Supporting Information

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SI Materials and Methods

Plasmids Used for Gene Knock-In. The plasmid used for introducing the floxed gene into the chromosome of *L. pneumophila* was constructed as follows: First, we removed the PstI site in plasmid pJB908 (1) by ligating PstI-digested DNA that had been treated with mung bean nuclease to give pJB908PstI$^-$. We then inserted a cassette containing two directly repeated *lox*P sites from pZLlox-PGm (2) into pJB908PstI$^-$ as a BamHI/SalI fragment to give pJBloxP. After removing the BamHI and SacI sites by enzyme digestion followed by mung bean nuclease treatment, we expanded the multiple cloning site of pJBloxP by cloning a DNA fragment obtained by annealing from oligomers 5′-CGGAGCTCGGT-ACCGGATCCGCGGCCGCGCTAGCTCTAGAATTTAA A-TCTCGAGTGCA-3′ and 5′-CTCGAGATTTAAATTCTAG-AGCTAGCGCGGCCGCGGATCC GGTACCGAGCTCCG-3′ into SmaI/PstI digested vector. This manipulation introduced the following restriction sites between the two *lox*P elements: SacI, KpnI, NotI, BamHI, NheI, XbaI, *Swa*I and XhoI. The new plasmid was named pDP1. Using primers 5′-CTAGCCCGGGCTGGGC-TATCTGGACAAGGG-3′ and 5′-CATTCCCGGGGGAATC-GAAATCTCGTGATGG-3′, we cloned a 1 kb DNA fragment coding for the kanamycin resistance gene from pBBR1MCS-2 (3) as SmaI fragment into the *Swa*I site of pZL505 to give pZL506. Finally, we made pZL788 by cloning the floxing cassette from pZL506 into pBluescript and this plasmid is the source of a floxed cassette for future experiments.

Using the π protein-dependent plasmid pSB890 (4), we constructed pZL790, which is the basis for introducing floxed gene into *L. pneumophila* chromosome as follows: Two 1 kb DNA fragments flanking the intergenic region of *L. pneumophila* genes lpg2528 and lg2529 were amplified with primer pairs Up5′NotI/Up3′SalI (5′-CCTGCGGCCGCAACGCAAATTTCAACATGCC-3′/5′-CCTGTCGACCCCGGGCAAAATAGCAACCGCAATG-3′) and Down5′SalI/Down3′EcoRV (5′-CCTGTCGACTTCCTCAC-CAATTAAGTCAC-3′/5′-CCTGATATCGTCTATGATCAT-GAAATTAT-3′). After digesting with the appropriate restriction enzymes, the two DNA fragments were ligated into NotI/SmaI digested pSB890 to give pZL790. From this point, constructs for introducing each of the knock-in genes were made individually according to the availability of restriction sites in the gene of interest. For example, the plasmid used for the *icmQ* knock-in was constructed as follows: We first cloned the *icmQ* gene into pZL788 as a SacI/XbaI fragment to give pZL789. We then inserted the promoter region of *icmQ* (5) as a 136 bp EcoRV/SalI DNA fragment by PCR using primers PicmQup (5′-CCTGATATCAT-GATGAACGT GGTTTCAA-3′) and PicmQ down (5′-CCT-GATATCATGATGAACGTGGTTTCAA-3′) into SmaI/SalI digested pZL790 to give pZL793. The floxed *icmQ* gene was then cloned intopZL793 to give pZL794. Finally, we cloned a 0.8 kb chloramphenicol resistance gene as a NotI fragment into pZL795 to give pZL795Cm, which was used to introduce *icmQ* into the intergenic region between lpg2528 and lpg2529. In each step, we verified the correctness of the inserts by DNA sequencing. Plasmids for introducing other genes were constructed with similar procedures. Details for these cloning steps and the sequences of primers used are available upon request.

Construction of *L. pneumophila* Knock-In Strains. After obtaining a plasmid designed for introducing a floxed gene into the chromosome of *L. pneumophila*, we mobilized the construct into the corresponding *L. pneumophila* deletion mutant of the gene by tri-parental mating (6). Transconjugants selected on media containing 20 μg/ml kanamycin and 100 μg/ml streptomycin were streaked onto CYET media containing 4% sucrose to obtain colonies in which the integrated plasmid had been looped out by a second recombination event. We then identified candidate knock-in strains by screening colonies that were resistant to kanamycin but sensitive to the plasmid backbone marker chloramphenicol. After verifying the candidate strains by PCR analysis, we examined the intracellular growth of several candidates in mouse bone marrow-derived macrophages. In each case, the wild-type strain, Lp02, and the original deletion mutant were used as controls. Knock-in strains that could fully complement the mutation were retained for future use.

To construct the *dotA* knock-in strain, instead of using the *dotA* deficient strain Lp03, which contains a point mutation (7), we constructed an in-frame deletion mutant by a standard procedure (2). Two appropriately digested fragments amplified by PCR with oligoes ΔdotAup5′SalI 5′-CTAGGTCGACGCCTAAGT-CAATTTTTTCAATA-3′ dotAup3′BamHI 5′-TTGCGGATC-CGAGATAACTCAAGGCGGCTTCC-3′ dotAdown5′BamHI 5′-CTGCGGATCCGGTGGGTATACAGG CTGGGCAG-3′ dotAdown3′SacI 5′-CAGAGAGCTCCAGGGTCAGTAAAC-GAATGAGC-3′ were inserted pSR47 (2) digested with SalI and SacI. The mutant was obtained following a protocol described elsewhere (2).

Plasmids for Inducible Expression of *cre*. We constructed plasmids that allow IPTG-inducible expression of *cre* by placing tandem copies of the *lac* operator between the P*trc* promoter and the *cre* gene. In the final phases of this multiple-step plasmid construction procedure, plasmids carrying different copies of the *lac* operator were introduced into the knock-in *L. pneumophila* strain and the efficiency of IPTG-induced gene deletion was empirically examined for each strain. Details for construction are as follows: First, we cloned a DNA fragment derived from annealing 5′-ATGT*GTGGAATTGTGAGCGGATAA-CAATTTCA*CACAGGGTACCC-3′ and 5′-GGGTACCCTGT-*GTGAAATTGTTATCCGCTCACAATTCCAC*ACATGTAC-3′ into KpnI/HincII (the elements of the *lac* operator were italicized) digested pBBR1MCS-2 (3) to give pOP2. Similarly, we constructed pOP3 by inserting the same DNA fragment into KpnI/EcoRV digested pOP2. We then increased the tandem repeats of the *lac* operator to 4 copies by inserting this DNA element into KpnI/SmaI digested pOP3 to give pOP4.

To prepare a source of the *cre* gene associated with the necessary restriction sites, we amplified the gene using the primer pair 5′-CGATAGATCTACAGGAAACAGCTAT-GTCCAATTTACTGACCGTACAC-3′ and 5′-CTAGTCTA-GACTAATCGCCATCTTCCAGCA-3′ and inserted the fragment into XbaI/BamHI digested pBluescript as a BglII/XbaI fragment to give pBluecre. A KpnI/XbaI DNA fragment removed from pBluecre was cloned into similarly digested pOP4 to give pZL500.

To add another four copies of operator upstream of the *cre* gene, we amplified the operator region of pOP4 by PCR with primers 5′-GACTCACTATAGGGCGAATTGG-3′ and 5′-CTAGCTCGAGGGCTTTACACTTTATGCTTCC-3′. The PCR product was digested with XhoI/SmaI and then inserted into similarly digested pZL500 to give pZL502. These plasmids were the sources of the *cre* gene controlled by different copies of the *lac* operator.

A series of plasmids used to express *cre* under the control of IPTG were made from pZL188, a derivative of pJB908 config-

ured for protein expression. First, a BglII/XbaI DNA fragment amplified from pZL500 or pZL502 with primer 5′-CAGAGATCTGGCTTTACACTTTATGCTTC-3′ and 5′-CTAGCTCGAGGGCTTTACACT TTATGCTTCC-3′ was inserted into BglII/XbaI digested pZL188 to give pZL638 and pZL639, respectively. To gain tighter control of the expression of the *cre* gene, we inserted the same DNA fragments into BamHI/XbaI digested pZL638 or pZL639 to produce pZL680 and pZL681, respectively. These two plasmids exhibited the ideal properties in controlling the expression of *cre* in some of the *dot/icm* or *sdhA* knock-in strains, i.e., a low level of Cre in the absence of IPTG (the strains grew normally in broth containing kanamycin), but efficient induction can be achieved by addition of IPTG (see main text).

Construction of Strains Used for Intracellular Growth Experiments. For each knock-in strain, after verifying its intracellular growth phenotype, several of the *cre*-expressing plasmids were individually introduced and the resulting strains were examined for IPTG-induced deletion of the floxed gene in broth grown and phagocytosized bacteria. Strains in which expression of *cre* was tightly controlled and responsive to IPTG were retained for further studies. For example, pZL680 exhibited the best control in the *icmQ* knock-in strain and thus was used for all experiments involving in this strain.

Antibodies, Immunoprecipitation, TCA Precipitation, and Immunoblotting. For immunoblotting, both the IcmQ- and the SdhA-specific antibodies (6, 8) were used at 1:1000. To detect IcmQ in infected cells, we added 4 μl of an IcmQ-specific antibody (6) to lysates prepared from 5×10^7 macrophages infected with the *icmQ* knock-in strain for the indicated time. Precipitates were prepared by incubating 40 μl of protein G conjugated agarose with the lysates for 12 h at 4°C. After washing 3 times with the lysis buffer, samples resolved by SDS/PAGE were subjected to detection by immunoblotting. Translocated LidA was detected by fractionation of 5×10^7 infected macrophages with 1% digitonin following a described procedure (9).

To detect SdhA in infected cells, we lysed 1×10^8 infected cells with 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.7 μg/ml pepstatin). The soluble fraction of the lysates obtained by centrifugation at 16,000 *g* at 4°C was incubated with 10% trichloroacetic acid at 4°C for 2 h. The pellet were then collected by centrifugation, washed twice with cold acetone, resuspended in 2× Laemmli buffer and separated by SDS/PAGE. Unless otherwise noted, proteins were transferred to nitrocellulose membranes after SDS/PAGE. After incubating with the appropriate primary antibodies, washed membranes were incubated with an appropriate IRDye infrared secondary antibody. The Odyssey infrared imaging system was used for signal detection and for quantitating the intensity of protein bands (Li-Cor Biosciences).

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Fig. S1. Deletion of the floxed *icmQ* gene in exponentially grown bacteria. Bacterial cultures of $OD_{600} = 1.6$ were split into two subcultures, and IPTG was added to one of them. At indicated time points, a fraction of each culture was withdrawn, washed with PBS, and plated onto nonselective media and media containing kanamycin, respectively. The ratios of kanamycin-resistant cells were obtained by dividing the number of resistant colonies by total cell number at each time point. Similar results were obtained from at least two independent experiments, and data shown are one representative experiment done in triplicate.

Fig. S2. Deletion of *dotA* (*A*), *dotG*, or *dotO* (*B*) 1 h after bacterial uptake did not affect first round intracellular replication of *L. pneumophila*. In each case, knock-in strains grown to the post exponential phase (OD_{600} = 3.4–3.8) were used to infect mouse bone marrow-derived macrophages at an MOI of 0.05. Thirty minutes after adding bacteria, infection was synchronized by washing the samples with warm PBS three times to remove extracellular bacteria. After a 30-min incubation, 1 mM IPTG was added to a subset of samples. Intracellular bacterial growth was monitored by plating cell lysates at indicated time points on bacteriological medium as described in *Materials and Methods*. Data shown are from one representative experiment performed in triplicate. Note that all strains replicated in the first 24 h are indistinguishable to that of wild-type or uninduced samples, but induced samples failed to grow after the first round of multiplication.

Fig. S3. Deletion of *icmQ* led to decrease in its protein level after bacterial replication. (*A* and *C*) Lysates prepared from cells infected for 24 h (*A*) or cells treated with Cm (*C*) were subjected to immunoprecipitation with an anti-IcmQ antibody and SDS/PAGE resolved samples were probed for IcmQ. (*B* and *D*) In each case, the strength of the signals was quantitated by measuring the intensity of protein bands with the Odyssey infrared imaging system and was shown to the right of the blots.

Fig. S4. Knock-in of *sdhA* fully complemented a deletion mutant. Bacterial strains grown to the pos exponential phase were used to infect mouse bone marrow derived macrophages at an MOI of 0.05. Intracellular replication of bacteria was monitored as described in *Materials and Methods*. Bacterial strains used are: Wild-type Lp02 (diamonds); A *sdhA* knock-in strain (triangles) and a *sdhA* deletion mutant (squares). Data shown are from one representative experiment performed in triplicate.

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Fig. S5. Deletion of *sdhA* in replicating bacteria caused cell death in macrophages. Bone marrow derived macrophages were infected with the *sdhA* knock-in strain. (*Upper*) IPTG was added 8 h after uptake and samples were processed for TUNEL staining after another 8 h of incubation. Bacteria were labeled with a *L. pneumophila*-specific antibody and a secondary conjugated to FITC. Representative images from induced samples, note the multiple bacteria in the vacuole. (*Lower*) Representative images from uninduced samples. Note the large *Legionella* vacuole containing many bacteria.

Table S1. Strains of *L. pneumophila* used in this study

Strains	Genotypes	Ref.
Lp02	Philadelphia-1 *rps*L *hsd*R *thy*A	10
Lp03	Lp02*dotA*$^{-}$	10
Lp02ΔdotA	*dotA* in-frame deletion in Lp02	This study
Lp02ΔicmQ	*icmQ* in-frame deletion in Lp02	6
Lp02ΔdotG	*dotG* in-frame deletion in Lp02	11
Lp02ΔdotO	*dotO* in-frame deletion in Lp02	11
Lp02ΔsdhA	*sdhA* in-frame deletin in Lp02	8
ZL84	The knock-in cassette containing the *npt*II gene in the intergenic site of lpg2528 and lpg2529 in Lp02	This study
ZL85	*icmQ* knock-in	This study
DotAin	*dotA* knock-in in Lp02ΔdotA	This study
ZL86	*dotG* knock-in in Lp02ΔdotG	This study
ZL87	*dotO* knock-in in Lp02ΔdotO	This study
ZL284	*sdhA* knock-in in Lp02ΔsdhA	This study
ZL91	pZL680 in DotAin	This study
ZL92	pZL680 in ZL86	This study
ZL94	pZL680 in ZL87	This study
ZL117	pZL680 in ZL85	This study
ZL249	pZL683 in ZL284	This study