Supporting Information

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SI Materials and Methods

Preparation of Osmotically Shocked *Legionella pneumophila* Cells. *L. pneumophila* grown on charcoal-yeast extract plates for 2 d at 37 °C were inoculated in 2.5 mL of N-(2-acetamido)-2-aminoethanesulfonic acid-buffered yeast extract broth media (OD_{600} = 0.2). The liquid culture was incubated for 24 h at 37 °C with rotary shaking (250 min^{-1}). Osmotically shocked bacterial cells were prepared as previously described (1) with modifications as follows: We harvested 1 mL of bacterial cultures by centrifugation with microfuge for 5 min at 4 °C. The pellets were suspended with 1 mL of 1 M Sucrose, 150 mM Tris·Cl (pH 7.5), and 1 mM EDTA, and centrifuged again at 15,700 × *g* for 5 min at room temperature. The supernatants were removed and the pellets were suspended homogeneously with a residual small amount (about 10 μL) of buffer in the tubes. After standing the tubes at room temperature for 5 min, 1 mL of ice-cold distilled water was rapidly added into the tubes and immediately mixed well with the bacterial cells. After standing the tubes on ice for 5 min, final 15 μg/mL of DNaseI and 10 mM $MgCl_2$ were added, and the tubes were incubated at room temperature for 10 min to digest chromosomal DNA. Final 20 mM EDTA and 150 mM Tris·HCl pH 7.5 were added to the tubes, and the bacterial cells were spun down at 15,700 × *g* for 30 min at 4 °C. The pellets were washed again with 1 mL of cold TE (10 mM Tris·Cl pH 7.5, 1 mM EDTA). The final pellets were suspended with 50 μL of TE as samples for electron microscopy.

Immunogold Labeling of T4SS Core Complexes. Anti-rabbit IgG-Gold antibody (Sigma G7402) was used for immunogold labeling of the purified type IV secretion system (T4SS) core complexes. Labeling was performed on glow-discharged carbon grids. Sample-applied grids were treated with 5 μL of 0.1% (wt/vol) BSA in PBS for 30 min in a sealed container at room temperature to decrease nonspecific binding. After removing the solution from grids using edges of small paper filters, the grids were incubated with 5 μL of 1 μg/mL of the primary antibodies (anti-DotF or anti-M45 antibodies) in 0.1% (wt/vol) BSA/PBS for 60 min in a sealed container at room temperature. The grids were washed by 10 μL of PBS six times (2 min each) at room temperature. Subsequently, the grids were incubated with 5 μL of 1/20 dilution of the IgG-Gold antibody in 0.1% (wt/vol) BSA/PBS for 60 min in a sealed container at room temperature. The grids were washed by 10 μL of PBS six times (2 min each) and by 10 μL of distilled water six times (2 min each) at room temperature and stained with 2% (wt/vol) uranyl acetate for transmission EM.

Cell Culture. U937 monocyte-like cells were cultured in RPMI-1640 (LifeScience) supplemented with heat-inactivated 10% FBS. Chinese hamster ovary (CHO)–FcγRII cells were cultured in α-MEM (LifeScience) supplemented with heat-inactivated 10% FBS as described (2).

Effector Translocation Assay. Translocation of adenylate cyclase (cya)-fused proteins into CHO–FcγRII cells after infection with *Legionella* was assayed as described previously (3, 4) with minor modifications. Briefly, CHO–FcγRII cells were replated in 24-well plates and challenged by *Legionella* strains expressing cya fusions at a multiplicity of infection (moi) of 30 in the presence of opsonizing antibody (1:3,000 dilution). Eight hours later, infected cells were lysed in 500 μL of lysis reagent 1B provided from a cAMP Biotrak EIA System (GE Healthcare, RPN2251); cAMP levels were determined according to the manufacturer's instructions.

Intracellular Growth Assay. Growth of *L. pneumophila* in U937 cells was determined using a standard intracellular growth assay as described previously (5). Briefly, confluent phorbol-12-myristate-13-acetate–treated U937 cells in 24-well tissue culture dishes were infected with *L. pneumophila* at an moi of 2. After incubation for 1 h at 37 °C and 5% CO_2, the extracellular bacteria were removed with 3× PBS washes, and fresh media was added to the cells. Immediately or after 1–3 d infection, total bacterial colony forming units were determined for individual wells.

Shotgun Liquid Chromatography Tandem-Mass Spectrometry Analysis. Protein samples were separated by 12.5% SDS gel until the dye front ran into the gel for ∼1 cm. After visualization of proteins by coomassie brilliant blue staining, gel pieces excised into five pieces per sample were subjected to trypsin digestion and liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis (LTQ Orbitrap Velos, ThermoScientific). The obtained data were merged and analyzed using Scaffold (version 3.4.5, Proteome Software). We used the sum of total ion currents of assigned MS/MS spectra as a proxy for protein level, because it was reported that a label-free method based on ion currents provides a level of accuracy comparable to labeling approaches (6).

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2. Kagan JC, Roy CR (2002) *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat Cell Biol* 4(12):945–954.
3. Kubori T, Hyakutake A, Nagai H (2008) *Legionella* translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions. *Mol Microbiol* 67(6):1307–1319.
4. Nagai H, et al. (2005) A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. *Proc Natl Acad Sci USA* 102(3): 826–831.
5. Zuckman DM, Hung JB, Roy CR (1999) Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. *Mol Microbiol* 32(5):990–1001.
6. Cappadona S, Baker PR, Cutillas PR, Heck AJ, van Breukelen B (2012) Current challenges in software solutions for mass spectrometry-based quantitative proteomics. *Amino Acids* 43(3):1087–1108.

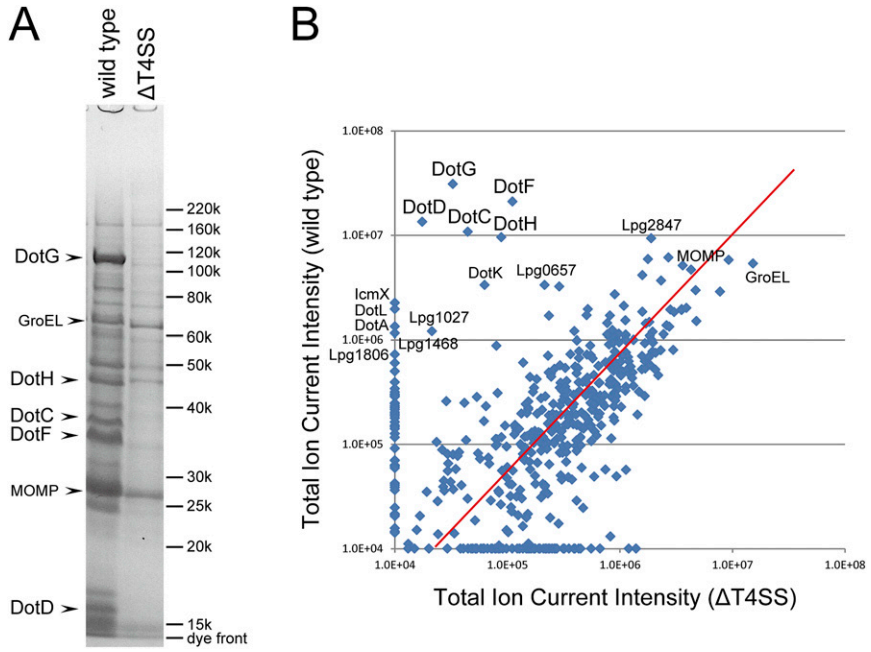

Fig. S1. Shotgun proteomics of the core-complex–enriched fractions from wild-type or ΔT4SS strains. Samples are isolated as in Fig. 2*E* and were subjected to 12.5% SDS gel analysis followed by coomassie brilliant blue staining (*A*). Excised gel pieces are subjected to trypsin digestion and LC-MS/MS analysis. The result was summarized by the scattered plot (*B*), where each detected *L. pneumophila* protein is plotted based on its sum of MS2 total ion currents, a protein abundance measure, in samples from wild-type (*y* axis) and the mutant (*x* axis) strains. It should be noted that in the condition used for the data analysis (minimum protein identification probability, 99.0%; minimum number of unique peptide, 2; minimum peptide identification probability, 95.0%) using Scaffold, a small number of peptides in the fraction isolated from the T4SS mutant strain are missassigned to some of the Dot/Icm proteins (for example, DotG has 623 and two MS spectra assigned in fractions isolated from wild-type and T4SS mutant strains, respectively), which give nonminimum ion current to Dot/Icm proteins even in the fraction isolated from the T4SS mutant. See Table S1 for the listing of selected proteins.

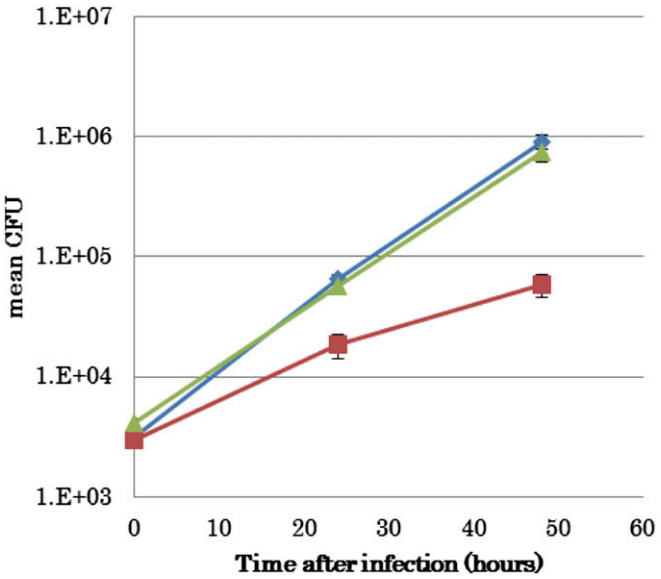

Fig. S2. M45 tagging at carboxy-terminus of DotD does not affect intracellular growth in U937 cells. Lp01 + vector, blue triangles; Δ*dotD* + vector, red squares; Δ*dotD* + p*dotD*–M45, green triangles.

Table S1. List of selected *L. pneumophila* proteins found in the core-complex–enriched fraction by shotgun proteomics

		Total ion currents (TICs)		
Proteins*	Molecular weight	Wild type	ΔT4SS	Fold change†
DotG	108 kDa	3.09E+07	3.27E+04	945
DotF	30 kDa	2.10E+07	1.11E+05	189
DotD	18 kDa	1.34E+07	1.75E+04	769
DotC	34 kDa	1.08E+07	4.45E+04	242
DotH	39 kDa	9.54E+06	8.83E+04	108
DotK	21 kDa	3.33E+06	6.26E+04	53
Lpg0657	28 kDa	3.33E+06	2.14E+05	16
YajC	12 kDa	3.22E+06	2.89E+05	11
IcmX	51 kDa	2.24E+06	1.00E+04	224
DotL	88 kDa	1.96E+06	1.00E+04	196
DotA	113 kDa	1.34E+06	1.00E+04	134
Lpg1027	50 kDa	1.20E+06	2.13E+04	56
Lpg1468	17 kDa	1.15E+06	1.00E+04	115

*Proteins with TIC(wild type) > 1.0×10^6 and fold change > 10 were shown.
†Fold change is defined as TIC(wild type)/TIC(ΔT4SS).

Table S2. A comparative table of selected T2SS/T4SS proteins

Type IVB		Type IVA				
Legionella	R64	*Agrobacterium*	pKM101	R388	Type II	Notes
DotD	TraH	VirB7	TraN	TrwH	GspD	Core component
DotC	TraI	—	—	—	—	Core component
DotB	TraJ	VirB11	TraG	TrwD	GspE	Secretion ATPase
DotH/IcmK	TraN	VirB9?	TraO?	TrwF?	—	Core component
DotG/IcmE	TraO	VirB10	TraF	TrwE	—	Core component
DotF/IcmG	TraP	—	—	—	—	Core component

T2SS, type II secretion system.