sibility of culturing each and every stage of the life cycle of the rodent parasite *P. berghei* immediately opens up important new areas of investigation in this useful model species.

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36. Cold Matrigel (90 μl) was pipetted into each culture well (*16*) and gelled at 37°C for 30 min. Ookinetes were co-cultured with *D. melanogaster* S2 cells (*17*) kindly supplied by J.-L. Imler, CNRS, Strasbourg, France. Ookinetes and S2 cells were suspended in either RPMI 1640 medium, supplemented as in (*15*, *19*), or Schneider's medium (Sigma), supplemented as in Table 1. Ookinete (10^4 per chamber)–S2 cell suspension was pipetted onto solidified Matrigel in the chamber plate and incubated at 19° ± 1°C. Most of the medium and S2 cells were changed every 48 to 72 hours.

37. We gratefully acknowledge the support of The Wellcome Trust and the Saudi Arabian Government (E.M.A.).

16 October 2001; accepted 12 December 2001

REPORTS

A Bacterial Guanine Nucleotide Exchange Factor Activates ARF on *Legionella* Phagosomes

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The intracellular pathogen *Legionella pneumophila* subverts vesicle traffic in eukaryotic host cells to create a vacuole that supports replication. The *dot/icm* genes encode a protein secretion apparatus that *L. pneumophila* require for biogenesis of this vacuole. Here we show that *L. pneumophila* produce a protein called RalF that functions as an exchange factor for the ADP ribosylation factor (ARF) family of guanosine triphosphatases (GTPases). The RalF protein is required for the localization of ARF on phagosomes containing *L. pneumophila*. Translocation of RalF protein through the phagosomal membrane is a *dot/icm*-dependent process. Thus, RalF is a substrate of the Dot/Icm secretion apparatus.

Legionella pneumophila are aquatic bacteria that infect and grow within protozoan hosts in most freshwater ecosystems (*1*). When these bacteria are inhaled by humans, *L. pneumophila* will replicate in alveolar macrophages, resulting in a severe pneumonia known as Legionnaires' disease (*2*, *3*). *Legionella pneumophila* replicate within phagocytes by first creating a specialized vacuole that is similar morphologically to the endoplasmic reticulum (ER) of its host (*4*, *5*). Biogenesis of this replicative vacuole requires the Dot/Icm transporter (*6*), which is a type IV protein secretion apparatus (*7*, *8*). Pathogens such as *Agrobacterium tumefaciens* and *Helicobacter pylori* use type IV transporters to inject bacterial proteins directly into the cytosol of eukaryotic host cells (*9*–*11*). It is thought that the Dot/Icm transporter is used by *L. pneumophila* to inject proteins into host cells in order to control the biogenesis of a replicative organelle by modulating the activity of host factors involved in vesicle traffic. However, genetic screens that have been successful in isolating virulence determinants required for growth of *L. pneumophila* in host cells, including the genes encoding the Dot/Icm secretion apparatus, have not revealed any injected proteins (*7*, *8*, *12*, *13*).

The host protein ADP ribosylation factor–1 (ARF1) is found on phagosomes containing wild-type *L. pneumophila* but is not localized to phagosomes containing *L. pneumophila dot/icm* mutants (*14*). ARF1 is a highly conserved small GTP-binding protein that acts as a key regulator of vesicle traffic from the ER and Golgi [reviewed in (*15*)]. Because ARF1 localization on phagosomes containing *L. pneumophila* requires the Dot/Icm transporter, an injected bacterial protein may be required for ARF1 recruitment. To find proteins that are injected into host cells by the Dot/Icm transporter, we focused on bacterial gene products that may play a direct role in localization of ARF1 to phagosomes containing *L. pneumophila*. Because the association of cytosolic ARF onto vesicle membranes is coincident with GTP activation, we searched the genome of *L. pneumophila* for proteins that had homology to ARF-specific guanine nucleotide exchange factors (GEFs) (*16*).

We identified a *L. pneumophila* gene that encodes a 374 amino acid protein with a Sec7-homology domain (Fig. 1A) (*17*). Sec7-homology domains are found in a diverse family of eukaryotic ARF-GEFs and are sufficient to stimulate the exchange of GDP for GTP [reviewed in (*18*)]. This *L. pneumophila* gene was called *ralF*. A gene from the intracellular pathogen *Rickettsia prowazekii* (*19*) that is predicted to encode a protein that has 42% identity to the full-length RalF protein was identified using the RalF protein sequence as a BLASTP query (Fig. 1A). Currently, the RalF protein and this *R. prowazekii* protein are the only two prokaryotic gene products known to contain a Sec7-homology domain.

The RalF protein was detected (*20*) in wild-type *L. pneumophila* (Fig. 1B, Lp01) and in an isogenic mutant that has a defective Dot/Icm transporter (Fig. 1B, Lp01 Δ*dotA*) but was not detected after the *ralF* gene was deleted from the *L. pneumophila* chromosome (Fig. 1B, Lp01 Δ*ralF*). The RalF protein was also produced by *L. pneumophila* Philadelphia-1 (Fig. 1B, Lp philadelphia-1), which is a clinical isolate obtained from the first documented *L. pneumophila* disease outbreak (*3*). These data demonstrate that a protein containing a Sec7-homology domain is produced by *L. pneumophila*.

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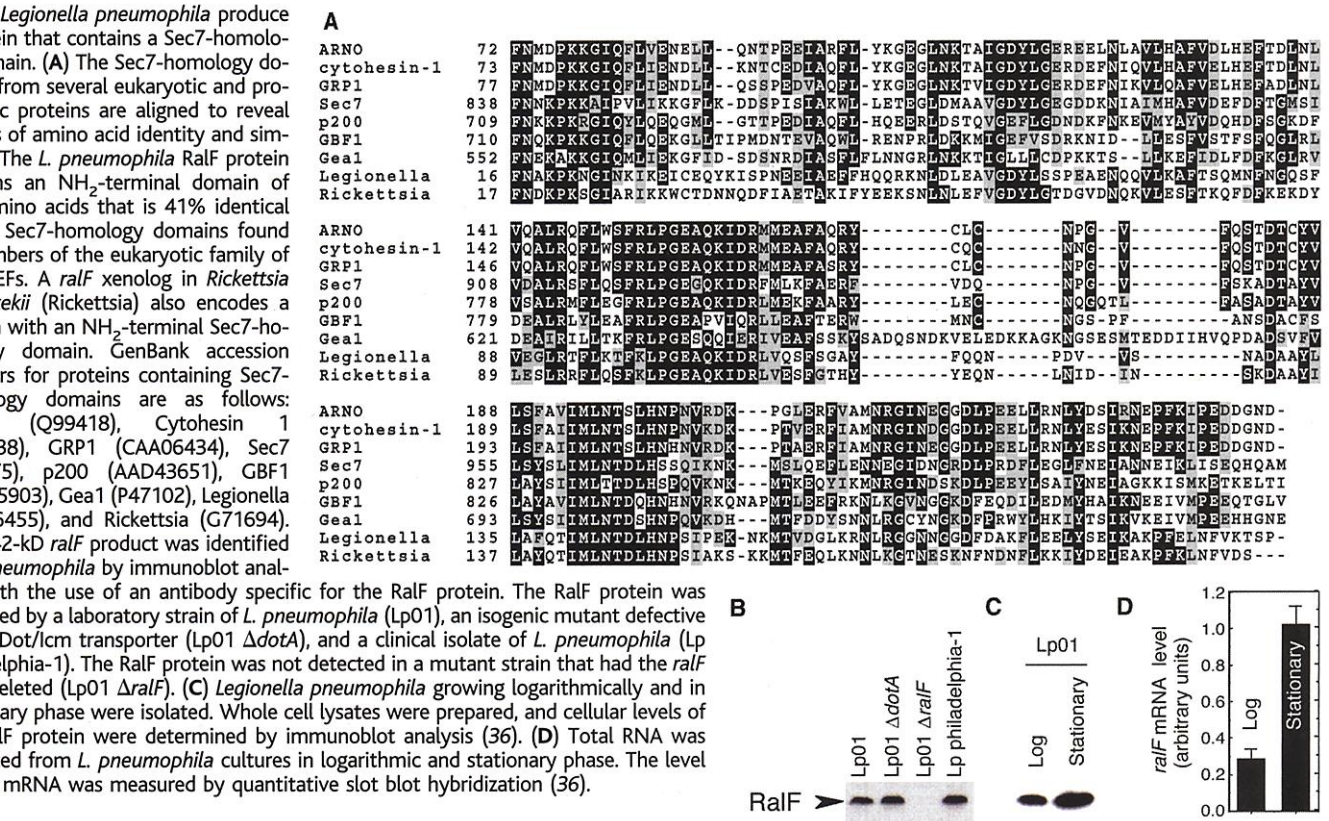


Fig. 1. *Legionella pneumophila* produce a protein that contains a Sec7-homology domain. (**A**) The Sec7-homology domains from several eukaryotic and prokaryotic proteins are aligned to reveal regions of amino acid identity and similarity. The *L. pneumophila* RalF protein contains an NH_2-terminal domain of 200 amino acids that is 41% identical to the Sec7-homology domains found in members of the eukaryotic family of ARF-GEFs. A *ralF* xenolog in *Rickettsia prowazekii* (Rickettsia) also encodes a protein with an NH_2-terminal Sec7-homology domain. GenBank accession numbers for proteins containing Sec7-homology domains are as follows: ARNO (Q99418), Cytohesin 1 (Q15438), GRP1 (CAA06434), Sec7 (P11075), p200 (AAD43651), GBF1 (AAD15903), Gea1 (P47102), Legionella (AY056455), and Rickettsia (G71694). (**B**) A 42-kD *ralF* product was identified in *L. pneumophila* by immunoblot analysis with the use of an antibody specific for the RalF protein. The RalF protein was produced by a laboratory strain of *L. pneumophila* (Lp01), an isogenic mutant defective in the Dot/Icm transporter (Lp01 Δ*dotA*), and a clinical isolate of *L. pneumophila* (Lp philadelphia-1). The RalF protein was not detected in a mutant strain that had the *ralF* gene deleted (Lp01 Δ*ralF*). (**C**) *Legionella pneumophila* growing logarithmically and in stationary phase were isolated. Whole cell lysates were prepared, and cellular levels of the RalF protein were determined by immunoblot analysis (*36*). (**D**) Total RNA was extracted from *L. pneumophila* cultures in logarithmic and stationary phase. The level of *ralF* mRNA was measured by quantitative slot blot hybridization (*36*).

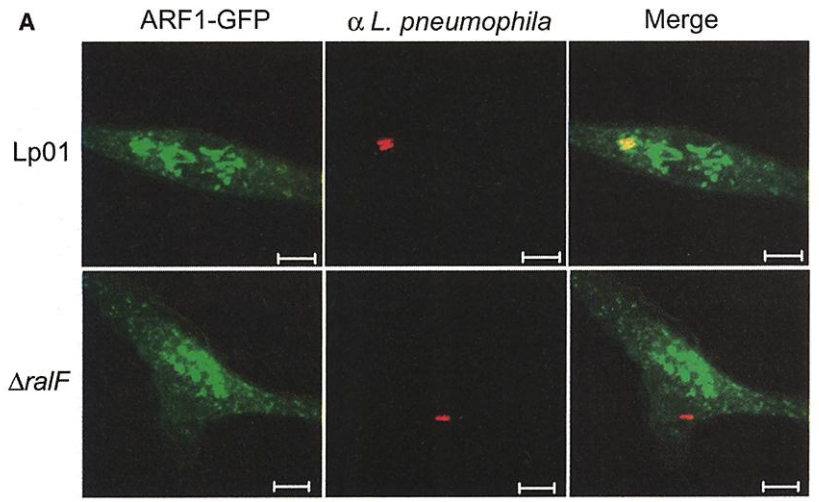
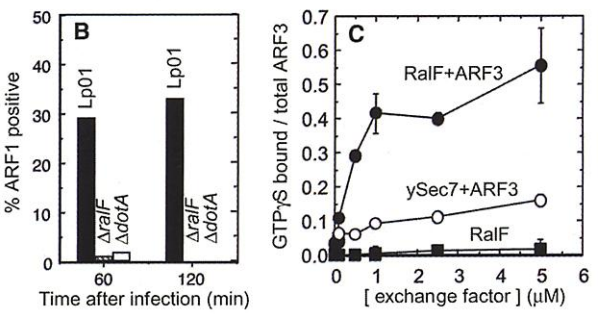


Fig. 2. The RalF protein is a guanine nucleotide exchange factor that recruits ARF1 to phagosomes containing *L. pneumophila*. (**A**) Mouse bone marrow–derived macrophages expressing ARF1-GFP were infected with wild-type *L. pneumophila* (Lp01) and an isogenic mutant that has the gene encoding RalF deleted (Δ*ralF*). Macrophages were fixed 1 hour after infection. Bacteria were stained with antibody to *L. pneumophila* and secondary antibody labeled with Texas Red. Stacked confocal images show that ARF1-GFP is found on phagosomes containing wild-type *L. pneumophila* but is not located on phagosomes containing the Δ*ralF* mutant. Bar, 5 μm. (**B**) Localization of ARF1-GFP was measured for phagosomes containing wild-type *L. pneumophila* (Lp01), mutants defective in Dot/Icm transporter function (Δ*dotA*), and *ralF* mutants (Δ*ralF*). The proportion of phagosomes that stain positive for ARF1-GFP at 60 and 120 min after infection is shown for each strain of *L. pneumophila*. Results are the average of two independent experiments in which at least 50 phagosomes were scored. The numbers obtained in each experiment varied by less than 20%. (**C**) ARF guanine nucleotide exchange activity of the GST-RalF protein was determined with the use of myristoylated ARF3. Exchange activity is presented as the stoichiometry of [^{35}S]GTPγS bound to ARF3 (1 μM) after a 10-min incubation with varying concentrations of GST-RalF (RalF + ARF3). The minimal Sec7 homology domain from *Saccharomyces cerevisiae* Sec7p was used as a positive control (ySec7 domain + ARF3), and reactions containing GST-RalF without ARF3 served as a negative control (RalF).



It has been hypothesized that proteins injected into host cells by the Dot/Icm apparatus are up-regulated as exponentially growing bacteria enter stationary phase (*21*). Immunoblot analysis showed that the cellular concentration of the RalF protein is greater in stationary phase bacteria than in bacteria growing exponentially (Fig. 1C). To examine whether the *ralF* gene is growth-phase regulated, mRNA levels from

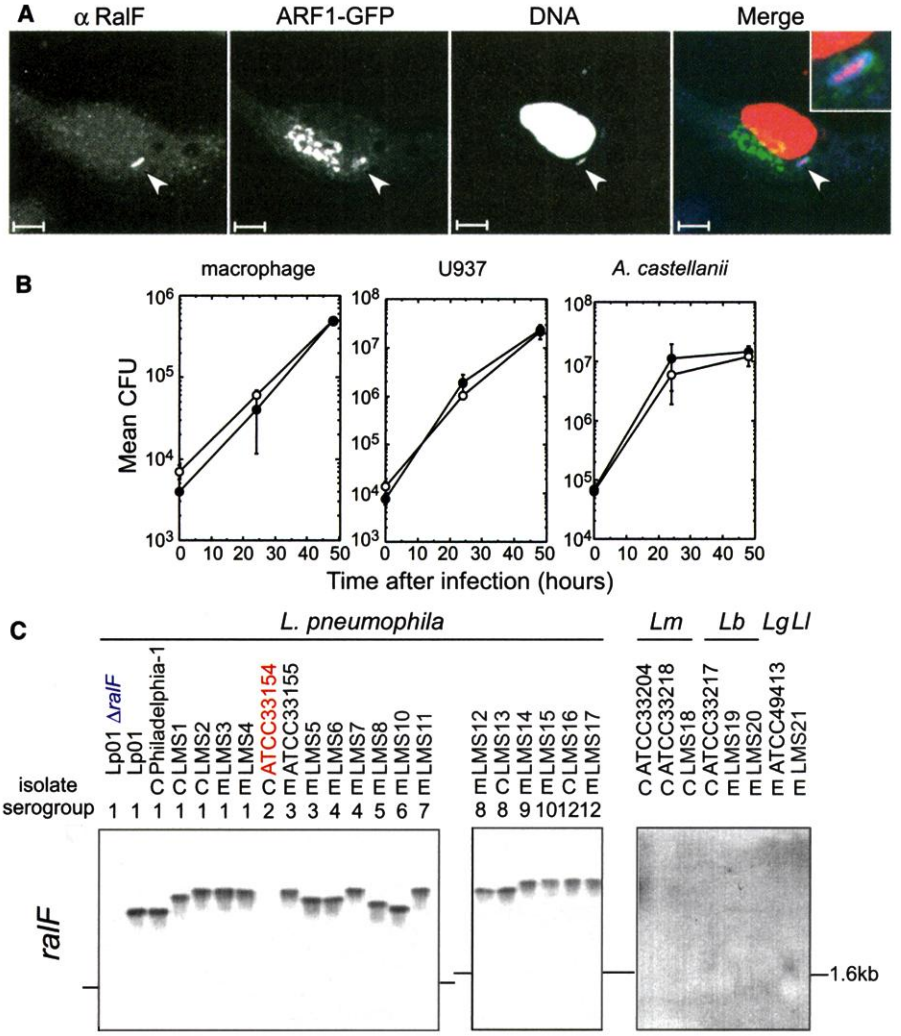


Fig. 3. The RalF protein is a substrate translocated into host cells by the Dot/Icm transporter. **(A)** Mouse bone marrow–derived macrophages expressing ARF1-GFP were infected with wild-type *L. pneumophila* and fixed 1 hour after uptake. Localization of the RalF protein was visualized by staining with an affinity-purified antibody specific for RalF and a secondary antibody conjugated to Cy5. DNA was stained with propidium iodide, which labels both the macrophage nucleus and bacterial cells. A projection of stacked confocal images shows that RalF (blue) and ARF1-GFP (green) co-localize on phagosomes containing *L. pneumophila* (red). Bar, 5 μm. **(B)** Intracellular growth of *L. pneumophila* strain Lp01 (closed circles) and the isogenic *ralF* mutant (open circles) was measured in eukaryotic host cells. The intracellular growth kinetics of these *L. pneumophila* strains were determined in mouse bone marrow–derived macrophages, the human macrophage-like cell line U937, and the protozoan host *A. castellanii*. Graphs show colony-forming units ± standard deviation of each strain recovered from infected host cells over 48 hours. **(C)** Southern analysis indicates that the *ralF* gene is present in most serogroups of *L. pneumophila* but is not detected in other *Legionella* species examined, which included isolates of *L. micdadei* (Lm), *L. bozemanii* (Lb), *L. gratiana* (Lg), and *L. longbeachae* (Ll). The strains of *Legionella* examined were obtained either as clinical specimens from infected patients (C) or were isolated from environmental sources (E). Blue type is used to highlight the *ralF* deletion mutant constructed in this study (Lp01 Δ*ralF*) and serogroup 2 strain of *L. pneumophila* (ATCC 33154) lacking the *ralF* gene is highlighted with red type. The film in the far right panel was exposed longer (12 hours) than films in the left and middle panels (4 hours).

bacteria in exponential and stationary phase were measured by slot-blot hybridization (Fig. 1D). There was a threefold increase in *ralF* expression as exponentially growing bacteria enter stationary phase. Thus, the RalF protein has an expression profile predicted for proteins injected into host cells by the Dot/Icm transporter.

To investigate the in vivo role of the RalF protein, the recruitment of ARF1 to phagosomes containing *L. pneumophila* was examined (*22*). ARF1-GFP co-localization was observed on phagosomes containing wild-type *L. pneumophila* but was not detected on phagosomes containing Δ*dotA* mutants of *L. pneumophila* (Fig. 2, A and B). ARF1-GFP was not observed on phagosomes containing Δ*ralF* mutants, indicating that this protein facilitates localization of ARF1 to phagosomes containing *L. pneumophila*. Thus, RalF protein and the Dot/Icm transporter are both required for the recruitment of ARF to the *Legionella* phagosome accounting for the acronym RALF.

It was predicted that the RalF protein could function as an ARF-GEF by virtue of its Sec7-homology domain. The six mammalian ARFs fall into three classes that have overlapping functions in regulating vesicle transport (*15*). Guanine nucleotide exchange assays (*23*) revealed that RalF was active on ARF1 and ARF3, which are class I proteins (Fig. 2C) (*24*). RalF was also active on the class III protein ARF6, but was less active on the class II protein ARF5 (*24*). These data are consistent with previous studies of eukaryotic proteins containing Sec7-homology domains, which often have guanine nucleotide exchange activity for more than one ARF protein family member (*18*). Thus, the RalF protein from *L. pneumophila* functions as an exchange factor that activates members of the ARF protein family.

To interact with ARF and to activate it directly, the RalF protein must be translocated out of the bacterium and across the phagosome membrane. Because bacteria use type IV transporters to inject proteins into foreign cells and because *dot/icm* mutants of *L. pneumophila* are unable to recruit ARF to their phagosomes, we predicted that the Dot/Icm apparatus was necessary for RalF export during host cell infection. This hypothesis was tested by immunofluorescent staining of *L. pneumophila* phagosomes with an affinity-purified antibody specific for the RalF protein (*25*). Co-localization of RalF and ARF1-GFP was apparent on phagosomes containing wild-type *L. pneumophila* (Fig. 3A). When RalF staining on *L. pneumophila* phagosomes was measured 30 min after internalization, we found that 38 ± 5.5% of phagosomes containing wild-type *L. pneumophila* stained positive for RalF (*24*). Specific staining with the RalF antibody was not observed on phagosomes containing Δ*dotA* mutants (2.0 ± 1.4%) or on phagosomes containing Δ*ralF* mutants (3.7 ± 3.6%). Thus, localization of RalF protein on phagosomes containing *L. pneumophila* requires a functional Dot/Icm transporter, which indicates that RalF is translocated into eukaryotic host cells by the Dot/Icm transporter. There are no obvious reasons why RalF protein staining remains localized to phagosomes containing *L. pneumophila*. Transmembrane or membrane interaction domains are not apparent in the RalF protein sequence. This may mean that RalF interacts with another protein or a lipid on the phagosome membrane or perhaps RalF is rapidly degraded in the host cytoplasm.

We were interested in whether *L. pneumophila* require RalF for replication inside eukaryotic cells. Growth of wild-type *L. pneumophila* and an isogenic Δ*ralF* mutant was measured in murine bone marrow–derived macrophages (*26*), in the human mac-

rophage-like cell line U937 (*27*), and in the protozoan host *Acanthamoeba castellanii* (*28*). There was no intracellular growth defect observed for the Δ*ralF* mutant in these three eukaryotic hosts (Fig. 3B). Thus, the RalF protein is not required for growth of *L. pneumophila* within these hosts, which explains why *ralF* had not been isolated previously in genetic screens for *L. pneumophila* intracellular growth mutants.

The high degree of sequence identity between the Sec7-homology domains in RalF and eukaryotic ARF-GEFs in addition to the observation that proteins with Sec7-homology domains have been found in only two prokaryotic organisms suggests that horizontal gene transfer of a eukaryotic gene gave rise to the *ralF* gene. If *ralF* were acquired recently by horizontal gene transfer, it might not be present in all *L. pneumophila* serogroups or in other *Legionella* species. To address this question, genomic DNA from several *L. pneumophila* serogroups was analyzed by Southern hybridization to determine the genetic distribution of *ralF* (*29*). The *ralF* gene was present in all *L. pneumophila* serogroups with the exception of the American Type Culture Collection (ATCC) *L. pneumophila* serogroup 2 strain #33154 (Fig. 3C, red type). The *ralF* gene was not detected in any other species of *Legionella* examined. However, the *dotA* gene was detected in these other species (*24*) and we have shown previously that the IcmX protein is produced by these bacteria (*30*), which means these species of *Legionella* have essential components of the Dot/Icm transporter but do not have the *ralF* gene. Although a gene similar to *ralF* is found in *R. prowazekii*, comparative genomic analysis indicates that there are no gene products containing a Sec7-homology domain in the related species *R. conorii* (*31*). Thus, the *ralF* gene was most likely acquired by *L. pneumophila* and *R. prowazekii* after speciation occurred within each genus.

Legionella pneumophila are found ubiquitously in freshwater environments where they parasitize protozoan hosts (*1*). It is reasonable to assume that natural conditions exist where *L. pneumophila* containing the *ralF* gene have a selective advantage. For instance, *ralF* may allow *L. pneumophila* to infect protozoan hosts that restrict the growth of *Legionella* lacking *ralF*, or perhaps *ralF* enables *L. pneumophila* to infect permissive protozoan host cells more efficiently during periods of environmental stress. There are over 35 different species of *Legionella*, yet most large outbreaks of community-acquired Legionnaires' disease worldwide are caused by *L. pneumophila* (*32*). This raises the question of whether acquisition of *ralF* makes *L. pneumophila* a more virulent human pathogen compared with the other *Legionella* species that are missing *ralF*. It is likely that genes acquired recently encoding proteins that are secreted by the Dot/Icm transporter may not only enhance replication of *Legionella* in new environments but could coincidentally increase virulence of those *Legionella* for humans. For example, a newly acquired substrate of the Dot/Icm transporter may allow *L. pneumophila* to evade host immune responses more effectively or permit replication of *L. pneumophila* in human cells that are not permissive for bacteria which lack this protein. Identifying and characterizing additional substrates of the Dot/Icm transporter, in combination with a comparative analysis of *Legionella* genomes, will provide valuable information on how an environmental organism has become a human pathogen.

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16. *Legionella pneumophila* genomic sequences were downloaded from the Columbia Genome Center (http://genome3.cpmc.columbia.edu/~legion/index.html) and a database was constructed that contained open reading frames greater than 225 bp. Each open reading frame was used as a query in a BLASTX search (*33*) and these results were examined to identify *L. pneumophila* proteins with homology to eukaryotic proteins known to interact with ARF.
17. The beginning of an open reading frame encoding a polypeptide with a Sec7-homology domain was located at the end of a short genomic contig. The complete *ralF* gene was sequenced directly from the *L. pneumophila* chromosome by priming BigDye Terminator reactions (Applied Biosystems, Foster City, CA) with oligonucleotides homologous to the end of this contig (*34*).
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20. A glutathione S-transferase (GST)–RalF fusion protein was constructed in the vector pGEX-KG (*35*) and purified from *Escherichia coli* as described (*30*). Rabbits were immunized with GST-RalF and antibodies were collected by the Yale Animal Resource Center. Immunoblot analysis to determine RalF protein levels in *L. pneumophila* was conducted as described (*36*).
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22. A restriction fragment encoding ARF1-GFP was ligated into the retroviral vector pCLXSN and packaged virus was produced in 293T cells as described (*37*). Retroviral particles were used to transfect murine bone marrow–derived macrophages from A/J mice (*38*) and macrophages were re-plated onto cover glass in 24-well tissue culture plates 36 to 48 hours after transfection. Macrophages were infected with *L. pneumophila* at a multiplicity of 50 and tissue culture plates were centrifuged for 5 min at 180*g*. Macrophages and *L. pneumophila* were co-incubated for 10 min at 37°C, then extracellular bacteria were removed by washing each well 3× with tissue culture medium. Macrophages were incubated for an additional 50 min or 110 min, then cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. Cells were permeabilized for 10 min at room temperature in PBS containing 0.2% Triton X-100, and then blocked for 30 min at 37°C in PBS containing 2% goat serum and 50 mM NH_4Cl. Intracellular bacteria were visualized by immunofluorescent staining with the use of a polyclonal rabbit antibody to *Legionella* followed by Texas Red–labeled goat anti-rabbit antibody (Molecular Probes, Eugene, OR). All fluorescent images were acquired with the use of a Zeiss LSM-510 laser scanning confocal microscope.
23. To measure guanine-nucleotide-exchange activity, myristoylated ARF proteins were purified and [^{35}S]GTPγS binding assays were conducted as described previously (*39*). The GST fusion protein containing the Sec7-homology domain of yeast Sec7p (amino acids 833–1020) was described previously (*40*).
24. Supplementary material is available at *Science* Online at www.sciencemag.org/cgi/content/full/295/5555/679/DC1.
25. Purified GST-RalF was conjugated to cyanogen bromide-activated sepharose-4B (Sigma, St. Louis, MO) and this matrix was used to affinity-purify RalF antibodies from rabbit immune serum. To examine RalF staining on phagosomes containing *L. pneumophila*, macrophages expressing ARF1-GFP were infected with *L. pneumophila* for the times indicated and fixed. Cover slips were stained with affinity-purified rabbit anti-RalF antibody (10 μg/ml) followed by a Cy5-labeled goat anti-rabbit secondary antibody (Zymed, South San Francisco, CA). Cover slips were then incubated for 30 min at 37°C in PBS containing 200 μg/ml RNase A (Sigma) and were then incubated for 15 min at 37°C in PBS containing 20 ng/ml propidium iodide (Sigma) to stain DNA.
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41. We thank members of the Columbia Genome Center (supported by NIH Grant R01 AI44371) for making *L. pneumophila* genomic data available to the public, Y. Abu Kwaik for providing *A. castellanii*, J. Donaldson for plasmids encoding ARF1, and D. Debbie for critical reading of this manuscript. This work was supported by NIH grant R29 AI41699.

11 October 2001; accepted 3 December 2001