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Sel1 Repeat Protein LpnE Is a *Legionella pneumophila* Virulence Determinant That Influences Vacuolar Trafficking▽

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The environmental pathogen *Legionella pneumophila* possesses five proteins with Sel1 repeats (SLRs) from the tetratricopeptide repeat protein family. Three of these proteins, LpnE, EnhC, and LidL, have been implicated in the ability of *L. pneumophila* to efficiently establish infection and/or manipulate host cell trafficking events. Previously, we showed that LpnE is important for *L. pneumophila* entry into macrophages and epithelial cells. In further virulence studies here, we show that LpnE is also required for efficient infection of *Acanthamoeba castellanii* by *L. pneumophila* and for replication of *L. pneumophila* in the lungs of A/J mice. In addition, we found that the role of LpnE in host cell invasion is dependent on the eight SLR regions of the protein. A truncated form of LpnE lacking the two C-terminal SLR domains was unable to complement the invasion defect of an *lpnE* mutant of *L. pneumophila* 130b in both the A549 and THP-1 cell lines. The *lpnE* mutant displayed impaired avoidance of LAMP-1 association, suggesting that LpnE influenced trafficking of the *L. pneumophila* vacuole, similar to the case for EnhC and LidL. We also found that LpnE was present in *L. pneumophila* culture supernatants and that its export was independent of both the Lsp type II secretion system and the Dot/Icm type IV secretion system. The fact that LpnE was exported suggested that the protein may interact with a eukaryotic protein. Using LpnE as bait, we screened a HeLa cell cDNA library for interacting partners, using the yeast two-hybrid system. Examination of the protein-protein interaction between LpnE and a eukaryotic protein, obscurin-like protein 1, suggested that LpnE can interact with eukaryotic proteins containing immunoglobulin-like folds via the SLR regions. This investigation has further characterized the contribution of LpnE to *L. pneumophila* virulence and, more specifically, the importance of the SLR regions to LpnE function.

Legionella pneumophila is an intracellular pathogen and the causative agent of Legionnaires' disease. The bacterium infects alveolar macrophages and establishes a replicative vacuole derived from the endoplasmic reticulum by utilizing the Dot/Icm type IV secretion system (48, 53). The ability of *L. pneumophila* to propagate itself within macrophages appears to have developed from its parasitic relationship with protozoa in aquatic environments. The genome sequences of three *L. pneumophila* strains have highlighted the large number of eukaryotic protein-like proteins that this pathogen has acquired (11, 13). These proteins are predicted to allow *L. pneumophila* to manipulate host cell processes, and several bacterial proteins with similarity to eukaryotic proteins have been identified as substrates of the Dot/Icm system (12, 19, 39, 41). Others, such as the ecto-nucleoside triphosphate diphosphohydrotase Lpg1905, have distinct roles in pathogenesis (47). The genome sequences have revealed that *L. pneumophila* possesses five genes encoding proteins with predicted tetratricopeptide repeat (TPR) motifs. At least two of these genes, *lpnE* and *enhC*, contribute to enhanced entry into human tissue culture cell lines (14, 42).

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The TPR motif was originally reported for cell division cycle proteins of *Saccharomyces cerevisiae* (32, 49). Today this motif is known to be ubiquitous in nature, as it is found within functionally unrelated proteins from all genera. A TPR is defined as a degenerate 34-residue motif with a consensus amino acid arrangement of alternate large and small residues and high amino acid conservation observed specifically at positions 8, 20, and 27 (49). These conserved residues allow the TPR to create a pair of antiparallel alpha helices. Multiple motifs, ranging from 3 to 16 in number among TPR-containing proteins, lead to the formation of an alpha superhelical structure (17). This complex and unique structure gives rise to distinct substrate grooves that facilitate specific protein-protein interactions. The ability of TPR proteins to interact with other proteins enables them to play a vital role in eukaryotic cell processes, such as mitosis, transcription repression, and protein import (20, 37, 52). Bacteria also utilize TPR proteins for a range of functions, including gene regulation, flagellar motor function, chaperone activity, and virulence (9, 16, 43, 54). Several chaperones required for type III secretion system-mediated translocation of virulence proteins into host cells contain TPR domains, including PcrH from *Pseudomonas aeruginosa*, LcrH from *Yersinia* species, and CesD from enteropathogenic *Escherichia coli* (8, 9, 54).

The Sel1 repeat (SLR) motif comprises a subtype of TPR, named after the extracellular protein from *Caenorhabditis elegans* for which it was first described (29). Sel1 and its homo-

TABLE 1. Bacterial and yeast strains and plasmids used in this study

Strain or plasmid	Serogroup and/or characteristics	Source or reference
Strains		
L. pneumophila strains		
130b (ATCC BAA-74)	O1; clinical isolate	22
130b *lpnE*::*km*	*lpnE* insertion mutant of 130b (Km^r)	42
130b *lpnE*::*km*/(pMIP:*lpnE*)	*lpnE*::*km* mutant carrying pMIP:*lpnE* (Km^r Cm^r)	42
130b *lspDE*::*km*	*lspDE* insertion mutant of 130b (Km^r)	47
130b *dotA*::*cm*	*dotA* insertion mutant of 130b (Cm^r)	47
E. coli strains		
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F′ [*proAB* $^+$ *lacI*^{q} *lacZ* Δ*M15* Tn*10*] (Tet^r)	Stratagene
BL-21 (DE3)	F^- *omp T hsd*S_B (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
KC8	*pyrF*::Tn*5 hsdR leuB600 trpC9830 lacD74 strA galK hisB436*	Clontech
S. cerevisiae strains		
PJ69-4a	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*Gal7-lacZ*	35
AH109	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2*::$GAL1_{TATA}$-*HIS3 MEL1* $GAL2_{UAS}$-$GAL2_{TATA}$-*ADE2 URA3*::$MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ*	35
Y187	*MAT*α *ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4*Δ *met*$^-$ *gal80*Δ *URA3*::$GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ MEL1*	30
Plasmids		
pMip	pMMB207 with the promoter region of *mip* cloned into SacI/XbaI sites	42
pMip:*lpnE*	pMip carrying *lpnE* and the ribosome binding site cloned into XbaI/PstI sites	42
pMIP:$lpnE_{1\text{-}51}$	pMIP with an *lpnE* truncation encoding the first 51 amino acids cloned into XbaI/PstI sites	This study
pMIP:$lpnE_{1\text{-}122}$	pMIP with an *lpnE* truncation encoding the first 122 amino acids cloned into XbaI/PstI sites	This study
pMIP:$lpnE_{1\text{-}266}$	pMIP with an *lpnE* truncation encoding the first 266 amino acids cloned into XbaI/PstI sites	This study
pMIP:$lpnE_{52\text{-}375}$	pMIP with an *lpnE* truncation encoding amino acid 52 to the stop codon cloned into XbaI/PstI sites	This study
pGBT9	$GAL4_{(1\text{-}147)}$ DNA binding domain; *TRP1* Amp^r	4
pGAD424	$GAL4_{(768\text{-}881)}$ activation domain; *LEU2* Amp^r	4
pGADT7	$GAL4_{(768\text{-}881)}$ activation domain; *LEU2* Amp^r; hemgglutinin epitope tag	Clontech
pMAL-c2x	Expression vector to generate translation fusions to *malE*; Ptac Amp^r	New England Biolabs

logues are involved in cell-to-cell interactions that specify the fate of *C. elegans* cells during development through binding of the membrane proteins Lin-12 and Glp-1 (29). The SLR motif has a less stringent definition than the TPR motif, with the length of an SLR ranging from 36 to 44 amino acids (40). However, the motif consensus sequences are comparable and, subsequently, the motif folding is considered to be equivalent. As such, SLR proteins are also predicted to mediate important protein-protein interactions, and the motif is found preferentially in eukaryotic proteins (40).

All EnhC, LpnE, and LidL proteins contain SLR-type TPR motifs and have been shown to be important for *L. pneumophila* -host interactions (14, 42). In this study, we investigated the contribution of LpnE to the infection of amoebae and A/J mice by *L. pneumophila*. In addition, we examined the involvement of LpnE in trafficking of the *Legionella*-containing vacuole and the basis of protein-protein interactions mediated by the SLR regions of LpnE to increase our understanding of how this protein is linked to *L. pneumophila* virulence.

MATERIALS AND METHODS

Bacterial and yeast strains, growth conditions, and plasmids. Bacterial and yeast strains and plasmids used in this study are listed in Table 1. Bacteria were cultured aerobically at 37°C. *L. pneumophila* strains were grown in ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid] buffered yeast extract broth or on buffered charcoal yeast extract (BCYE) plates (23), and unless otherwise stated, *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth or agar. Antibiotics were added to media at the following final concentrations for *L. pneumophila* (or *E. coli*): kanamycin, 25 μg/ml (100 μg/ml); chloramphenicol, 6 μg/ml (12.5 μg/ml); and ampicillin, 100 μg/ml (100 μg/ml). *Saccharomyces cerevisiae* was cultured in YPD (1% yeast extract, 2% peptone, and 2% dextrose) or synthetic dropout medium (Clontech, CA) lacking appropriate nutrients at 30°C.

DNA, PCR, and cloning techniques. Plasmid DNA was purified using a QIAprep spin miniprep kit (QIAGEN, Hilden, Germany), and bacterial genomic DNA was isolated as described previously (2). DNA-modifying enzymes were used according to the manufacturer's instructions (Promega, WI). PCR amplification was performed using 200 ng of template DNA and approximately 0.25 μg of each oligonucleotide. Oligonucleotide sequences and annealing conditions used are listed in Table 2. Plasmid constructs were confirmed by DNA sequencing using a PRISM ready reaction dye deoxy terminator cycle sequencing kit and a 3730 DNA analyzer (Applied Biosystems, CA). DNA sequences were assembled using Sequencher 3.1.1 (Gene Codes Corp., MI).

Coculture of *Acanthamoeba castellanii* and *L. pneumophila*. *A. castellanii* ATCC 50739 was grown in 75-mm^2 tissue culture flasks (Sarstedt, Leicestershire, United Kingdom) with PYG 712 medium at 20°C without agitation (10). Cultures of *A. castellanii* that were 72 h old were harvested and seeded into 24-well tissue culture trays (Sarstedt) at a density of 2.5×10^5 cells/well in A.c. buffer (0.1% trisodium citrate, 0.4 mM $CaCl_2$, 2.5 mM KH_2PO_4, 4 mM $MgSO_4$, 2.5 mM Na_2HPO_4, 0.05 mM ferric pyrophosphate). Stationary-phase *L. pneumophila* strains, also resuspended in A.c. buffer, were added at a multiplicity of infection (MOI) of 0.01, and the coculture was incubated at 37°C with 5% CO_2. At specific time points, samples from duplicate wells for each strain were plated onto BCYE agar and assessed for replication of *L. pneumophila*. Results were expressed as mean $\log_{10}$ CFU ± standard deviations for three independent experiments.

Pulmonary infection of A/J mice with *L. pneumophila*. To examine the comparative virulence of *L. pneumophila* 130b and the *lpnE*::*km* derivative within an established animal model, mixed infections of mutant and wild-type *L. pneumo-*

TABLE 2. Sequences of oligonucleotides used in this study

Primer	Sequence (5′–3′)	Annealing temp (°C)
lpnE F	GCT CTA GAG ATA GCT CTT AAA AAT AAG G	46
$lpnE_{1\text{-}51}$ R	TTC TGC AGT TAA TCT CCT TTA TCC GCT TC	46
$lpnE_{1\text{-}122}$ R	TTC TGC AGT TAC GCA TTA TTT TGA TCC GC	46
$lpnE_{1\text{-}266}$ R	TTC TGC AGT TAG GCA TTC CCC AGA TCT G	46
$lpnE_{52\text{-}375}$ F	GCT CTA GAA AGG AAT TAT AAA TGG CCA AGG CTC AAT ATT TGC TGG GTA AG	46
lpnE R	AAC TGC AGG AAA ACA GGT AAC AGG C	46
MBP-*lpnE* F	GCT CTA GAA TGG ACA TGA AAA AAT ATA TT	44
lpnE-Y2HF	CGG GAT CCG TAT GGA CAT GAA AAA ATA TAT T	44
OBLS1 IgF	CGA ATT CAT GGC AGG TCT GGA GGA TGT G	56
OBLS1 IgR	GG GAT CCC GAC TCA TCG TCA ATT TCA C	56
OBLS1 FnF	CGA ATT CAT GCC CCG GAA GCT CGA CGT C	56
OBLS1 FnR	CGG GAT CCA CTA CGG CCA TGT CCG CTG	56
OBLS1 $Ig_{Fn}R$	CGG GAT CCT TAT TTT ACT CTG AGA AGC GTA G	50

phila were performed as described previously (45, 46). Briefly, 6- to 8-week-old female A/J mice (Jackson Laboratory, ME) were anesthetized and inoculated intratracheally with 10^6 CFU of wild-type and mutant bacteria at a ratio of 1:1. Twenty-four and 72 h after inoculation, mice were sacrificed and their lung tissue isolated. Tissue was homogenized using a Pro 200 homogenizer (Pro Scientific Inc., CT), and complete host cell lysis was attained by incubation of the homogenized tissue in 0.1% saponin for 10 min at 37°C. Serial dilutions were plated onto both plain and antibiotic-selective BCYE to obtain the total number of viable bacteria and the number of *lpnE* mutant bacteria colonizing the lung tissue. Mixed infections were also performed with *lpnE*::*km*/(pMip:*lpnE*) and *lpnE*::*km*/(pMIP:$lpnE_{52\text{-}375}$) strains in competition with *L. pneumophila* 130b/(pMIP).

Transcomplementation of *L. pneumophila* 130b *lpnE*::*km* with truncated *lpnE*. Truncated forms of *lpnE* were amplified using the oligonucleotides listed in Table 2 and cloned into the XbaI/PstI sites of pMIP (42). These plasmids were introduced into *L. pneumophila* 130b *lpnE*::*km* via electroporation as described previously (21). Briefly, 50-ml logarithmic-phase cultures were harvested via centrifugation (10,000 × *g*, 4°C, 15 min) and washed with cold phosphate-buffered saline (PBS) and then cold 10% (vol/vol) glycerol before being resuspended in 10% glycerol with approximately 500 ng of plasmid DNA and subjected to electrophoresis at 2,300 V, 100 Ω, and 25 mF.

Tissue culture conditions and *L. pneumophila* invasion assays. The human monocytic cell line THP-1 and the human alveolar epithelial cell line A549 were propagated and prepared for infection as described previously (42). Stationary-phase strains of *L. pneumophila* were added at an MOI of 5 for THP-1 cells and an MOI of 100 for A549 cells and allowed to infect cells for 2 h in 5% CO_2 at 37°C. Cells were then treated with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria and washed with PBS before being lysed with 0.01% digitonin. Serial dilutions of the inoculum and bacteria recovered from lysed cells were plated on BCYE agar, and results are expressed as percentages of the inoculum that resisted killing by gentamicin (means ± standard deviations for at least three independent experiments).

Avoidance of LAMP-1 by *L. pneumophila*-containing vacuoles. Immunofluorescence was used to investigate the ability of *Legionella*-containing vacuoles to avoid lysosomal fusion, using the lysosome-associated membrane protein LAMP-1 as a marker for lysosomal membranes, in both A549 and THP-1 cells. Immunofluorescence was performed as described previously (47). Briefly, 10^5 A549 cells were seeded onto 12-mm glass coverslips (Menzel-Glaser, Braunschweig, Germany) and grown overnight before being infected with stationary-phase *L. pneumophila* at an MOI of 100. Alternatively, 10^5 THP-1 cells were allowed to differentiate in the presence of phorbol 12-myristate 13-acetate for 72 h, ensuring differentiation into adherent, elongated cells, before being infected with stationary-phase *L. pneumophila* at an MOI of 5. Infection proceeded for 5 h or 24 h before cells were washed with PBS and fixed with 4% (wt/vol) paraformaldehyde (pH 7.4). Following fixation, the coverslips were blocked for 1 h with PBS containing 10% fetal bovine serum and then stained with monoclonal mouse anti-*L. pneumophila* (6026; ViroStat, ME) and, subsequently, anti-mouse immunoglobulin G (IgG)–Alexa Fluor 594 (Invitrogen, CA). Following labeling of the extracellular bacteria, cells were permeabilized for 1 h with PBS containing 10% fetal bovine serum and 0.05% saponin. Cells were washed with PBS and then stained with anti-*L. pneumophila* and rabbit anti-human LAMP-1 H-288 (Santa Cruz Biotechnology, CA). Anti-rabbit–Alexa Fluor 488 and anti-mouse–Alexa Fluor 594 or –Alexa Fluor 405 were used as secondary antibodies, and all antibodies were diluted in PBS with 10% fetal bovine serum, used at 1:50 (*Legionella*), 1:100 (LAMP-1), or 1:200 (secondary antibodies), and incubated with the cells for 1 h at 37°C. At the end of this staining procedure, intracellular bacteria appeared green, LAMP-1 appeared red, and extracellular bacteria appeared red or blue. Coverslips were mounted in DAKO fluorescent mounting medium (DAKO Corporation) and stored at 4°C in the dark. Slides were examined under a 100× objective on an Olympus BX51 microscope (Olympus, Tokyo, Japan). Images were acquired using an Olympus DP-70 digital camera and merged using DP controller software, version 1.1.1.71. LAMP-1 avoidance was scored blind for various strains of *L. pneumophila*, and data for at least 50 intracellular bacteria from three independent coverslips were expressed as mean percentages of LAMP-1 avoidance (± standard deviations). Differences in LAMP-1 avoidance were assessed for significance using an unpaired two-tailed *t* test.

Production of recombinant LpnE and polyclonal anti-LpnE antibodies. Full-length *lpnE* was amplified by PCR, using MBP-*lpnE* F and *lpnE* R (Table 2), and cloned into the XbaI/PstI sites of the pMAL-c2x expression vector (New England Biolabs, MA) to generate a maltose binding protein (MBP) fusion with LpnE. Production of this fusion protein was induced in *E. coli* BL21 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and subsequently purified using amylose agarose as described by the manufacturer (Scientifix, Australia). Polyclonal antibodies were generated against column-purified MBP-LpnE resuspended in incomplete Freund's adjuvant by inoculation of 500 μg of protein into a rabbit on days 0, 28, and 49 before exsanguination on day 66 (Chemicon International, Inc., CA). Immune serum was adsorbed against *L. pneumophila* 130b *lpnE*::*km* and diluted 1:500 for immunoblotting in 0.05% Tween (vol/vol) in Tris-buffered saline.

TCA precipitation of culture supernatants. *L. pneumophila* extracellular proteins were precipitated from bacterial culture supernatants as described previously (38). Fifty-milliliter samples of stationary- or late-logarithmic-phase broth cultures of *L. pneumophila* were subjected to centrifugation (10,000 × *g*, 4°C, 15 min), and the supernatants were filtered through 0.22-μm filters (Millipore, MA). A 10% (wt/vol) final concentration of trichloroacetic acid (TCA) was added to precipitate the proteins, which were pelleted by centrifugation (10,000 × *g*, 4°C, 1 h) and washed with 100% (vol/vol) cold methanol before being dried and resuspended in 2× sodium dodecyl sulfate (SDS) sample buffer. TCA precipitation included the use of previously described *lspDE* and *dotA* mutants (47). TCA-precipitated culture supernatants and whole-cell lysate samples of strains of interest were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for immunoblotting. Rabbit anti-*E. coli* RpoB antibodies (34) were used at 1:4,000 as a control for cytoplasmic protein contamination of supernatant samples, and rabbit anti-Lpg1905 (1:500) (47) was used to control for loading of TCA-precipitated proteins. When investigating the presence of truncated forms of LpnE in culture supernatants, Novex 4 to 20% acrylamide-Tris-glycine gels were utilized to ensure the recovery of small peptides.

Yeast two-hybrid system. *lpnE* was amplified with the *lpnE*-Y2HF and *lpnE* R oligonucleotides (Table 2) and cloned into pGBT9 digested with BamHI and PstI. The resulting plasmid was transformed into *S. cerevisiae* AH109 by the lithium acetate method as described previously (28). This strain was mated with *S. cerevisiae* Y187 pretransformed with a human HeLa Matchmaker GAL4 library according to the manufacturer's protocol (Clontech). Protein-protein

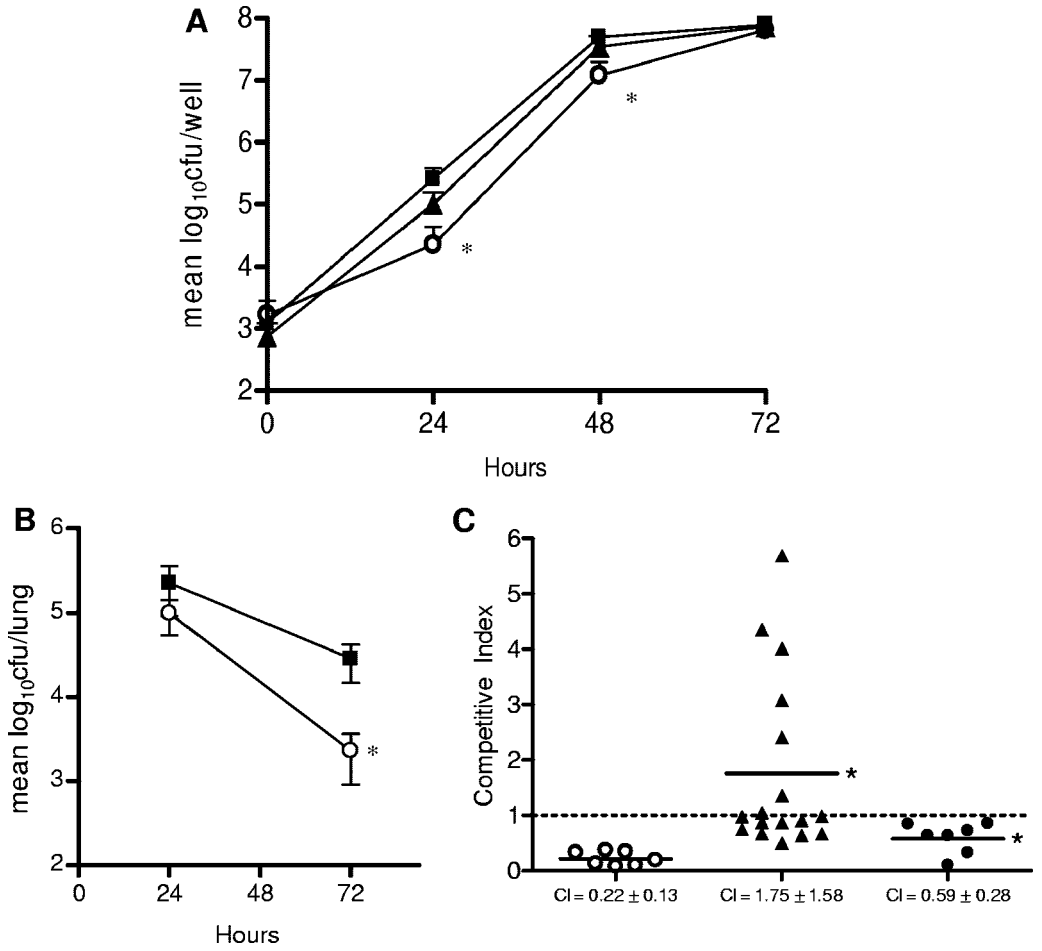

FIG. 1. (A) *L. pneumophila* infection of *A. castellanii*. Amoebae were infected at an MOI of 0.01 with wild-type *L. pneumophila* 130b (■) or the *lpnE*::*km* (○) or *lpnE*::*km*/(pMIP:*lpnE*) (▲) mutant. Bacterial CFU were determined at 24, 48, and 72 h postinfection and are represented as mean $\log_{10}$ CFU ± standard deviations for three independent experiments. *, significantly different from 130b ($P = 0.034$ at 24 h and $P = 0.003$ at 48 h [unpaired, two-tailed *t* test]). (B) Lung colonization of A/J mice by *L. pneumophila* 130b (■) and an *lpnE*::*km* mutant (○). Strain 130b and the *lpnE*::*km* mutant were introduced into the lungs of A/J mice via intratracheal inoculation at a ratio of 1:1. Twenty-four and 72 h following infection, the numbers of CFU for 130b and the *lpnE*::*km* mutant were determined. Data are expressed as means ± standard deviations of the $\log_{10}$ CFU recovered per lung ($n = 7$ or 8). *, significantly different from 130b at 72 h ($P = 0.018$; unpaired, two-tailed *t* test). (C) CIs of derivatives of *L. pneumophila* 130b in mixed infections with the wild-type parent strain. The pairs tested were as follows: *lpnE*::*km* mutant versus 130b (○), *lpnE*::*km*/(pMIP:*lpnE*) mutant versus 130b/(pMIP) (▲), and *lpnE*::*km*/(pMIP:$lpnE_{52\text{-}375}$) mutant versus 130b/(pMIP) (●). *, CI was significantly different from that for the *lpnE*::*km* mutant versus 130b ($P < 0.05$; unpaired, two-tailed *t* test).

interactions were selected by plating the mating mixture onto synthetic dropout medium plates lacking adenine, histidine, tryptophan, and leucine. Library plasmids were isolated from resulting yeast colonies and rescued in *E. coli* KC8 on M9 minimal medium containing the required nutrients, except for tryptophan. Library plasmids and pGBT9:*lpnE* were transformed into *S. cerevisiae* PJ69-4A, and β-galactosidase liquid culture assays (*o*-nitrophenyl-β-D-galactoside) were performed in triplicate according to the Clontech manual for the Matchmaker yeast two-hybrid system. The interaction between LpnE and specific regions of obscurin-like protein 1 (OBSL1) was examined in the same manner. A region of OBSL1 encompassing multiple immunoglobulin (Ig)-like domains and a second region encoding an Ig-like domain and a fibronectin (Fn) domain were cloned into pGAD424 digested with EcoRI and BamHI. The single Ig domain of the Fn fragment (Ig_{Fn}) was also cloned into the EcoRI/BamHI fragment of pGAD424. The resulting plasmids were introduced into *S. cerevisiae* PJ69-4A, and the yeast strains were assessed for β-galactosidase activity.

RESULTS

Contribution of *lpnE* to infection of *A. castellanii* and A/J mice. Previously, we reported that *lpnE* was required for efficient uptake of *L. pneumophila* into A549 epithelial cells and THP-1 macrophages (42). In this work, we extended our virulence studies to examine the contribution of *lpnE* to replication of *L. pneumophila* in *A. castellanii* and in the lungs of A/J mice. Coculture of *L. pneumophila lpnE*::*km* and *A. castellanii* demonstrated that *lpnE* was necessary for efficient infection of the amoeba (Fig. 1A). For up to 48 h postinfection, the *lpnE* mutant was recovered in significantly smaller numbers than wild-type *L. pneumophila* 130b. Bacterial numbers 72 h after infection were comparable between 130b and the *lpnE* mutant. The small reduction in CFU observed for the *lpnE* mutant during the first 48 h of *A. castellanii* infection was restored to wild-type levels upon transcomplementation of the *lpnE* mutant with full-length *lpnE* (Fig. 1A).

The reduced ability of the *lpnE* mutant to infect macrophages, alveolar epithelial cells, and amoebae highlighted the importance of LpnE for host-pathogen interactions in both

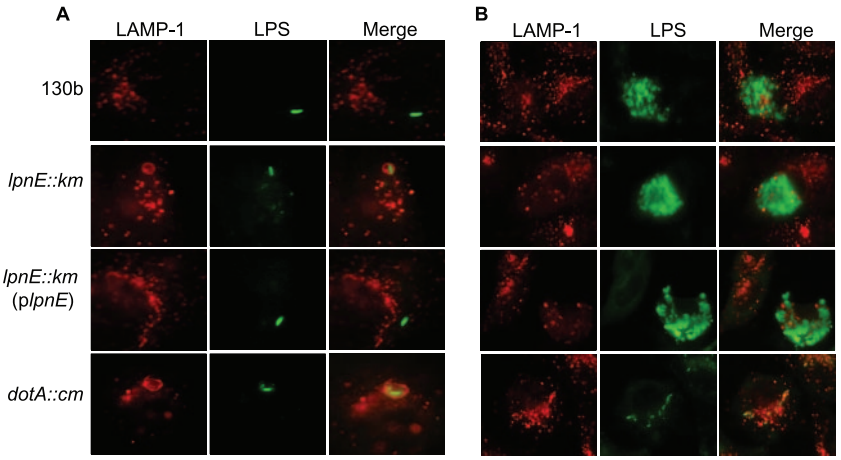

FIG. 2. (A) Immunofluorescence of LAMP-1 in A549 cells infected for 5 h with wild-type *L. pneumophila* 130b or the *dotA*::*cm*, *lpnE*::*km*, or *lpnE*::*km*/(pMIP:*lpnE*) mutant. LAMP-1 was detected with an anti-LAMP-1 mouse monoclonal antibody diluted 1/100 followed by the secondary antibody, anti-mouse–Alexa Fluor 488, diluted 1/200. Bacteria were detected with anti-lipopolysaccharide (anti-LPS) antibodies raised in rabbits and diluted 1/50, followed by the secondary antibody Alexa Fluor 594 diluted 1/200. (B) Immunofluorescence of LAMP-1 in A549 cells infected for 24 h with wild-type *L. pneumophila* 130b or the *dotA*::*cm*, *lpnE*::*km*, or *lpnE*::*km*/(pMIP:*lpnE*) mutant. Primary and secondary antibodies were the same as for panel A.

mammalian and protozoan hosts. The contribution of *lpnE* to infection of A/J mice was also examined by evaluating the ability of the *lpnE* mutant strain to compete with wild-type *L. pneumophila* 130b following intratracheal infection of A/J mice. Lung colonization was examined 24 and 72 h after infection (Fig. 1B). The mutant strain was recovered in similar numbers to wild-type *L. pneumophila* 130b 24 h after inoculation, but at 72 h significantly fewer mutant bacteria than *L. pneumophila* 130b bacteria were recovered. We also calculated the competitive index (CI) of the *lpnE* mutant by comparing the ratio of mutant to wild-type CFU in the lungs of mice at 72 h to the ratio of mutant to wild-type CFU in the inoculum. In general, a mutant with a CI of <0.5 is considered attenuated (5). At 72 h, the mean CI of the *lpnE* mutant was 0.22 ± 0.13 (Fig. 1C). Complementation of the *lpnE* mutant with full-length *lpnE* restored the CI of the mutant strain to 1.75 ± 1.58. These values were significantly different ($P = 0.019$; unpaired two-tailed *t* test), indicating that *lpnE* was needed for efficient colonization of the lungs of A/J mice (Fig. 1C).

Trafficking of the *lpnE* mutant in tissue culture cells. Although we showed previously that despite an initial invasion defect the *lpnE* mutant can replicate in THP-1 and A549 cells to wild-type levels (42), we did not examine the ability of the mutant to avoid trafficking to late endosomes and lysosomes. Here we investigated the capacity of the *lpnE* mutant vacuole to avoid fusion with late endosomes by determining the percentage of *lpnE* vacuoles that avoided the late endosomal marker LAMP-1 5 h after infection. We performed these experiments with A549 cells and THP-1 cells, with the latter undergoing extended treatment with phorbol 12-myristate 13-acetate to ensure differentiation into elongated cells, which are easier to view using immunofluorescence microscopy. We found that 5 h after infection, the majority of vacuoles containing a *dotA* mutant were positive for LAMP-1 in both A549 and THP-1 cells (Fig. 2 and 3). In contrast, the majority of *L. pneumophila* 130b vacuoles avoided LAMP-1 (Fig. 2 and 3). Interestingly, the *lpnE* mutant exhibited an intermediate trafficking phenotype. The percentage of *lpnE* mutant vacuoles that avoided LAMP-1 was significantly different from those observed for both the wild-type and the *dotA* mutant in A549 cells and for the wild-type in THP-1 cells (Fig. 3). This indicated that the inactivation of *lpnE* altered vacuole trafficking in tissue culture cells. Complementation of the *lpnE* mutant with full-length *lpnE* restored the ability of *L. pneumophila* to evade fusion with LAMP-1 (Fig. 3). Interestingly, the percentage of LAMP-1 avoidance for wild-type *L. pneumophila* was notably lower for THP-1 cells than for A549 cells 5 h after infection. This suggested that THP-1 cells had a greater capacity to drive

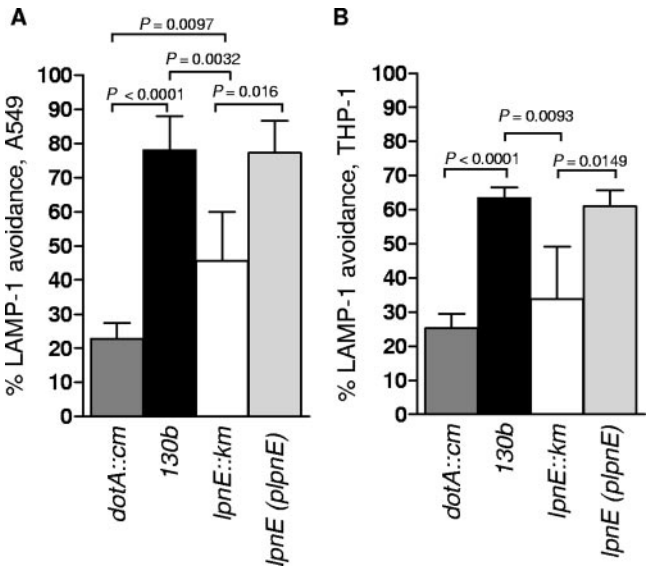

FIG. 3. Percentage of *Legionella*-containing vacuoles that avoided LAMP-1 after infection of A549 cells (A) and THP-1 cells (B) for 5 h. *P* values of <0.05 (unpaired two-tailed *t* test) are indicated. LAMP-1 avoidance was scored blind according to the staining patterns indicated in Fig. 4A.

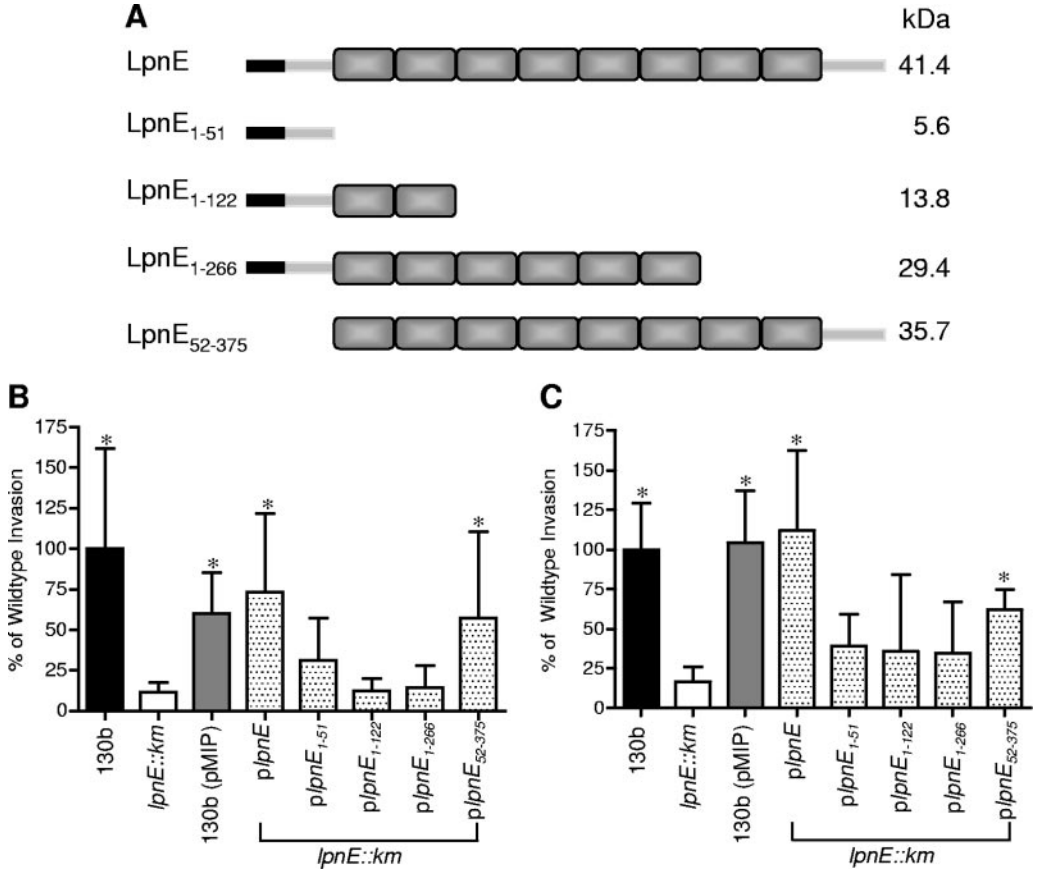

FIG. 4. (A) Schematic representation of *L. pneumophila* SLR protein LpnE and truncated variants created for this study. Shaded rectangles represent the SLR regions, and black rectangles signify the predicted N-terminal 22-amino-acid signal peptide. The truncations were used to complement *L. pneumophila lpnE*::*km*, and resulting strains were examined for uptake by THP-1 macrophages (B) and A549 epithelial cells (C). Data are expressed as percentages of the amount of inoculum that was intracellular following a 2-h infection and 1-h gentamicin treatment and are means ± standard deviations for at least three independent experiments. *, significantly different from the *lpnE*::*km* mutant ($P < 0.05$; unpaired two-tailed *t* test).

endosome fusion, which may relate to their monocytic lineage and/or the extended differentiation time used to produce flat cells for microscopy. Since the *lpnE* mutant showed decreased avoidance of LAMP-1, we also examined the ability of the *lpnE* mutant to form vacuoles containing multiple (>10) bacteria. Similar to wild-type *L. pneumophila*, the *lpnE* mutant was still able to form vacuoles containing multiple bacteria, and these did not associate with LAMP-1 (Fig. 2B).

Transcomplementation of the *lpnE* mutant with truncated forms of *lpnE*. To investigate the putative role of the eight SLR regions of LpnE in host-pathogen interactions, four truncated versions of *lpnE* were designed to encompass the N terminus (amino acids 1 to 51), the N terminus plus two SLR domains (amino acids 1 to 122), the N terminus and the first six SLR domains (amino acids 1 to 266), and the eight SLR domains plus the C terminus (amino acids 52 to 375) (Fig. 4A). These *lpnE* truncations were cloned into the pMIP complementation vector, which utilizes the constitutively active *mip* promoter region (56), and introduced into the *lpnE* mutant. The ability of these derivatives of *L. pneumophila* to establish infection of both THP-1 macrophages and A549 alveolar epithelial cells was investigated (Fig. 4B and C). As reported previously, disruption of *lpnE* significantly reduced the ability of *L. pneumophila* to infect both cell lines, which was restored upon transcomplementation with full-length *lpnE* (Fig. 4B and C) (42). In contrast, transcomplementation of the mutant strain with truncated forms of *lpnE* did not restore wild-type levels of invasion. The introduction of $lpnE_{1\text{-}51}$, $lpnE_{1\text{-}122}$, or $lpnE_{1\text{-}266}$ had no influence on the compromised ability of the *lpnE* mutant to infect THP-1 or A549 cells. However, $lpnE_{52\text{-}375}$, encoding all of the SLR regions of LpnE but lacking the N-terminal 51 amino acids, was able to partially complement the *lpnE* mutant defect in both cell types (Fig. 4B and C). We also examined the ability of the *lpnE* mutant complemented with $lpnE_{52\text{-}375}$ to colonize the lungs of A/J mice in mixed infections with wild-type *L. pneumophila*. The CI of the *lpnE*::*km* strain carrying pMIP:$lpnE_{52\text{-}375}$ in competition with wild-type *L. pneumophila*/(pMIP) at 72 h was 0.59 ± 0.11, indicating partial complementation of the virulence defect (Fig. 1C).

Presence of LpnE in culture supernatants. The reduced invasion phenotype of the *lpnE* mutant in THP-1 and A549 cells and the replication defect of the mutant in *A. castellanii* and A/J mice suggested that LpnE may play a direct role in host-pathogen interaction. To determine the localization of LpnE in *L. pneumophila*, rabbit polyclonal antibodies were raised against purified MBP-LpnE and used to detect LpnE

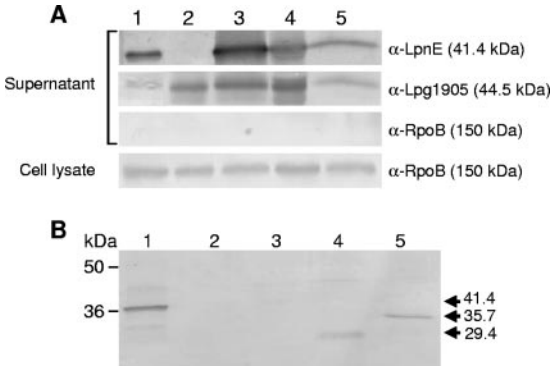

FIG. 5. Immunoblot analysis of culture supernatants precipitated with TCA and detected with anti-LpnE, anti-Lpg1905, and anti-RpoB antibodies. (A) Stationary-phase TCA-precipitated culture supernatants and whole-cell lysate samples from derivatives of *L. pneumophila* 130b were separated by SDS-polyacrylamide gel electrophoresis. Lane 1, *L. pneumophila* 130b; lane 2, *lpnE*::*km* mutant; lane 3, *lpnE*::*km*/(pMIP:*lpnE*) mutant; lane 4, Δ*dotA* mutant; lane 5, Δ*lspDE* mutant. (B) TCA-precipitated stationary-phase culture supernatants. Lane 1, *L. pneumophila lpnE*::*km*/(pMIP:*lpnE*) mutant; lane 2, *lpnE*::*km*/(pMIP:$lpnE_{1\text{-}51}$) mutant; lane 3, *lpnE*::*km*/(pMIP:$lpnE_{1\text{-}122}$) mutant; lane 4, *lpnE*::*km*/(pMIP:$lpnE_{1\text{-}266}$) mutant; lane 5, *lpnE*::*km*/(pMIP:$lpnE_{52\text{-}375}$) mutant. The presence of both $LpnE_{1\text{-}266}$ and $LpnE_{52\text{-}375}$ in the culture supernatant is indicated by arrows and the predicted molecular size of each protein.

within stationary-phase *L. pneumophila* cultures. Immunoblot analysis showed that LpnE was present in the culture supernatant of *L. pneumophila* 130b (Fig. 5A), indicating that the protein was extracellular. Interestingly, the presence of LpnE in culture supernatants appeared to be independent of the virulence-related Lsp type II secretion system (46) and also independent of the Dot/Icm type IV secretion system, as LpnE was found in culture supernatants derived from both *lspDE* and *dotA* mutant strains of *L. pneumophila* 130b (Fig. 5A). To confirm that the presence of LpnE in the culture supernatant was not due to cell breakdown and contamination with cytoplasmic proteins, the same samples were incubated with anti-RpoB to detect a known cytoplasmic protein. In addition, we used antibodies to the secreted protein Lpg1905 (47) to control for protein loading (Fig. 5A). Apart from a lack of LpnE expression in the *lpnE* mutant and overexpression of LpnE from the complementing pMIP plasmid, LpnE was present in equivalent amounts in all other strains tested, including wild-type *L. pneumophila* 130b and the secretion system mutants (Fig. 5A).

We also examined export of the truncated forms of LpnE. The predicted products of $lpnE_{1\text{-}51}$ and $lpnE_{1\text{-}122}$ were not detected with anti-LpnE, even in whole-cell lysate samples (data not shown). This indicated that these small truncations of LpnE were either not recognized by our anti-LpnE sera or, alternatively, that $lpnE_{1\text{-}51}$ and $lpnE_{1\text{-}122}$ were not expressed in vitro. However, both $LpnE_{1\text{-}266}$ and $LpnE_{52\text{-}375}$ were expressed and present in culture supernatants (Fig. 5B). This demonstrated that neither the C terminus, absent in $LpnE_{1\text{-}266}$, nor the predicted N-terminal signal sequence, absent in $LpnE_{52\text{-}375}$, was essential for LpnE export. At this stage, the mechanism by which LpnE reaches the culture supernatant is unknown.

LpnE interacts with Ig-like domains of eukaryotic proteins via the SLR regions. The presence of LpnE in culture supernatants provided further evidence that during infection, the protein may interact with host proteins to stimulate the uptake of *L. pneumophila*. Since TPR regions in general are known to mediate protein-protein interactions, the ability of LpnE to bind to eukaryotic proteins was examined using the yeast two-hybrid system. Screening of full-length LpnE against a HeLa cell cDNA library identified several putative binding partners of LpnE (Table 3). Recovery of the interacting clones revealed that the most prevalent binding partner, accounting for 28.6% of rescued clones, was OBSL1. This eukaryotic protein, ranging in molecular mass from 130 to 230 kDa, is a member of the Unc-89/obscurin gene family, exhibiting homology to the N-terminal region of the giant muscle protein obscurin, which is known to interact with titin, small ankyrin 1, and myosin within vertebrate skeletal muscle to mediate muscle contraction (3, 27, 57). The function of OBSL1 is unknown, although, similar to obscurin, the protein possesses a number of putative Ig-like domains and an Fn domain (Fig. 6A). While other putative

TABLE 3. Eukaryotic proteins identified as possible binding partners of LpnE from a yeast-two hybrid HeLa cell cDNA library screen

Protein	Function	% of recovered clones	Accession no.
OBSL1	Putative cytoskeletal adaptor protein	28.6	KIAA0657
N-Acetylneuraminic acid phosphate synthase	Sialic acid synthesis	14.3	AAF75261
Map kinase kinase (kinase 7)	Role in cell signaling	10.7	AAV38460
Phosphoserine aminotransferase	L-Serine biosynthesis	10.7	AAD42052
Coatomer protein I (COPI) epsilon subunit	Membrane trafficking, possesses TPR domain	7	CAA10316
Ras-related GTPase	Small GTPase	7	NP_005393
Neuronal protein	Unknown	7	AAP97253
Thyroid receptor-interacting protein 6 (TRIP6)	Organization of actin filaments and control of cell migration	3.6	AAK21007.1
Solute carrier family 3, isoform A	Transmembrane protein that can mediate integrin-dependent signaling	3.6	NP_001012679
ATPase 13A1	Putative role in cation transport	3.6	AAH69211
Na^+/K^+ ATPase beta-3 chain (ATPB-3)	Maintenance of electrochemical gradient across the plasma membrane	3.6	NP_001670

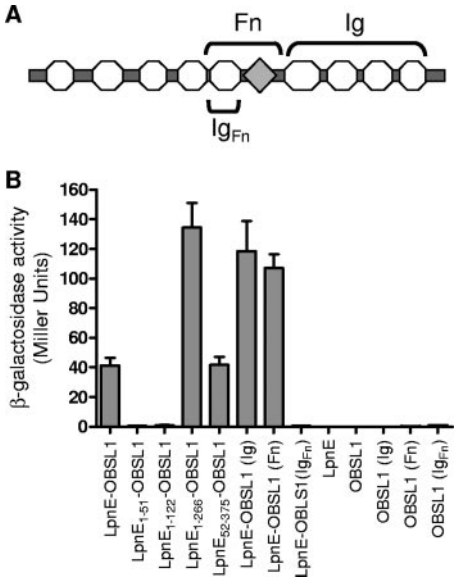

FIG. 6. (A) Domain organization of OBSL1, comprising several Ig-like domains (circles) and one Fn domain (diamond). The regions of OBSL1 subcloned for protein-protein interaction studies, namely, Ig, Fn, and Ig_{Fn}, are indicated. (B) Protein-protein interactions were assessed using the yeast two-hybrid system β-galactosidase reporter assay. Full-length LpnE, $LpnE_{1-266}$, and $LpnE_{52-375}$ showed strong interactions with OBSL1, as did full-length LpnE with two of the truncated forms of OBSL1 but not with Ig_{Fn}.

eukaryotic binding partners identified here may warrant further investigation, particularly the epsilon subunit of COPI involved in eukaryotic endosome trafficking and phagosome maturation (6), we initially examined the interaction between LpnE and OBSL1 as the most frequently recovered target. The specificity of the interaction between OBSL1 and LpnE was confirmed and quantified within *S. cerevisiae* PJ69-4a by using liquid culture β-galactosidase assays (Fig. 6B). The truncated forms of LpnE were also tested for the ability to bind to OBSL1. $LpnE_{1-51}$ and $LpnE_{1-122}$ could not bind OBSL1, but $LpnE_{1-266}$ and $LpnE_{52-375}$, with six and eight SLR regions, respectively, were able to interact with OBSL1. Curiously, $LpnE_{1-266}$ exhibited the strongest interaction with OBSL1, but full-length LpnE and $LpnE_{52-375}$ showed an equivalent strength of interaction with OBSL1, demonstrating that the full repertoire of SLR domains is needed for wild-type-strength protein-protein interaction (Fig. 6B). To investigate the basis of the interaction of LpnE with OBSL1 in more detail, two sections of OSBL1, one comprising the Fn type 3 domain and an Ig domain (Fn) and a second comprising the C-terminal Ig domains of OBSL1 (Ig) (Fig. 6A), were cloned into pGAD424 and examined for the ability to interact with LpnE (Fig. 6B). Both fragments of OBSL1 exhibited strong binding to LpnE, suggesting that the protein-protein interaction occurred through the Ig-like domains common to both fragments. In an attempt to confirm that LpnE interacted specifically with Ig-like domains, we cloned the 8.3-kDa predicted Ig_{Fn} domain into pGAD424 and examined its interaction with LpnE. β-Galactosidase assays for *S. cerevisiae* carrying pGBT9:LpnE and pGAD424:Ig_{Fn} demonstrated no interaction between the Ig_{Fn} peptide and LpnE. Although this lack of interaction may indicate that the Ig domains do not mediate an interaction between OBSL1 and LpnE, it is more likely that such a small peptide is unable to establish the tertiary structure required for protein-protein interactions.

DISCUSSION

Bacterial attachment and subsequent entry into host cells are required for *L. pneumophila* infection. Furthermore, the ability of *L. pneumophila* to subvert host cell trafficking pathways occurs immediately upon uptake, indicating that this process is likely linked to mechanisms of entry (36). *L. pneumophila* uptake by host cells occurs via coiling and conventional phagocytosis. Coiling phagocytosis, which has been observed during *L. pneumophila* entry into human monocytes, alveolar macrophages, and polymorphonuclear leukocytes and into amoebae, involves the formation of a long, asymmetrical pseudopod that coils around and engulfs the bacterium (1, 7, 33). This is an unusual mechanism of phagocytosis observed only in the uptake of *L. pneumophila* and some spirochetes, trypanosomatids, and yeasts (44, 51). Recently, it was shown, using *Dictyostelium discoideum*, that *L. pneumophila* utilizes specific phagocytic processes that abrogate the use of host cell phosphoinositol 3-kinase, which is traditionally important for host cell membrane dynamics during phagocytosis (55). A range of *L. pneumophila* virulence factors have been identified as being important for entering host cells. These include pili, the heat shock protein Hsp60, RtxA, the Dot/Icm type IV secretion system, and other novel factors, including the SLR proteins EnhC and LpnE (14, 24, 26, 31, 42, 50). In this study, we extended our previous observations that LpnE contributes to host cell invasion by *L. pneumophila* and compared the growth characteristics of wild-type *L. pneumophila* and the *lpnE* mutant in *A. castellanii* and in the lungs of A/J mice. In both virulence models, the *lpnE* mutant was significantly attenuated compared to the wild-type strain. In particular, the difference observed between wild-type *L. pneumophila* and the *lpnE* mutant in bacterial replication in the lungs of A/J mice indicated that *lpnE* plays a significant role in the interaction of *L. pneumophila* with a mammalian host. Interestingly, a significant difference in lung colonization between the two strains was evident only at 72 h, which did not appear to correlate with the early defect seen in tissue culture cells. However, it is possible that the early infection defect of the *lpnE* mutant in mammalian cells in vitro did not result in an observable colonization defect until later times in infection, after more than one round of bacterial entry and egress had occurred. Therefore, the virulence defect of the *lpnE* mutant may reflect a reduced capacity to invade eukaryotic cells and establish a replicative vacuole that, over time, results in reduced mutant numbers compared to wild-type growth.

The presence of five genes encoding SLR proteins in the *L. pneumophila* genome may point to functional redundancy (11, 13). This is a common feature of *L. pneumophila*, with functional redundancy clearly present among substrates of the Dot/Icm system (39). However, possession of variant forms of virulence determinants may also point to the ability of *L. pneumophila* to promote growth within a divergent range of environmental hosts. Further evidence that all of the SLR-containing proteins may be important for *L. pneumophila*

propagation is that their expression is upregulated during the transmissive phase within *A. castellanii* (10). The transmissive phase is induced late during intracellular replication and is characterized by the upregulation of most virulence determinants, including flagellar components and substrates of the Dot/Icm secretion system. Induction of these factors facilitates bacterial egress from host cells and efficient spread of the infection. Both EnhC (21 SLRs) and LpnE (8 SLRs) contribute to host cell entry, and it is assumed that LidL (12 SLRs), Lpg1356 (8 SLRs), and Lpg1062 (8 SLRs) may also contribute to *L. pneumophila* virulence. Both EnhC and LidL play a role in early signaling events that regulate trafficking of the *Legionella* -containing vacuole. Vacuoles containing mutants of these proteins show a reduced ability to evade acquisition of LAMP-1, providing further evidence that the *L. pneumophila* SLR proteins are important for the initial establishment of infection (15). In this study, we also observed reduced LAMP-1 avoidance by vacuoles containing the *lpnE* mutant compared to those containing wild-type *L. pneumophila*.

Complementation with full-length *lpnE* restored vacuole trafficking to the wild-type phenotype. The shared phenotype of the *enhC*, *lidL*, and *lpnE* mutants may indicate that the SLR proteins act together or in combination to inhibit vacuolar acquisition of LAMP-1, although the mechanism behind this is currently unknown. These data may also indicate that the SLR proteins are not specifically involved in the invasion of host cells, but rather in the mechanisms that immediately follow, namely, establishment of the intracellular replicative niche. The reduced recovery of SLR protein mutants at the initial stages of infection may indicate increased lysosomal degradation of these strains.

Using antibodies generated to recombinant LpnE, we found that LpnE was present in the culture supernatant of *L. pneumophila*. The presence of LpnE in the culture supernatant appeared to be independent of both the Lsp and Dot/Icm secretion systems and supports the finding that LpnE was not present in the type II secretome, despite the presence of a putative N-terminal signal peptide (18). Of further curiosity is the fact that both $LpnE_{1\text{-}266}$, which lacks the C-terminal region of LpnE, including the last two SLRs, and $LpnE_{52\text{-}375}$, which retains only the SLR regions of LpnE, omitting the putative N-terminal signal peptide, were observed in culture supernatants. This suggested that LpnE uses a unique mechanism of export, independent of N- or C-terminal secretion signals, or that LpnE export may occur through more than one mechanism. At this stage, the mechanism by which LpnE reaches the extracellular environment is unknown.

While the expression of $LpnE_{1\text{-}51}$ and $LpnE_{1\text{-}122}$ could not be detected by Western blotting, it was clear that $LpnE_{1\text{-}266}$ and $LpnE_{52\text{-}375}$ were both localized similarly to full-length LpnE. Despite both of these truncations being present in the culture supernatant, $lpnE_{52\text{-}375}$, encoding eight SLRs, was the only truncation able to partially complement the invasion defect of the *lpnE* mutant. $lpnE_{52\text{-}375}$ was also able to partially complement the virulence defect of the *lpnE* mutant in A/J mice. While $lpnE_{52\text{-}375}$ could not completely restore wild-type levels of invasion or lung colonization, this investigation nevertheless highlighted the importance of the full SLR repertoire for function.

Given that SLR domains mediate protein-protein interactions, we examined the ability of LpnE to bind with eukaryotic proteins by using the yeast two-hybrid system. A range of putative eukaryotic binding partners were isolated, the most frequent of which was OBSL1. LpnE and two truncated forms, $LpnE_{1\text{-}266}$ and $LpnE_{52\text{-}375}$, were shown to interact strongly with OBSL1. More specifically, LpnE bound fragments of OBSL1 containing multiple Ig-like domains or an Fn and an Ig-like domain. However, using this technique, we were unable to demonstrate an interaction between LpnE and a single Ig domain. Recently, OBSL1 expression was observed in a range of tissues, and localization of the protein to intercalated discs and the perinuclear region of rat cardiac myocytes indicated its likely function as a cytoskeletal adaptor protein (27). While the interaction between LpnE and OBSL1 may not occur in vivo, it allows speculation that LpnE may interact with other proteins that possess Ig-like folds. Ig-like domains are present in a range of proteins of eukaryotic organisms with roles in cell-cell recognition and in cell surface receptors. Many eukaryotic surface-exposed cell adhesion molecules possess Ig domains to which LpnE may bind, leading to the initiation of host cell signaling events that enhance *L. pneumophila* internalization and/or establishment of the *L. pneumophila* replicative vacuole.

There is evidence within eukaryotic systems that proteins containing SLRs are involved in multiprotein complexes with specific and distinct biological functions. For example, the SLR protein Hrd3 is a key component of the endoplasmic reticulum-associated degradation complex that identifies and processes misfolded proteins for ubiquitination (25). Furthermore, it is clear that SLR regions are crucial to the formation of such complexes, and they are considered to act as scaffolding or receptor domains linking components via multiple substrate binding sites. Hence, it is attractive to propose that SLR-containing proteins of *L. pneumophila*, particularly LpnE, EnhC, and LidL, may form a multiprotein complex linking the bacterium to the host cell via cell adhesion molecules and that they may act in concert to achieve this. However, this study also identified a range of other eukaryotic proteins that may interact with LpnE in vivo. To establish which of these putative binding partners is important during *L. pneumophila* infection, the in vivo localization of LpnE and the other SLR proteins of *L. pneumophila* must be investigated. In addition, further elucidation of the interactions between *L. pneumophila* SLR proteins and both host and bacterial proteins is therefore important to discern the functions of these virulence determinants in *L. pneumophila* infection.

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REFERENCES

1. **Abu Kwaik, Y.** 1996. The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. Appl. Environ. Microbiol. **62:**2022–2028.

2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1995. Current protocols in molecular biology. John Wiley and Sons, Inc., New York, NY.
3. **Bagnato, P., V. Barone, E. Giacomello, D. Rossi, and V. Sorrentino.** 2003. Binding of an ankyrin-1 isoform to obscurin suggests a molecular link between the sarcoplasmic reticulum and myofibrils in striated muscles. J. Cell Biol. **160:**245–253.
4. **Bartel, P. L., C.-T. Chien, R. Sternglanz, and S. R. Fields.** 1993. Using the two-hybrid system to detect protein-protein interactions, p. 153–179. *In* D. A. Hartley (ed.), Cellular interactions in development: a cellular approach. Oxford University Press, Oxford, United Kingdom.
5. **Beuzon, C. R., and D. W. Holden.** 2001. Use of mixed infections with *Salmonella* strains to study virulence genes and their interactions in vivo. Microbes Infect. **3:**1345–1352.
6. **Botelho, R. J., D. J. Hackman, A. D. Schreiber, and S. Grinstein.** 2000. Role of COPI in phagosome maturation. J. Biol. Chem. **275:**15717–15727.
7. **Bozue, J. A., and W. Johnson.** 1996. Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii* : uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. Infect. Immun. **64:**668–673.
8. **Broms, J. E., P. J. Edqvist, A. Forsberg, and M. S. Francis.** 2006. Tetratricopeptide repeats are essential for PcrH chaperone function in *Pseudomonas aeruginosa* type III secretion. FEMS Microbiol. Lett. **256:**57–66.
9. **Broms, J. E., A. Forslund, A. Forsberg, and M. S. Francis.** 2003. PcrH of *Pseudomonas aeruginosa* is essential for secretion and assembly of the type III translocon. J. Infect. Dis. **188:**1909–1921.
10. **Bruggemann, H., A. Hagman, M. Jules, O. Sismeiro, M. A. Dillies, C. Gouyette, F. Kunst, M. Steinert, K. Heuner, J. Y. Coppee, and C. Buchrieser.** 2006. Virulence strategies for infecting phagocytes deduced from the in vivo transcriptional program of *Legionella pneumophila*. Cell. Microbiol. **8:**1228–1240.
11. **Cazalet, C., C. Rusniok, H. Bruggemann, N. Zidane, A. Magnier, L. Ma, M. Tichit, S. Jarraud, C. Bouchier, F. Vandenesch, F. Kunst, J. Etienne, P. Glaser, and C. Buchrieser.** 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. Nat. Genet. **36:**1165–1173.
12. **Chen, J., K. S. de Felipe, M. Clarke, H. Lu, O. R. Anderson, G. Segal, and H. A. Shuman.** 2004. *Legionella* effectors that promote nonlytic release from protozoa. Science **303:**1358–1361.
13. **Chien, M., I. Morozova, S. Shi, H. Sheng, J. Chen, S. M. Gomez, G. Asamani, K. Hill, J. Nuara, M. Feder, J. Rineer, J. J. Greenberg, V. Steshenko, S. H. Park, B. Zhao, E. Teplitskaya, J. R. Edwards, S. Pampou, A. Georghiou, I. C. Chou, W. Iannuccilli, M. E. Ulz, D. H. Kim, A. Geringer-Sameth, C. Goldsberry, P. Morozov, S. G. Fisher, G. Segal, X. Qu, A. Rzhetsky, P. Zhang, E. Cayanis, P. J. De Jong, J. Ju, S. Kalachikov, H. A. Shuman, and J. J. Russo.** 2004. The genomic sequence of the accidental pathogen *Legionella pneumophila*. Science **305:**1966–1968.
14. **Cirillo, S. L. G., J. Lum, and J. D. Cirillo.** 2000. Identification of novel loci involved in entry by *Legionella pneumophila*. Microbiology **146:**1345–1359.
15. **Conover, G. M., I. Derre, J. P. Vogel, and R. R. Isberg.** 2003. The *Legionella pneumophila* LidA protein: a translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity. Mol. Microbiol. **48:**305–321.
16. **Core, L., and M. Peregro.** 2003. TPR-mediated interaction of RapC with ComA inhibits response regulator-DNA binding for competence development in *Bacillus subtilis*. Mol. Microbiol. **49:**1509–1522.
17. **Das, A. K., P. T. W. Cohen, and D. Barford.** 1998. The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. EMBO J. **17:**1192–1199.
18. **DebRoy, S., J. Dao, M. A. Soderberg, O. Rossier, and N. P. Cianciotto.** 2006. *Legionella pneumophila* type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. Proc. Natl. Acad. Sci. USA **103:**19146–19151.
19. **de Felipe, K. S., S. Pampou, O. S. Jovanovic, C. D. Pericone, S. F. Ye, S. Kalachikov, and H. A. Shuman.** 2005. Evidence for acquisition of *Legionella* type IV secretion substrates via interdomain horizontal gene transfer. J. Bacteriol. **187:**7716–7726.
20. **Dodt, G., and S. J. Gould.** 1996. Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. J. Cell Biol. **135:**1763–1774.
21. **Doyle, R. M., T. W. Steele, A. M. McLennan, I. H. Parkinson, P. A. Manning, and M. W. Heuzenroeder.** 1998. Sequence analysis of the *mip* gene of the soilborne pathogen *Legionella longbeachae*. Infect. Immun. **66:**1492–1499.
22. **Engleberg, N. C., D. J. Drutz, and B. I. Eisenstein.** 1984. Cloning and expression of *Legionella pneumophila* antigens in *Escherichia coli*. Infect. Immun. **44:**222–227.
23. **Feeley, J. C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Rasheed, D. C. Mackel, and W. B. Baine.** 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J. Clin. Microbiol. **10:**437–441.
24. **Gao, L. Y., O. S. Harb, and Y. Abu Kwaik.** 1998. Identification of macrophage-specific infectivity loci (mil) of *Legionella pneumophila* that are not required for infectivity of protozoa. Infect. Immun. **66:**883–892.
25. **Gardener, R. G., G. M. Swarbrick, N. W. Bays, S. R. Cronin, S. Wilhovsky, L. Seelig, C. Kim, and R. Y. Hampton.** 2000. Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. J. Cell Biol. **151:**69–82.
26. **Garduno, R. A., E. Garduno, and P. S. Hoffman.** 1998. Surface-associated hsp60 chaperonin of *Legionella pneumophila* mediates invasion in a HeLa cell model. Infect. Immun. **66:**4602–4610.
27. **Geisler, S. B., D. Robinson, M. Hauringa, M. O. Raeker, A. B. Borisov, M. V. Westfall, and M. W. Russell.** 2007. Obscurin-like 1, OBSL1, is a novel cytoskeletal protein related to obscurin. Genomics **89:**521–531.
28. **Gietz, R. D., R. H. Schiestl, A. R. Willems, and R. A. Woods.** 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast **11:**355–360.
29. **Grant, B., and I. Greenwald.** 1996. The *Caenorhabditis elegans sel-1* gene, a negative regulator of *lin-12* and *glp-1*, encodes a predicted extracellular protein. Genetics **143:**237–247.
30. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Ellege.** 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805–816.
31. **Hilbi, H., G. Segal, and H. A. Shuman.** 2001. Icm/Dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. Mol. Microbiol. **42:**603–617.
32. **Hirano, T., M. Kinoshita, K. Morikawa, and M. Yanagida.** 1990. Snap helix with knob and hole: essential repeats in *Schizosaccharomyces pombe* nuclear protein *nuc2* +. Cell **60:**319–328.
33. **Horwitz, M. A.** 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell **36:**27–33.
34. **Ishihama, A., N. Fujita, and R. E. Glass.** 1987. Subunit assembly and metabolic stability of *E. coli* RNA polymerase. Proteins **2:**42–53.
35. **James, P., J. Halladay, and E. A. Craig.** 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:**1425–1436.
36. **Kagan, J. C., M. P. Stein, M. Pypaert, and C. R. Roy.** 2004. *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle. J. Exp. Med. **199:**1201–1211.
37. **King, R. W., J. M. Peters, S. Tugendreich, M. Rolfe, P. Hieter, and M. W. Kirschner.** 1995. A 20*S* complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell **81:**279–288.
38. **Leyton, D. L., J. Sloan, R. E. Hill, S. Doughty, and E. L. Hartland.** 2003. Transfer region of pO113 from enterohemorrhagic *Escherichia coli* : similarity with R64 and identification of a novel plasmid-encoded autotransporter, EpeA. Infect. Immun. **71:**6304–6319.
39. **Luo, Z. Q., and R. R. Isberg.** 2004. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. Proc. Natl. Acad. Sci. USA **101:**841–846.
40. **Mittl, P. R. E., and W. Schneider-Brachert.** 2007. Sel1-like repeat proteins in signal transduction. Cell. Signal. **19:**20–31.
41. **Nagai, H., J. C. Kagan, X. Zhu, R. A. Kahn, and C. R. Roy.** 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science **295:**679–682.
42. **Newton, H. J., F. M. Sansom, V. Bennett-Wood, and E. L. Hartland.** 2006. Identification of *Legionella pneumophila* -specific genes by genomic subtractive hybridization with *Legionella micdadei* and identification of *lpnE*, a gene required for efficient host cell entry. Infect. Immun. **74:**1683–1691.
43. **Okabe, M., T. Yakushi, and M. Homma.** 2005. Interactions of MotX with MotY and with the PomA/PomB sodium ion channel complex of the *Vibrio alginolyticus* polar flagellum. J. Biol. Chem. **280:**25659–25664.
44. **Rittig, M. G., K. Schroppel, K. H. Seack, U. Sanders, E. N. N'Diaye, I. Maridonneau-Parini, W. Solbach, and C. Bogdan.** 1998. Coiling phagocytosis of trypanosomatids and fungal cells. Infect. Immun. **66:**4331–4339.
45. **Robey, M., W. A. O'Connell, and N. P. Cianciotto.** 2001. Identification of *Legionella pneumophila rcp*, a *pagP* -like gene that confers resistance to cationic antimicrobial peptides and promotes intracellular infection. Infect. Immun. **69:**4276–4286.
46. **Rossier, O., S. R. Starkenburg, and N. P. Cianciotto.** 2004. *Legionella pneumophila* type II protein secretion promotes virulence in the A/J mouse model of Legionnaires' disease pneumonia. Infect. Immun. **72:**310–321.
47. **Sansom, F. M., H. J. Newton, S. Crikis, N. P. Cianciotto, P. J. Cowan, A. F. d'Apice, and E. L. Hartland.** 2007. A bacterial ecto-triphosphate diphosphohydrolase similar to human CD39 is essential for intracellular multiplication of *Legionella pneumophila*. Cell. Microbiol. **9:**1922–1935.
48. **Segal, G., M. Purcell, and H. A. Shuman.** 1998. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc. Natl. Acad. Sci. USA **95:**1669–1674.
49. **Sikorski, R. S., M. S. Boguski, M. Goebl, and P. Hieter.** 1990. A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. Cell **60:**307–317.
50. **Stone, B. J., and Y. Abu Kwaik.** 1998. Expression of multiple pili by *Legionella pneumophila* : identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. Infect. Immun. **66:**1768–1775.
51. **Szczepanski, A., and H. B. Fleit.** 1988. Interaction between *Borrelia burgdor-*

feri and polymorphonuclear leukocytes: phagocytosis and the induction of the respiratory burst. Ann. N. Y. Acad. Sci. **539:**425–428.
52. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature **369:**758–761.
53. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.
54. **Wattiau, P., B. Bernier, P. Deslee, T. Michiels, and G. R. Cornelis.** 1994. Individual chaperones required for Yop secretion by *Yersinia*. Proc. Natl. Acad. Sci. USA **91:**10493–10497.
55. **Weber, S. S., C. Ragaz, K. Reus, Y. Nyfeler, and H. Hilbi.** 2006. *Legionella pneumophila* exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole. PLoS Pathog. **2:**418–430.
56. **Wieland, H., M. Faigle, F. Lang, H. Northoff, and B. Neumeister.** 2002. Regulation of the *Legionella mip-* promotor during infection of human monocytes. FEMS Microbiol. Lett. **212:**127–132.
57. **Young, P., E. Ehler, and M. Gautel.** 2001. Obscurin, a giant sarcomeric Rho guanine nucleotide exchange factor protein involved in sarcomere assembly. J. Cell Biol. **154:**123–136.

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