An in vivo gene deletion system for determining temporal requirement of bacterial virulence factors

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Analysis of phenotypes associated with specific mutants has been instrumental in determining the roles of a bacterial gene in a biological process. However, this technique does not allow one to address whether a specific gene or gene set is necessary to maintain such a process once it has been established. In the study of microbial pathogenesis, it is important but difficult to determine the temporal requirement of essential pathogenic determinants in the entire infection cycle. Here we report a Cre/loxP-based genetic system that allowed inducible deletion of specific bacterial genes after the pathogen had been phagocytosed by host cells. Using this system, we have examined the temporal requirement of the Dot/Icm type IV protein transporter of Legionella pneumophila during infection. We found that deletion of single essential dot/icm genes did not prevent the internalized bacteria from completing one cycle of intracelluar replication. Further analyses indicate that the observed phenotypes were due to the high stability of the examined Dot/Icm protein. However, postinfection deletion within 8 h of the gene coding for the Dot/Icm substrate, SdhA, abolishes intracellular bacterial growth. This result indicates that the Dot/Icm transporter is important for intracellular bacterial growth after the initial biogenesis of the vacuole. Our study has provided a technical concept for analyzing the temporal requirement of specific bacterial proteins or protein complexes in infection or development.

Study of the function of a bacterial protein often is achieved by analyzing the distinct phenotypes of mutants lacking the corresponding gene. Due to technical barriers, it is difficult, if not impossible, to analyze whether any of these genes are required for maintaining established development or infection status. For instance, genes critical for the formation of biofilm had been identified in numerous bacteria, but whether or how long these genes are necessary for a biofilm structure to persist is unknown. Similarly, many pathogens use specialized protein secretion systems to deliver panels of virulence factors into host cells to construct a permissive niche necessary for multiplication. In most cases, however, it is not known whether these pathogenic elements are necessary for the entire infection cycle.

Legionella pneumophila, the etiological agent of the Legionnaires’ disease, is a Gram-negative bacterium ubiquitously found in freshwater environments. This bacterium uses the Dot/Icm type IV secretion system to transfer a large cohort of effectors into the host cell. The initial biogenesis of the vacuole takes place before Dot/Icm substrate genes are transcribed (1). The presence of functional Dot/Icm proteins is necessary for intracellular bacterial growth after the pathogen had been phagocytosed by host cells. Using this system, we have examined the temporal requirement of the Dot/Icm transporter during intracellular bacterial growth.

Results

The Development of a Genetic System for Inducible Gene Deletion After Bacterial Uptake. To develop a system that allows the deletion of a specific bacterial gene at any time during infection, we constructed two plasmids suitable for isopropyl β-D-thiogalactoside (IPTG)-inducible bacterial gene knockout by a multistep cloning process (Materials and Methods). The first was a π protein-dependent plasmid used for recombination-based introduction of a gene flanked by two loxP sites (referred to as flanked throughout the text) into a neutral site on the L. pneumophila chromosome. In this construct, a floxed multiple cloning site and a kanamycin resistance gene were flanked by two 1.0-kb DNA fragments from the site of L. pneumophila genes lpg2528 and lpg2529 (Fig. 1A). Insertion of the floxed cassette into the 154-bp intergenic region between the convergently transcribed genes lpg2528 and lpg2529 did not interfere with intracellular growth.

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of *L. pneumophila* (data not shown). To ensure wild-type level gene expression, the construct was designed so that the floxed gene was expressed from its cognate promoter (Fig. 1B).

The second plasmid supplied the Cre recombinase, which was expressed from a promoter tightly regulated by the Lac repressor LacI. In this plasmid, multiple copies of the lac operator were placed between the Ptc promoter and the cre gene (Fig. 1C). In the absence of IPTG, no Cre was synthesized. Thus, the floxed gene was expressed to complement the corresponding chromosomal deletion. Addition of IPTG, a compound that can efficiently penetrate some eukaryotic cell membranes, would inactivate the LacI repressor. This inactivation would lead to the production of Cre, which would subsequently induce recombination between the two *loxP* sites, resulting in the deletion of the floxed gene (Fig. 1B and C). The positionning of the *npII* gene between the two *loxP* sites allowed for easy testing of the efficiency of the deletion based on the sensitivity of the bacteria to kanamycin. Introduction of several *dot/icm* genes, including *icmQ*, *dotA*, *dotG*, and *dotO*, into the chromosomal site fully restored virulence of the corresponding mutant (see Results; data not shown). We first examined the time required for IPTG-induced gene deletion in broth-grown bacteria using the *icmQ* knock-in strain. In the absence of IPTG, all of the cells exhibited resistance to kanamycin, indicating the presence of *icmQ* in these bacteria (Fig. 1D). However, within 1 h of the addition of 1 mM IPTG, >80% of the bacteria became sensitive to the antibiotics, and the ratio of kanamycin-resistant bacteria dropped to <1% after 7 h of induction (Fig. 1E). The relatively low rates of deletion may be due to the less active metabolism in postexponentially grown bacteria, because induction of exponentially grown bacteria led to almost complete deletion within 3 h [supporting information (SI) Fig. S1]. These data indicated that IPTG-induced gene deletion could be achieved in broth-grown *L. pneumophila*.

To examine the system’s effectiveness in deleting the floxed genes in host cells, we determined the extent of IPTG-induced deletion of *icmQ* after bacterial uptake. Thirty minutes after infection, IPTG was added to infected macrophages and the deletion of *icmQ* was determined. We found that 2 h of IPTG induction was sufficient to delete the floxed gene in >99% of the bacteria (Fig. 1E). In contrast, nearly 100% of the bacteria from uninduced samples remained kanamycin-resistant (Fig. 1E). Similar deletion efficiency was observed in strains harboring other floxed genes (data not shown). Taken together, our efforts have produced a genetic system that allows inducible deletion of bacterial genes after pathogen phagocytosis.

**Deletion of Individual *dot/icm* Genes Within 1 h After Bacterial Uptake Did Not Abolish *L. pneumophila* Intracellular Growth.** To examine the usefulness of our Cre/loxP-based in vivo gene deletion system, we attempted to determine the temporal requirement of the Dot/Icm type IV secretion system by *L. pneumophila* during infection. We first tested the *icmQ* knock-in strain. Thirty minutes after bacterial uptake, we induced a gene deletion by incubating infected cells with 1 mM IPTG for 5 h. After removing the inducer, we determined total bacterial counts at several time points. Twenty-four hours after infection, there was a 10-fold increase in bacterial growth in infections using the wild-type *L. pneumophila* strain Lp02 (Fig. 2A). A similar rate of multiplication was obtained in uninduced infections by using the *icmQ* knock-in strain (Fig. 2A). In the same time, however, IPTG-treated samples also exhibited robust growth, increasing by ~9-fold (Fig. 2A), but no further growth was observed in the IPTG-induced samples beyond the first 24 h of incubation (Fig. 2A). We also examined the growth of strains harboring floxed *dotA*, *dotG*, or *dotO*. Deletion of none of these genes after bacterial internalization led to a significant growth defect in the first 24 h of infection (Fig. S2). These data were reminiscent of earlier observations that repression of *dotA* expression in internalized *L. pneumophila* did not affect its first growth cycle (3).

To determine whether the observed phenotypes were due to the stability of IcmQ, we examined its protein levels in *L. pneumophila* after IPTG induction. In broth-grown bacteria, when the inducer was added to cultures at early exponential phase (OD < 1.0), induction for 15 h resulted in bacterial cells that did not contain detectable IcmQ (Fig. 2B, last lane). As expected, these bacteria were unable to replicate intracellularly (data not shown). In contrast, addition of IPTG to bacteria grown to late exponential phase (OD_{600} = 3.0) did not lead to a detectable decrease in IcmQ (Fig. 2B). In phagocytosed bacteria, 24 h after infection, the level of IcmQ in IPTG-induced samples decreased but still was readily detectable (Fig. 2C). These data suggested that protein stability is responsible for the observed growth after gene deletion.

**The IcmQ Protein Is Sufficient to Maintain a Functional Transporter for 20 h After Gene Deletion.** To address whether protein stability indeed accounted for the phenotypes, we further examined the temporal requirement of IcmQ during *L. pneumophila* infection. We chose to focus on this gene because its expression is driven by a promoter that is the weakest among the examined *dot/icm*
genes and its protein level in bacterial cells is low (6, 13). Moreover, a high-quality, anti-IcmQ antibody is available to monitor its levels under different conditions (13). Earlier studies showed that bacteriostatic antibiotics such as chloramphenicol (Cm) and erythromycin did not significantly alter the route of intracellular trafficking of internalized \textit{L. pneumophila} (14, 15). We hypothesized that after gene deletion, halting the bacterial growth with inhibitory concentrations of such antibiotics would provide more time for the protein to decay, perhaps leading to more severe growth defects. Thus, after IPTG induction for 5 h, we treated the samples with 5 \mu g/ml Cm for 10 h. After removing the antibiotic, the bacteria were then allowed to resume growth for an additional 19 h. The drug treatment did not detectably affect intracellular growth of the wild type or the uninduced \textit{icmQ} knock-in strain (Fig. 3A). Unexpectedly, bacteria in IPTG-treated samples also exhibited robust growth but consistently displayed 2- to 3-fold defect (Fig. 3A). Thus, inhibition of bacterial growth for 10 h after deleting the \textit{icmQ} gene significantly affected but did not abolish intracellular replication of \textit{L. pneumophila}. Furthermore, infected cells in Cm-treated samples appeared healthy, probably because the antibiotic inhibits the expression of both pro-death and pro-survival effectors.

To examine whether the Dot/Icm transporter was still active after the gene deletion, we first examined the association of the endoplasmic reticulum protein calnexin with bacterial vacuoles (7). At all time points examined, similar rates of calnexin-positive vacuoles were detected in wild-type strain infections and in uninduced \textit{icmQ} knock-in strain infections (Fig. 3B). IPTG induction and Cm treatment caused lower rates of calnexin-positive vacuoles, but such defect was restored after removing the antibiotic (Fig. 3B). These data further suggested that postphagocytic deletion of the \textit{icmQ} gene followed by Cm treatment for 10 h did not abolish the activity of the Dot/Icm system.

To confirm the activity of the Dot/Icm system, we examined its ability to translocate the effector LidA (6) after these treatments. Without Cm treatment, the LidA-positive vacuoles were ~65\% and the rate was maintained at the same level at all time points examined (data not shown). However, Cm treatment for 10 h markedly decreased the association of LidA with the bacterial vacuoles (Fig. 3C). Furthermore, 10 h of recovery incubation fully restored the rates of LidA-positive vacuoles in samples infected with the wild-type bacterium or with the uninduced \textit{icmQ} knock-in strain (Fig. 3C). In IPTG-induced samples, however, LidA-positive vacuoles also increased, but only marginally (Fig. 3C).

Because detection of translocated effectors by immunostaining often required higher levels of proteins, we examined the levels of injected LidA by digitonin fractionation of infected cells (16). Before Cm treatment, translocated LidA was readily detectable in infected cells; the drug treatment led to a decrease in its levels, but the protein was still detectable (Fig. 3D). Furthermore, after the removal of Cm and recovery, translocated LidA increased in both induced samples and uninduced samples, with the latter at a higher level (Fig. 3D). These data further indicated that the Dot/Icm system is still functional for substrate transfer after gene deletion followed by 10 h of growth inhibition.

Finally, we examined the IcmQ protein under these conditions. Immediately after Cm treatment, the amount of IcmQ was indistinguishable between induced and uninduced samples (Fig. 3E). However, after the 10-h recovery incubation, IcmQ in induced samples decreased to ~20\% of that of the uninduced samples (Fig. 3E and Fig. S3). Taken together, these results indicated that the IcmQ protein is extremely stable and that its cellular levels may be lowered only by dilution associated with cell division. The latter property is likely responsible for the robust growth of \textit{L. pneumophila} after gene deletion.

**Deletion of the \textit{sdhA} Gene in Internalized Bacteria Within 8 h After Infection Severely Affected Intracellular Growth of \textit{L. pneumophila}.**

Clearly the Dot/Icm transporter was extremely stable, and disruption of one of its component genes after phagocytosis did not abolish the multiplication of internalized bacteria. Next we considered the possibility that some effectors of the Dot/Icm may have a lower stability, especially in host cell cytosol. Furthermore, continuous translocation of the effector after gene deletion could quickly deplete the protein from the bacterial cytoplasm, which could likely lead to more severe growth defects. Thus, we examined the temporal requirement for \textit{sdhA}, a Dot/Icm substrate essential for intracellular growth in mouse macrophages (9). In the absence of IPTG, the \textit{sdhA} knock-in strain underwent an almost 10-fold growth in 24 h, a rate indistinguishable from that of the wild-type strain; such growth continued throughout the entire 3-day experiment (Fig. 4A and Fig. 4B). Importantly, in samples that received IPTG 30 min after infection, no bacterial growth was detected (Fig. 4A). In analyses in which samples were induced at different times after uptake, adding IPTG at 4 h completely abolished bacterial replication (Fig. 4A and B). Similarly, initiation of the induction at 8 h led to significant disruption of bacterial multiplication. Consider-
able inhibition of growth also occurred when IPTG was added 12 h after uptake (Fig. 4A and B). However, when the induction time was extended to 16 h, no disruption of growth was observed (Fig. 4).

To correlate the growth phenotypes with the presence of the SdhA protein, we attempted to examine SdhA in infected cells after IPTG-induced gene deletion. We were unable to detect SdhA in cells infected with any of the testing strains in the first 8 h of incubation (data not shown). However, this protein became detectable when infections had proceeded for >12 h, but only in uninduced samples (Fig. 4C). The inability to detect SdhA in the early phases of infection likely is due to the low level expression of this protein (9).

**Deletion of sdhA in Internalized Bacteria Led to Cell Death.** SdhA functions to protect infected macrophages from apoptosis (9),
but whether deletion of this gene in actively replicating bacteria also leads to cell death is unknown. We therefore examined the apoptotic status of infected macrophages after IPTG-induced deletion of sdhA. One hour after uptake, we added IPTG to a subset of infected cells, and samples withdrawn at different time points were analyzed for apoptosis. During the entire experimental duration, <3% of the uninfected cells appeared apoptotic (data not shown). In infections using the wild-type strain or in uninduced infections using the sdhA knock-in strain, the fraction of apoptotic cells increased slightly over the 16-h incubation but never exceeded 10% (Fig. 5A). Similar to an earlier study (9), in infections using the sdhA mutant, up to 50% of the infected cells appeared apoptotic at 8 h after infection (Fig. 5A). However, further incubation to 16 h resulted in a drop of the cell death rates to ~40% (Fig. 5A). Importantly, deletion of sdhA in intracellular bacteria led to cell death, and the rates peaked at 8 h after induction. A lower (27%) cell death rate was detected when the induction was extended to 16 h (Fig. 5A). The decrease in apoptosis rates in prolonged infections most likely was due to the loss of infected cells (ref. 9 and Y.L., and Z.-Q.L., unpublished data).

We also determined whether deletion of sdhA in actively replicating bacteria led to cell death by initiating gene deletion at several points after bacterial uptake. Approximately 38% of the infected cells were apoptotic when IPTG was added at 1 h after uptake followed by an additional 8 h of incubation (Fig. 5B). The percentage of apoptotic infected cells decreased when the infection was allowed to proceed for longer time before the initiation of gene deletion (Fig. 5B). Cell death occurred in cells containing either small (one to two bacteria) or medium (approximately five bacteria) vacuoles (Fig. S5). Thus, deletion of sdhA in replicating L. pneumophila also led to cell death, although to a lesser extent. Taken together, these data indicated that in mouse macrophages L. pneumophila needs the SdhA protein, and thus the Dot/Icm transporter, to maintain a replicative vacuole even when the bacteria are actively replicating.

Discussion

The experimental versatility of the Cre/loxP system has been exploited for both temporal and spatial control of gene deletions in various murine systems, leading to the elucidation of the functions of many proteins in diseases and development (17). Similarly, the combination of site-specific recombinase and the in vivo expression technology (known as IVET) (18) has led to the identification of genes important for different phases of infection (2). In some cases, the requirement of a certain gene at a specific infection stage has been elucidated by using the in vivo inducible gene expression method (19). Such analyses are of particular importance in the clarification of possible cross-talk between virulence determinants that appear to control different stages of infection. However, few studies have used site-specific recombination systems for inducible gene deletion in prokaryotic cells to analyze the temporal requirement of specific genes or gene sets. In this report, we have developed such a system to demonstrate that during bacterial intracellular growth, the Dot/Icm type IV transporter is required by L. pneumophila for a longer time than previously thought.

Consistent with its constitutive expression pattern (8), we showed that the SdhA protein was required for at least 8 h for L. pneumophila to complete a productive infection. Considering the fact that it took at least 2 h of IPTG induction for gene deletion to occur in most of the bacterial cells, the relatively lower cell death rates, and thus substantial multiplication, very likely were a result of the activity of SdhA synthesized before gene deletion (Fig. 5A). Similarly, SdhA contributed by multiple bacterial cells could account for the low cell death rates observed in infections induced after bacterial replication had begun (Fig. 5B). Clearly, macrophages infected by the sdhA mutant or the knock-in strain (induced) did not immediately become apoptotic. The several hours of delay may be necessary for the putative cell-death-inducing factors to accumulate to a critical level or such toxic proteins may be induced only when the bacteria have entered active growth phase.

Several lines of evidence suggested that the stability of the IcmQ protein is accounted for by the observed short time needed for the Dot/Icm transporter after gene disruption. Gene deletion and Cm treatment clearly led to a lower IcmQ protein level, but this reduction did not translate into the abolishment of intracellular growth, because the transporter was still capable of transferring effectors into host cells (Fig. 3). It is most likely that the decrease in IcmQ protein levels is accounted for by dilution associated with cell division (Figs. 2 B and C and 3E). These results are consistent with an earlier study showing that a single Dot/Icm-competent L. pneumophila cell was able to initiate and maintain a vacuole to support full replication of a coresiding Dot/Icm-deficient bacterium (4). Similarly, lower levels of DotA did not cause significant loss of intracellular growth (7). These data suggested that effectors of the Dot/Icm system are very efficient in the establishment and maintenance of the replicative vacuoles. Although we did not exhaustively examine every single Dot/Icm protein, an earlier study on DotA (3) and our analysis of IcmQ suggest that this transporter is extremely stable, probably due to the lack of a mechanism for disassembling the apparatus.

Some type IV secretion systems have been shown to remain active after conditions for expression of their component genes are no longer favorable. In Agrobacterium tumefaciens, the transfer of the tumor-inducing plasmid continues for many hours after removing the inducing signals (20). After being assembled, a stable transporter would be beneficial to the microorganism when null mutations occurred in its component genes. A functional transporter would allow the transfer of the genetic elements to complete or the production of a large number of progenies to occur. Such outcomes could increase the probabil-
ity of repairing the mutated gene by recombination or other mechanisms. Whether other type IV secretion systems involved in pathogenicity exhibit similar stability remains to be determined.

In summary, we have developed a genetic system that allows inducible deletion of specific bacterial genes even when the bacteria are at a specialized development stage or are associated with their host. As long as the biological membranes of the model organism are permeable to IPTG and the organism does not efficiently metabolize this compound, this method should be useful for analyzing the temporal requirement of microbial factors in other systems.

Materials and Methods

Bacterial Strains and Plasmids. All L. pneumophila strains used in this study were derivative of the Philadelphia 1 strain Lp02 (21) and are listed in Table S1. In-frame deletion mutants of L. pneumophila were described previously (13, 22). Plasmids designed to introduce floxed L. pneumophila genes into the corresponding mutant were constructed by a multistep cloning procedure detailed in the SI Materials and Methods. Similarly, a series of plasmids for inducible expression of the Cre recombinase were constructed as described in the SI Materials and Methods. For each strain, the efficiency of IPTG-induced gene deletion in bacteria grown in broth or in macrophages was empirically determined by testing several of these plasmids in the knock-in strain. In each case, a cre-expressing plasmid that not only exerted tight control in the absence of IPTG (did not detectably affect the growth of bacteria on media containing kanamycin) but also allowed efficient IPTG-induced gene deletion was used.

Infection, Gene Deletion, and Intracellular Growth Analysis. L. pneumophila strains grown to appropriate phases were used for induction or for infection at indicated multiplicities of infection (MOIs). Intracellular growth of L. pneumophila was analyzed in previously published procedures (23). In all experiments, IPTG was used at a final concentration of 1 mM. For in vitro induction, samples withdrawn at the time points indicated in Fig. 1D were subjected to extensive washes (five times) to remove the inducer, and the diluted bacteria were plated onto appropriate charcoal yeast extract plates. To determine the time required for efficient gene deletion in phagocytosed bacteria, we washed infected cells with PBS eight times to remove extracellular bacteria and then added IPTG. When required, Cm and kanamycin were used at 5 μg/ml and 20 μg/ml, respectively.

Immunoprecipitation, Trichloroacetic Acid (TCA) Precipitation, and Immunoblotting. Details for protein sample preparation, antibody, immunoprecipitation, digitonin fractionation, TCA precipitation, and immunodetection are in the SI Materials and Methods.

Immunostaining, TUNEL Staining, and Fluorescence Microscopy. The translocation of LiDa was determined by immunostaining with a LiDa-specific antibody (7). The association of calnexin with Legionella-containing vacuoles was assayed as previously described (8). The cell death status of infected cells was assayed by TUNEL staining as described previously (9, 24). Samples were inspected and scored under a fluorescence microscope.

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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 PsiI site in plasmid pJB908 (1) by ligating PsiI-digested DNA that had been treated with mung bean nuclease to give pJB908PsiI. We then inserted a cassette containing two directly repeatedloxP sites from pZLoxP-Gm (2) into pJB908PsiI - as a BamHI/Sall fragment to give pBloxP. After removing the BamHI and Sall sites by enzyme digestion followed by mung bean nuclease treatment, we expanded the multiple cloning site of pBloxP by cloning a DNA fragment obtained by annealing from oligomers 5’-CGAGCTCGGT-ACCGGATCCCGGAGGCTAGCTAGCTAATTCCAGTCGA-3’ and 5’-CTCGAGATTAAAAATTTCTAGAGCTACGGGATCCCGGATCCCTGGAATTTGTTACCA-3’. We then inserted a BamHI/Sall fragment derived from the kanamycin resistance gene from pBBR1MCS-2 (3) into the multiple cloning site of pBloxP to give pZL500. Finally, we made pZL789 by cloning the floxing cassette from pZL500 into pBluescript and this plasmid is a source of a floxed cassette for future experiments.

Using the 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 lpg2529 were amplified with primer pairs Up5/Down5, which gave 1 kb DNA fragments treated with mung bean nuclease to give pZL508. We then inserted a 1 kb DNA fragment coding for the kanamycin resistance gene from pBBR1MCS-2 (3) as SmaI fragment into the SmaI site of pZL505 to give pZL506. Finally, we made pZL789 by cloning the floxing cassette from pZL506 into pBluescript and this plasmid is a source of a floxed cassette for future experiments.

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 5’-CTGGATATCGTCTATGATCAT-CTCGAGATTTAAATTCTAG-3’ and 5’-CTCGAGATTAAAAATTTCTAGAGCTACGGGATCCCGGATCCCTGGAATTTGTTACCA-3’ into NotI/Sall digested pZL500. We then inserted a 1 kb DNA fragment coding for the kanamycin resistance gene from pBBR1MCS-2 (3) as SmaI fragment into the SmaI site of pZL505 to give pZL506. Finally, we made pZL789 by cloning the floxing cassette from pZL506 into pBluescript and this plasmid is a source of a floxed cassette for future experiments.

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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 function of the deleted gene were selected.

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/Sall 5’-CTAGGTCGAGCCCTAAGT-CAATTTTTCATAA-3’ dotAup3/BamHI 5’-TTGCGAGTC- CGAGATACTCAAGGGCGCTTCC-3’ dotAdown5/BamHI 5’-CTCGAGTTGACGTGTGTTACAGG CGTGCGGATCCGGTGGGTATACAGG CTGGGCAG-3’ dotAdown3/SacI 5’-CAGAGAGCTCCAGGGTGTTACAAAC-GAATGACC-3’ were inserted into pSR47 (2) digested with Sall 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 5’-ATGTTGAGATTCTGAGGGATCCCA-CAATTCTACAGGGTACCA-3’ and 5’-GGTACCGTGGTGGATATTTTCGCCCTATC-3’ into KpnI/BamHI digested pZL500. We then inserted the tandem repeats of the lac operator to 4 copies by inserting this DNA element into KpnI/Sall digested pOP4. We then increased the tandem repeats of the lac operator to 4 copies by inserting this DNA element into KpnI/Sall 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’-CTGGATATCGTCTATGATCAT-CTCGAGATTTAAATTCTAG-3’ and 5’-CTCGAGATTAAAAATTTCTAGAGCTACGGGATCCCGGATCCCTGGAATTTGTTACCA-3’. After digesting with the appropriate restriction enzymes, the two DNA fragments were ligated into NotI/Sall 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 pZL789 as a Sall/BstXI fragment. We then inserted the promoter region of *icmQ* (5) as a 136 bp EcoRV/Sall DNA fragment by PCR using primers *P*cmQup (5’-CCTGATCTATCATGATTGAGAAGCTTGGTTTCA-3’) and *P*cmQdown (5’-CCTGATCTATCATGATTGAGAAGCTTGGTTTCA-3’) into NotI/Sall digested pZL790 to give pZL791. The cloned *icmQ* gene was then cloned into pZL793 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 function of the deleted gene were selected.
ured for protein expression. First, a BglII/XbaI DNA fragment amplified from pZL500 or pZL502 with primer 5'-CA-
GAGATCTGGCTTTACACTTTATGCTTC-3' and 5'-CTAGTCGAGGGCTTTACACTTTATGCTTC-3' was in-
ersted 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 indi-
vidually 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 Immunoblot-
ing. 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 × 107 macrophages infected with the icmQ
knock-in strain for the indicated time. Precipitates were pre-
pared 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 × 107 infected macrophages with 1% digitonin
following a described procedure (9).

To detect SdhA in infected cells, we lysed 1 × 108 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 Na3VO4, 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
samples resolved by SDS/PAGE were subjected to de-
tection by immunoblotting. Translocated LidA was detected
by fractionation of 5 × 107 infected macrophages with 1% digitonin
following a described procedure (9).

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8. Laguna RK, et al. (2006) A Legionella pneumophila-translocated substrate that is
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73:4370–4380.
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.
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 (OD₆₀₀ = 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.
Knock-in of \textit{sdhA} fully complemented a deletion mutant. Bacterial strains grown to the post 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 \textit{sdhA} knock-in strain (triangles) and a \textit{sdhA} deletion mutant (squares). Data shown are from one representative experiment performed in triplicate.
Fig. 55. 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

<table>
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<tr>
<th>Strains</th>
<th>Genotypes</th>
<th>Ref.</th>
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<tr>
<td>Lp02</td>
<td>Philadelphia-1 rpsL hsdR thyA</td>
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<tr>
<td>Lp03</td>
<td>Lp02dotA</td>
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<tr>
<td>Lp02ΔdotO</td>
<td>dotO in-frame deletion in Lp02</td>
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<tr>
<td>Lp02ΔsdhA</td>
<td>sdhA in-frame deletion in Lp02</td>
<td>8</td>
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<tr>
<td>ZL84</td>
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