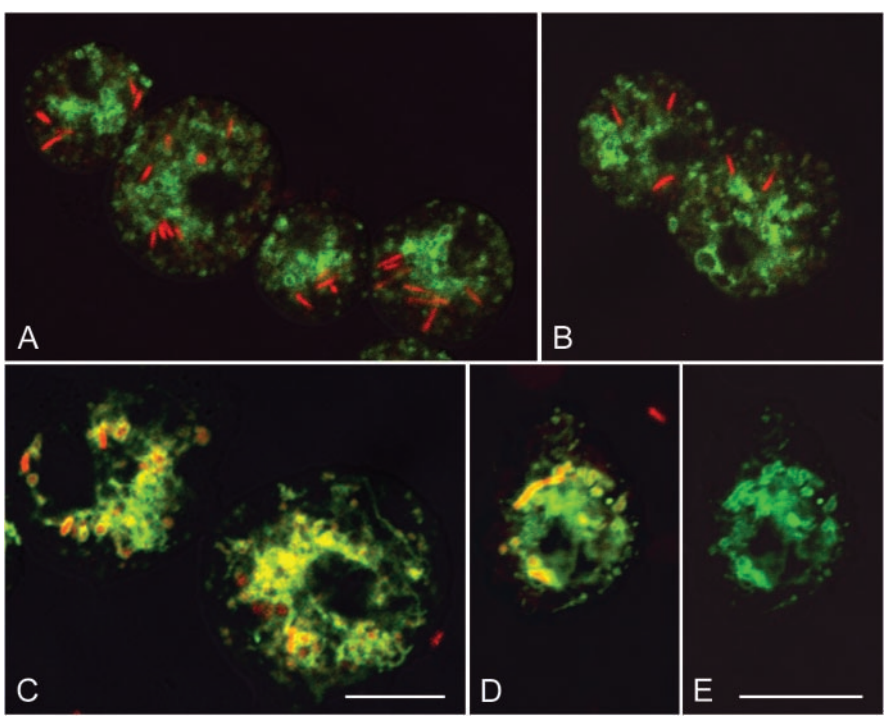

Fig. 2. Trafficking of vacuolar proton pumps to phagosomes containing three strains of *L. pneumophila*. (**A**), JR32 (wild-type); (**B**), JC51 (*lepA::Gm*, *lepB::Km*); (**C** to **E**), GS3011 (*icmT::Km*). (A and B), cells 3 to 4 hours after infection; (C), cells after 50 min of infection; and (D), the same preparation 24 hours later, still in the presence of *L. pneumophila*. (E) The same cell as in (D), but minus the red channel, to reveal more clearly VatM-GFP in the phagosome membrane. Scale bars, 10 μm. (A and B), same magnification as (C); (D) same as (E.)

References and Notes

1. M. Steinert, K. Birkness, E. White, B. Fields, F. Quinn, *Appl. Environ. Microbiol.* **64**, 2256 (1998).
2. A. Essig, M. Heinemann, U. Simnacher, R. Marre, *Appl. Environ. Microbiol.* **63**, 1396 (1997).
3. H. Abd, T. Johansson, I. Golovliov, G. Sandstrom, M. Forsman, *Appl. Environ. Microbiol.* **69**, 600 (2003).
4. O. S. Harb, L. Y. Gao, Y. Abu Kwaik, *Environ. Microbiol.* **2**, 251 (2000).
5. R. F. Breiman *et al.*, *JAMA* **263**, 2924 (1990).
6. T. J. Rowbotham, *Isr. J. Med. Sci.* **22**, 678 (1986).
7. J. M. Solomon, R. R. Isberg, *Trends Microbiol.* **8**, 478 (2000).
8. M. A. Horwitz, *J. Exp. Med.* **158**, 2108 (1983).
9. M. A. Horwitz, F. R. Maxfield, *J. Cell Biol.* **99**, 1936 (1984).
10. J. Coers, C. Monahan, C. R. Roy, *Nature Cell Biol.* **1**, 451 (1999).
11. M. A. Horwitz, *J. Exp. Med.* **158**, 1319 (1983).
12. J. C. Kagan, C. R. Roy, *Nature Cell Biol.* **4**, 945 (2002).
13. S. Sturgill-Koszycki, M. S. Swanson, *J. Exp. Med.* **192**, 1261 (2000).
14. G. Segal, M. Purcell, H. A. Shuman, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1669 (1998).
15. J. P. Vogel, H. L. Andrews, S. K. Wong, R. R. Isberg, *Science* **279**, 873 (1998).
16. G. Segal, H. A. Shuman, *Mol. Microbiol.* **33**, 669 (1999).
17. H. Nagai, J. C. Kagan, X. Zhu, R. A. Kahn, C. R. Roy, *Science* **295**, 679 (2002).
18. G. M. Conover, I. Derre, J. P. Vogel, R. R. Isberg, *Mol. Microbiol.* **48**, 305 (2003).
19. T. H. Sollner, *Dev. Cell* **2**, 377 (2002).
20. M. P. Sory, G. R. Cornelis, *Mol. Microbiol.* **14**, 583 (1994).
21. K. S. de Felipe, H. A. Shuman, unpublished observations.
22. S. G. Berk, R. S. Ting, G. W. Turner, R. J. Ashburn, *Appl. Environ. Microbiol.* **64**, 279 (1998).
23. M. Molmeret *et al.*, *Mol. Microbiol.* **43**, 1139 (2002).
24. M. Clarke *et al.*, *J. Cell Sci.* **115**, 2893 (2002).
25. M. Maniak, *Int. Rev. Cytol.* **221**, 257 (2002).

26. See *Amoeba proteus* symbiotic bacterium: www.ncbi.nlm.nih.gov:80/Taxonomy.
27. K. W. Jeon, *J. Eukaryot. Microbiol.* **42**, 1 (1995).
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A Putative Ca^{2+} and Calmodulin-Dependent Protein Kinase Required for Bacterial and Fungal Symbioses

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Legumes can enter into symbiotic relationships with both nitrogen-fixing bacteria (rhizobia) and mycorrhizal fungi. Nodulation by rhizobia results from a signal transduction pathway induced in legume roots by rhizobial Nod factors. *DMI3*, a *Medicago truncatula* gene that acts immediately downstream of calcium spiking in this signaling pathway and is required for both nodulation and mycorrhizal infection, has high sequence similarity to genes encoding calcium and calmodulin-dependent protein kinases (CCaMKs). This indicates that calcium spiking is likely an essential component of the signaling cascade leading to nodule development and mycorrhizal infection, and sheds light on the biological role of plant CCaMKs.

The legume-rhizobia symbiosis fixes as much nitrogen worldwide as the chemical fertilizer industry, owing to the ability of rhizobial bacteria to induce the morphogenesis of a new plant organ, the legume root nodule, in which they fix nitrogen. Rhizobial signals, the lipochito-oligosaccharidic Nod factors, initiate symbiotic responses on the roots of legume hosts and are required for recognition, controlled infection, and nodule formation (*1*–*4*). These molecules induce a variety of responses, including rapid calcium influx and calcium spiking in root hair cells, specific gene induction, alterations in epidermal cell morphology, and cortical cell mitosis (*4*).

Genetic studies in the model legumes *Medicago truncatula* and *Lotus japonicus* have led to the identification of genes involved in the perception and transduction of Nod factors. Two types of transmembrane receptor–like serine/threonine kinases with putative extracellular regions containing LysM domains (LysM-RLKs), required for Nod factor responses and rhizobial infection, are hypothesized to form heterodimeric Nod factor receptors (*5*–*7*). In *M. truncatula*, three downstream genes, *DMI1*, *DMI2*, and *DMI3*, are required for both nodulation and the formation of arbuscular mycorrhizae (the AM symbiosis). The AM symbiosis occurs in most land plants (a notable exception is the Brassicaceae family, which includes *Arabidopsis thaliana*) and involves fungi of the order Glomales (*8*–*10*). Both the fungal and bacterial symbiosis partners trigger plant host genetic programs that permit controlled and localized infection. *DMI2* encodes a leucine-rich repeat receptor–like protein kinase (NORK) (*11*). Mutants of *DMI1*, *DMI2*, and *DMI3* are blocked for most responses to Nod factors: They do not exhibit induction of root hair branching, early nodulin gene expression, and cortical cell division (*3*). However, they differ in their ability to respond to Nod factors by the induction in root hair cells of sharp oscillations in the concentration of cytoplasmic calcium (calcium spiking), which is lost in *dmi1* and *dmi2* but not in *dmi3* mutants (*12*, *13*). *DMI3* thus seems to act immediately downstream of calcium spiking.

DMI3 maps to the south arm of chromosome 8 (*14*), between the two markers SDP1 and PU01, located 1 and 2 cM, respectively, from *DMI3*. These two markers were used to isolate primary bacterial artificial chromosomes (BACs), and an 800-kb contig of BACs that spanned the *DMI3* region was assembled using a combination of chromosome walking from BAC end sequences and restriction endonuclease fingerprinting of the clones (fig. S1). Genetic markers originating from the BAC contig were developed and recombination events were used to position the *DMI3* locus to a 190-kb interval. Two BACs encompassing this region were sequenced (GenBank accession numbers AY508218 and AY508219), which allowed the identification of candidate genes on the basis of sequence homologies. Among them we noted several genes coding for transcription factors (fig. S1). A putative calcium and calmodulin-dependent protein kinase (CCaMK) detected in the sequenced region appeared as a promising candidate for *DMI3* (Fig. 1A). Using specific primers, we amplified this gene from genomic DNA of two *dmi3* mutant alleles, TRV25 and T1-5, and sequenced it. The wild-type and TRV25 allele differed by a 14–base pair (bp) deletion in the predicted kinase domain of the mutant, leading to a premature stop codon, whereas the T1-5 allele exhibited a point mutation in the kinase domain, leading to a stop codon. Identification of mutations in two independent mutant alleles together with the physical and genetic location of *DMI3* provides strong evidence that this candidate gene is *DMI3*.

To test this hypothesis, we attempted to complement the Nod^- mutation in TRV25 using the wild-type genomic sequence of the candidate gene. Because the nodulation phenotype of TRV25 depends on the root genotype (*14*), we used *Agrobacterium rhizogenes* for root transformation. Roots appearing on TRV25 seedlings after *A. rhizogenes* infection were inoculated with the rhizobial symbiont *Sinorhizobium meliloti*. Eighty percent of plants transformed by *A. rhizogenes* carrying the candidate gene developed nodules (an average of five nodules per plant) (Fig. 2). Microscopic examination of a representative sample of mature nodules revealed the presence of bacteria within the central tissue. No nodules could be detected on roots of plants transformed by *A. rhizogenes* carrying the vector (*15*). This complementation, together with the above described genetic evidence,

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