JOURNAL OF BACTERIOLOGY, Dec. 2006, p. 8231–8243 Vol. 188, No. 23
0021-9193/06/$08.00+0 doi:10.1128/JB.00937-06

Identification of Non-*dot*/*icm* Suppressors of the *Legionella pneumophila* Δ*dotL* Lethality Phenotype▽

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Received 28 June 2006/Accepted 13 September 2006

***Legionella pneumophila*, a causative agent of bacterial pneumonia, survives inside phagocytic cells by avoiding rapid targeting to the lysosome. This bacterium utilizes a type IVB secretion system, encoded by the *dot*/*icm* genes, to replicate inside host cells. DotL, a critical component of the Dot/Icm secretion apparatus, functions as the type IV coupling protein. In contrast to most *dot*/*icm* genes, which are dispensable for growth on bacteriological media, *dotL* is required for the viability of wild-type *L. pneumophila*. Previously we reported that Δ*dotL* lethality could be suppressed by inactivation of the Dot/Icm complex via mutations in other *dot*/*icm* genes. Here we report the isolation of non-*dot*/*icm* suppressors of this phenotype. These Δ*dotL* suppressors include insertions that disrupt the function of the *L. pneumophila* homologs of *cpxR*, *djlA*, *lysS*, and two novel open reading frames, lpg0742 and lpg1594, that we have named *ldsA* and *ldsB* for *l*ethality of Δ*dotL* *s*uppressor. In addition to suppressing Δ*dotL* lethality, inactivation of these genes in a wild-type strain background causes a range of defects in *L. pneumophila* virulence traits, including intracellular growth, implicating these factors in the proper function of the Dot/Icm complex. Consistent with previous data showing a role for the *cpx* system in regulating expression of several *dot*/*icm* genes, the *cpxR* insertion mutant produced decreased levels of three Dot/Icm proteins, DotA, IcmV, and IcmW. The remaining four suppressors did not affect the steady-state levels of any Dot/Icm protein and are likely to represent the first identified factors necessary for assembly and/or activation of the Dot/Icm secretion complex.**

Legionella pneumophila is a gram-negative, facultative intracellular parasite of freshwater protozoa. Although 48 species of *Legionella* have been described, *L. pneumophila* is the most common human pathogen in the genus *Legionella* and is the primary causative agent of Legionnaires' disease (15). Humans can become infected with *L. pneumophila* when they are exposed to aerosols from contaminated water sources (15). Upon internalization by alveolar macrophages, *L. pneumophila* prevents the acidification and lysosomal fusion of its phagosomal compartment and co-opts secretory vesicles from the endoplasmic reticulum (18–20, 36). After these early steps, the phagosome matures into a unique intracellular compartment, the replicative phagosome, where the organism multiplies (17). After the conclusion of a replicative cycle, *L. pneumophila* lyses its host cell and infects surrounding phagocytic cells.

Numerous cell biological processes have been implicated in the intracellular survival and replication of *L. pneumophila*, including modulation of both apoptosis and autophagy (1, 2). Although the role of these processes in *L. pneumophila* virulence is not fully understood, it is clear that alteration of the host cell endocytic pathway is critical to bacterial multiplication, as most avirulent *L. pneumophila* mutants are unable to replicate inside host cells because they mistarget and fail to form a replicative phagosome (5, 38). Complementation experiments utilizing these mutants revealed that *L. pneumophila* employs a specialized secretion system encoded by 26 "*dot*" or "*icm*" genes to prevent nascent *L. pneumophila*-containing phagosomes from entering into the endocytic pathway (39, 49).

The *dot*/*icm* genes encode an adapted conjugation apparatus that has been classified as a type IVB secretion system (T4SS) (10, 45). The *L. pneumophila* Dot/Icm T4SS is responsible for injecting a dozen or more bacterial proteins into the cytoplasm of host cells (4, 13, 14, 26, 30, 32). Although the specific biological functions of most Dot/Icm substrates have yet to be identified, many of these secreted proteins are expressed only in the early stationary phase of growth (4, 26). This may explain why the pathogen is not infectious during exponential growth and must differentiate into a transmissive form prior to infecting a new host cell (7).

Although critical to intracellular replication, most of the *L. pneumophila dot*/*icm* genes are not required for in vitro growth on bacteriological media (6, 40). However, it was recently reported that three *dot* genes, *dotL*, *dotM*, and *dotN*, are essential for the viability of *L. pneumophila* strain Lp02 (6). The *dotL* gene is also essential for the viability of an unrelated *L. pneumophila* strain, AA100, suggesting that this phenotype may be a conserved trait (C. D. Vincent and J. P. Vogel, unpublished data). Interestingly, it was discovered that the Δ*dotL* lethality phenotype could be suppressed by mutations in the majority of the *dot*/*icm* genes (6). These observations led to the model that loss of *dotL* is lethal to *L. pneumophila* due to the accumulation of a toxic complex, perhaps an unregulated secretion pore, in the envelope of the bacterium (6). According to this model, suppression of Δ*dotL* lethality could be achieved either directly or indirectly. For example, mutation

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▽ Published ahead of print on 22 September 2006.

of a component of the T4SS would directly disrupt the toxic secretion channel and would thus restore viability. Alternatively, the mechanism of suppression could be indirect, for example, by inactivation of genes needed for the proper assembly and/or activity of the secretion channel.

To test this, we performed a screen for transposon mutants that were able to survive in the absence of *dotL* (6). As predicted, the screen yielded a number of insertions in known *dot*/*icm* genes, including four in *dotA*, two in *dotG*, one in *dotI*, five in *dotO*, three in *icmF*, and one in *icmX* (6) and three in a new *dot* gene, *dotV* (J. A. Sexton et al., unpublished data). We report here the identification of non-*dot*/*icm* suppressor mutations, including disruptions of the *L. pneumophila* homologs of *cpxR*, *lysS*, *djlA*, and two novel genes, *ldsA* and *ldsB*. Although inactivation of each of these genes suppresses Δ*dotL* lethality, their inactivation in the presence of wild-type DotL results in varying effects on *L. pneumophila* virulence. These findings indicate that multiple genes are likely to play key roles in regulating the assembly and/or activation of the Dot/Icm secretion complex.

MATERIALS AND METHODS

Bacterial strains and media. Strains used in this study are listed in Table 1. All *L. pneumophila* strains used are derived from Lp02 (*hsdR rpsL thyA*), a wild-type strain derived from the clinical isolate Philadelphia 1 (5). *L. pneumophila* strains were grown on charcoal yeast extract agar (CYE) buffered with *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) or in ACES-buffered yeast extract broth (AYE). Antibiotics (kanamycin, 30 μg/ml; chloramphenicol, 5 μg/ml; streptomycin, 50 μg/ml; and gentamicin, 7.5 μg/ml), sucrose (5%), and thymidine (100 μg/ml) were added as necessary. *Escherichia coli* strains were grown in Luria-Bertani (LB) liquid medium or on LB agar plates. Antibiotics (ampicillin, 100 μg/ml; chloramphenicol, 5 μg/ml, and kanamycin, 20 μg/ml) were added as necessary.

Assay to determine suppression of Δ*dotL* lethality. The assay used to screen for Δ*dotL* lethality suppression has been described previously (6). Plasmid pJB1005 was transferred to *L. pneumophila* by using the RP4 conjugation system encoded on plasmid pRK600 (22). *L. pneumophila* strains that had integrated the plasmid onto the chromosome were selected by plating on medium containing streptomycin and kanamycin. The resulting *dotL*/Δ*dotL*::Cm^r merodiploid strains were plated on medium containing sucrose to select for resolution of the merodiploid to either *dotL* or Δ*dotL*::Cm^r. Sucrose-resistant colonies were streaked on medium containing kanamycin to confirm that the integrated plasmid had been lost and on medium containing chloramphenicol to screen for *dotL* or Δ*dotL*::Cm^r.

Plasmid construction. All complementing clones were made in the RSF1010 cloning vector pJB908 (43). The *cpxR cpxA* clone (pJB3243) was generated by amplifying the *cpxR-cpxA* operon, using primers JVP1147 and JVP1148. The *yitW lysS* clone (pJB3197) was generated by amplifying the *yitW-lysS* operon, using primers JVP1159 and JVP1218. The *lysS* clone (pJB3198) was generated by amplifying *lysS*, using primers JVP1217 and JVP1218. The *ldsA* clone (pJB3411) was generated by amplifying *ldsA*, using primers JVP1229 and JVP1230. The *djlA* clone (pJB3230) was generated by amplifying *djlA*, using primers JVP1149 and JVP1150. To generate a *djlA*-complementing clone in a plasmid that could be transferred by the Dot/Icm complex, the *djlA* gene from pJB3230 was cloned into pJB1627, generating plasmid pJB3192. All constructs were sequenced to confirm that no errors were introduced during PCR amplification.

Growth of *L. pneumophila* strains in mouse bone marrow-derived macrophages. Mouse bone marrow-derived macrophages were isolated from the femurs of female A/J mice as described previously (47). Intracellular growth of *L. pneumophila* strains was assayed as described previously (4, 47).

Western analysis. Western blotting was performed using standard techniques (4). Whole-cell extracts were generated from *L. pneumophila* cells grown to early stationary phase in AYE. Western blotting was performed using antibodies specific to DotL (diluted 1:10,000), DotB (diluted 1:500), DotG (diluted 1:1,000), DotO (diluted 1:3,000), IcmR (diluted 1:3,000), IcmX (diluted 1:10,000), RalF (diluted 1:3,000), SdeC (diluted 1:3,000), or isocitrate dehydrogenase (ICDH) (diluted 1:10,000). Generation of DotL-, DotB-, and SdeC-specific antibodies has been described previously (4, 6, 43). Antibodies to DotG, IcmX, IcmR, and RalF were generated against purified amino-terminal His_6 fusions injected into rabbits (Cocalico, Inc.). Antibody specific to ICDH was kindly provided by L. Sonenshein. Antibody specific to DotO was generously provided by R. Isberg. All antibodies were detected using goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) diluted 1:10,000 and an ECL detection kit (Amersham Biosciences).

Conjugation assay. Conjugation assays were performed as described previously (42). *L. pneumophila* strains were grown to early stationary phase, and 1×10^9 *L. pneumophila* donor cells were mixed with 1×10^{10} *E. coli* (strain ER1821) recipient cells. Cell mixtures were incubated for 2 h at 37°C on 0.45 μm-pore-size filters (Millipore) placed on CYE plus thymidine agar plates. Cells were resuspended in sterile water and plated on LB plus ampicillin (100 μg/ml) to determine the number of *E. coli* transconjugants. The conjugation frequency was determined by dividing the number of *E. coli* plasmid recipients by the number of *L. pneumophila* donors in each reaction. Shown are the averages and standard deviations for triplicate conjugation reactions.

Cytotoxicity assay. Contact-dependent cytotoxicity assays were performed as described previously (23, 46). *L. pneumophila* strains were added at the indicated multiplicities of infection (MOI) to mouse bone marrow macrophages plated on glass coverslips in 24-well tissue culture plates at a density of 1.5×10^5 bone marrow macrophages per coverslip. Bacteria were pelleted onto the macrophages at $168 \times g$ for 5 min at room temperature and then incubated for 1 hour at 37°C in 5% CO_2. The coverslips were then inverted onto a drop of phosphate-buffered saline (PBS) containing 25 μg/ml ethidium bromide and 5 μg/ml acridine orange (23) and were immediately observed. To calculate cytotoxicity, the number of ethidium bromide-positive macrophages was divided by the total number of macrophages observed in each field of view. Shown are the averages and standard deviations for four randomly selected fields of view scored for each strain at each MOI.

Intracellular targeting assay. Targeting assays were performed essentially as described previously (48). *L. pneumophila* cells grown to stationary phase were added to the macrophages at an MOI of approximately 5, and infections were allowed to proceed for 1 h at 37°C in 5% CO_2. Infected macrophages were fixed by the addition of paraformaldehyde-lysine-periodate containing 5% sucrose, followed by incubation at room temperature for 20 min. Extracellular bacteria were stained with polyclonal serum to *L. pneumophila* (3, 49) diluted 1:10,000 in PBS containing 2% goat serum (PBSG), followed by goat anti-rabbit secondary antibody conjugated to Cascade Blue (Molecular Probes) diluted 1:10,000 in PBSG. Macrophages were then permeabilized with methanol, and intracellular bacteria were decorated with polyclonal serum to *L. pneumophila* diluted 1:10,000 in PBSG and goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (Molecular Probes) diluted 1:10,000 in PBSG. LAMP-1 was labeled with the antibody ID4B, which was developed by J. Thomas August and was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City. It was diluted 1:2.5 in PBSG and goat anti-rat secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes) diluted 1:100 in PBSG. Only intracellular bacteria were scored for colocalization with the endocytic marker LAMP-1. The averages and standard deviations of numbers obtained from three sets of 100 intracellular bacteria are reported for each strain.

Immunofluorescence assay of SdeC secretion. Immunofluorescence staining of secreted SdeC was performed as described previously (4). Cells were stained with affinity-purified polyclonal serum raised to SdeC diluted 1:10, followed by incubation with goat anti-rabbit antibody conjugated to Oregon green (Molecular Probes) diluted 1:100. Bacterial and macrophage DNAs were stained with 4′,6-diamidino-2-phenylindole (DAPI). For each strain, three counts of 100 bacteria were averaged. Results were normalized to wild-type secretion of SdeC set at 100%.

RESULTS

Δ*dotL* lethality can be suppressed by *dot*/*icm* and non-*dot*/*icm* mutations. Although the *dot*/*icm* genes are required for the intracellular growth of *L. pneumophila*, most of these genes are dispensable for growth on bacteriological media (39, 40, 49). In contrast, *dotL* is essential for the viability of the *L. pneumophila* strain Lp02 under all growth conditions (6). We previously took advantage of Δ*dotL* lethality to perform a screen for mutations that allow growth in the absence of *dotL* (6). The assay was based on resolution of a *dotL*/Δ*dotL*::Cm^r

TABLE 1. Strains, plasmids and primers used

Strain, plasmid, or primer	Relevant genotype or sequence	Reference or source
E. coli strains		
DH5αλpir	DH5α(λpir) *tet*::Mu	24
ER1821	F$^-$ *glnV44* e14$^-$ (McrA$^-$) *endA1 thi-1* Δ*(mcrC-mrr)114*::IS*10*	New England Biolabs (Beverly, MA)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene
L. pneumophila strains		
Philadelphia 1	Wild-type strain	5, 27
Lp02	Philadelphia-1 *rpsL hsdR thyA*	5
Lp03	Lp02 *dotA*	5
JV1003	Lp02 *dotL*$^+$/Δ*dotL*::Cm^r	6
JV1005	Lp02 *dotA dotL*$^+$/Δ*dotL*::Cm^r	6
JV1139	Lp02 + pJB908	4
JV1141	Lp03 + pJB908	4
JV1372	Lp02 *djlA dotL*$^+$/Δ*dotL*::Cm^r	
JV1484	Lp02 *ldsB*::Kan^r	This study
JV1485	Lp02 *yitW*::Kan^r	This study
JV1807	Lp02 *cpxR*::Kan^r	This study
JV1811	Lp02 *djlA*::Kan^r	This study
JV1817	Lp02 *djlA*::Kan^r	This study
JV1835	Lp02 *ldsA*::Kan^r	This study
JV1837	Lp02 *ldsA*::Kan^r	This study
JV4044	Lp02 lacking all 26 *dot*/*icm* genes	This study
JV4070	JV1484 + pJB908	This study
JV4073	JV1485 + pJB908	This study
JV4075	JV1807 + pJB908	This study
JV4077	JV1811 + pJB908	This study
JV4078	JV1811 + pJB3230	This study
JV4157	Lp02 + pKB5	This study
JV4158	Lp03 + pKB5	This study
JV4163	JV1484 + pKB5	This study
JV4164	JV1485 + pKB5	This study
JV4165	JV1807 + pKB5	This study
JV4166	JV1811 + pKB5	This study
JV4172	JV1835 + pKB5	This study
JV4196	JV1807 + pJB3243	This study
JV4313	JV1811 + pJB3192	This study
JV4321	JV1485 + pJB3197	This study
JV4322	JV1485 + pJB3198	This study
JV4358	JV1835 + pJB908	This study
JV4359	JV1835 + pJB3411	This study
Plasmids		
pJB908	pKB5 Δ*oriT*	43
pJB1005	Δ*dotL*::Cm^r in pSR47S	6
pJB1627	pKB5 + Cm^r	This study
pJB3192	pJB1627 + *djlA*$^+$	This study
pJB3197	pJB908 + *yitW*$^+$ *lysS*$^+$	This study
pJB3198	pJB908 + *lysS*$^+$	This study
pJB3230	pJB908 + *djlA*$^+$	This study
pJB3243	pJB908 + *cpxR*$^+$ *cpxA*$^+$	This study
pJB3411	pJB908 + *ldsA*$^+$	This study
pJK211-2	Mini-Tn10 transposon	6
pKB5	RSF1010 cloning vector	5
pRK600	RP4 helper plasmid	22
pSR47S	*ori*R6K *oriT*RP4 *kan sacB*	29
Primers		
JVP1147	CCCGGATCCAGGATAAGTAATTCATGAGCAGC	
JVP1148	CCCGTCGACTAGTCAATAAGGAGGAACTCG	
JVP1149	CCCGGATCCAGGTAATTTCTGATAATGAACTTACG	
JVP1150	CCCGTCGACAACAAATTGGACTACCAACCC	
JVP1159	CCCGGATCCGGCAATAGTGGCAGAGCTCG	
JVP1217	CCCGGATCCGGGAATATTTTATTGATCATGTCTGAT AACTCCTGG	
JVP1218	CCCGTCGACCAGGATTTAGTGATTATGTCTGC	
JVP1229	CCCGGATCCGCGAGTGATTGAGCATTTGG	
JVP1230	CCCGTCGACGAACCCTCATGTCATTTTAGAAGC	

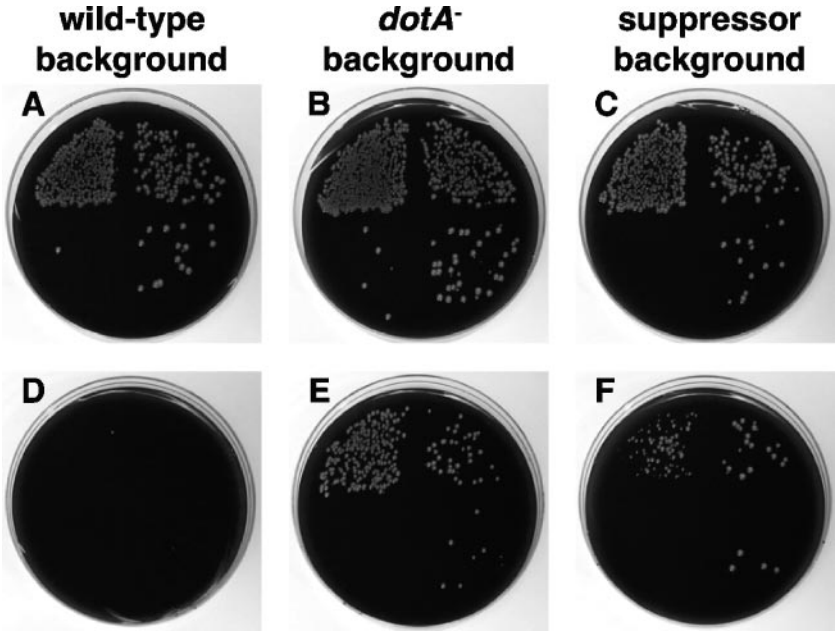

FIG. 1. *dotL* is an essential gene, and mutations that suppress Δ*dotL* lethality can be isolated. The essential nature of *dotL* can be demonstrated by the failure of a *dotL*$^+$/Δ*dotL*::Cm^r merodiploid JV1003 to resolve to the Δ*dotL*::Cm^r locus when grown on CYE-sucrose plates containing chloramphenicol (D). Resolution of the *dotL*$^+$/Δ*dotL*::Cm^r merodiploid to either the wild-type *dotL*$^+$ or the Δ*dotL*::Cm^r locus can be detected by colony formation on CYE-sucrose plates lacking chloramphenicol (A, B, and C). Growth on CYE-sucrose plates containing chloramphenicol (E and F) is indicative of the presence of a mutation capable of suppressing the lethality caused by loss of *dotL*. Shown are the resolution abilities of three *dotL*$^+$/Δ*dotL*::Cm^r merodiploid strains (the wild-type strain Lp02 JV1003, the *dotA* mutant JV1005, and a strain containing a novel suppressor mutation, JV1372). In each case, resolution was assayed by plating 10-fold serial dilutions in a clockwise manner starting in the upper left quadrant on CYE-sucrose plates (A, B, and C) or CYE-sucrose plates containing chloramphenicol (D, E, and F).

merodiploid strain, as observed in Fig. 1. Total resolution events can be detected by plating on CYE-sucrose plates, as the merodiploid cassette contains a counterselectable marker that confers sensitivity to sucrose. Specific resolution to the Δ*dotL*::Cm^r locus can be identified by growth of the strain on CYE plates containing sucrose and chloramphenicol. Therefore, by comparing growth of the merodiploid on the two types of media, it can be determined if a gene is essential or dispensable. The essentiality of *dotL* can be observed by the lowered plating efficiency of the wild-type *dotL*/Δ*dotL*::Cm^r merodiploid on CYE-sucrose plates containing chloramphenicol (Fig. 1D). Of the total resolution events (Fig. 1A), fewer than 1 in 1,000 resolved to the Δ*dotL*::Cm^r locus.

Although *dotL* is an essential gene, plating the *dotL*/Δ*dotL*::Cm^r merodiploid strain on sucrose-chloramphenicol plates occasionally did result in growth of a few colonies. These colonies were confirmed to contain *dotL* deletions and were deduced to be alive because they contained an additional mutation(s) capable of suppressing Δ*dotL* lethality (6). Based on the frequency at which these suppressors were observed, it seemed likely that there must be a large number of genes whose inactivation could alleviate the loss of *dotL*. This prediction proved true, as inactivation of almost any *dot*/*icm* gene, including *dotA*, is sufficient to suppress loss of *dotL* (Fig. 1B and E).

To identify additional suppressors, we previously reported a screen for mini-Tn10 insertions that allowed *L. pneumophila* to survive without the *dotL* gene (6). We identified 33 independent strains containing insertions that suppressed the loss of *dotL*. Nineteen of these strains possessed insertions in known *dot*/*icm* genes (6) or in *dotV* (Sexton et al., unpublished data). The remaining 14 suppressors contained insertions in open reading frames (ORFs) that did not resemble known *dot*/*icm* genes. These insertion mutations were able to suppress Δ*dotL* lethality at a frequency similar to that of a *dotA* mutation (Fig. 1C and 1F). In summary, *dotL* is an essential gene, but loss of *dotL* can be suppressed by insertions in *dot*/*icm* genes and non-*dot*/*icm* genes.

Identification of non-*dot*/*icm* suppressors of Δ*dotL* lethality. We report the sites of transposon insertion for 7 of the 15 non-*dot*/*icm* suppressors (Fig. 2 and Table 2). These seven insertions are in five genes: the *L. pneumophila* homologs of *cpxR*, *djlA*, and *yitW*, as well as two previously uncharacterized *L. pneumophila* open reading frames, lpg0742 and lpg1594 (9). Based on their ability to suppress loss of *dotL*, we have named these genes *ldsA* and *ldsB* for *l*ethality of Δ*dotL s*uppressor. We obtained a single insertion in *ldsB*, *cpxR*, and *yitW* and two independent insertions in *ldsA* and *djlA* (Fig. 2). However, it is worth noting that the original screen described by Buscher et al. was not performed to saturation (6).

Of the known genes, *cpxR* encodes the regulatory portion of a two-component system that responds to the stress of misfolded proteins in the periplasm (34, 37). The *djlA* gene encodes a DnaJ homolog and is involved in sensing and repairing misfolded proteins in the bacterial inner membrane (11). A DjlA homolog has been shown to be required for the efficient intracellular growth of *Legionella dumoffii*, although its specific role was not determined (33). Finally, the *yitW* gene (lpg0606) encodes a protein with similarity to metal-sulfur cluster bio-

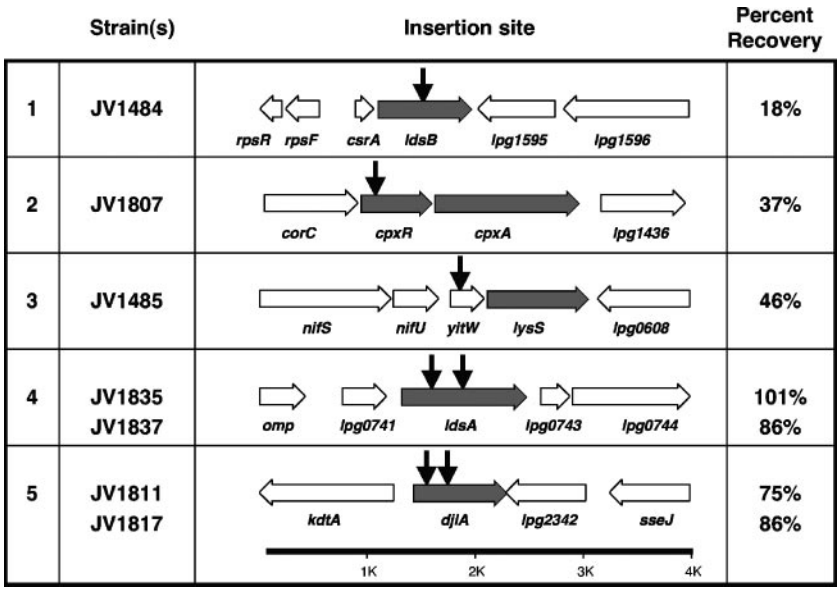

FIG. 2. Seven transposon insertions in non-*dot*/*icm* genes that suppress the lethality caused by loss of *dotL* were isolated. The insertions were found in five genes, i.e., *ldsB*, *cpxR*, *yitW*, *ldsA*, and *djlA*. A map of the region surrounding the site of the insertion(s) is shown, with the gene responsible for suppressing Δ*dotL* lethality indicated with gray arrows, the genes surrounding the site of insertion indicated with white arrows, and the sites of the transposon insertions shown with vertical black arrows. For each strain, the ability of the *dotL*$^+$/Δ*dotL*::Cm^r merodiploid to resolve to the Δ*dotL*::Cm^r locus is indicated in the far right column, with the frequency of Δ*dotL* mutant recovery normalized to the Δ*dotA* strain JV1005 (100%).

synthetic enzymes in the DUF59 Pfam family. However, we were able to determine that the suppression phenotype is not due to disruption of *yitW* but is instead solely due to a polar effect of the insertion on the expression of the downstream gene, *lysS* (see below). *lysS* encodes lysyl tRNA synthetase, which catalyzes the formation of Lys-tRNA^{Lys} (31).

TABLE 2. Description of the genes surrounding the Δ*dotL* suppressors

lpg no.[a]	Gene name	Protein product/homology
lpg1591	*rpsR*	30S ribosomal protein S18
lpg1592	*rpsF*	30S ribosomal protein S6
lpg1593	*csrA*	Carbon storage regulator
lpg1594	***ldsB***	**Lethality Δ*dotL* suppressor B**
lpg1595	ND[b]	Hypothetical protein, ErfK homology
lpg1596	*yfcX*	Enoyl coenzyme A hydratase
lpg1439	*corC*	Mg^{2+} and Co^{2+} transporter
lpg1438	***cpxA***	**Cpx two-component sensor**
lpg1437	*cpxR*	Cpx two-component regulator
lpg1436	ND	Hypothetical protein[c]
lpg0604	*nifS*	Aminotransferase
lpg0605	*nifU*	Nitrogen fixation protein
lpg0606	***yitW***	**Metal-sulfur cluster biosynthetic enzyme**
lpg0607	*lysS*	Lysyl tRNA synthetase
lpg0608	ND	*S*-Adenosylmethionine-dependent methyltransferase
lpg0740	*omp*	*Rickettsia* 17-kDa surface-exposed antigen (lipoprotein)
lpg0741	ND	Cystathionine-beta-synthase domain
lpg0742	***ldsA***	**Lethality Δ*dotL* suppressor A**
lpg0743	ND	Glutamate synthetase
lpg0744	ND	Sensory transduction (GGDEF domain)
lpg2340	*kdtA*	3-Deoxy-D-manno-octulosonic acid transferase
lpg2341	***djlA***	**DnaJ-like protein**
lpg2342	ND	Hypothetical protein
lpg2343	*sseJ* like	Lysophospholipase A

[a] Sites of insertion are indicated in boldface.
[b] ND, no gene name designated.
[c] Found only in *Legionella* species.

Of the novel suppressors, *ldsA* codes for a 417-amino-acid protein that is predicted to be localized to the inner membrane via eight transmembrane domains (9). Although homologs to LdsA are conserved in other *L. pneumophila* strains, including Paris and Lens (Lpp0807 and Lpl0778, respectively) (8), there do not appear to be highly homologous *ldsA* genes in any other bacterial species, including the closely related pathogen *Coxiella burnetii* (41). Four ORFs with very limited homology to *ldsA* can be detected in GenBank, including SO0975, GSU1615, MCA2787, and AdehDRAFT_0196 from *Shewanella*, *Geobacter*, *Methylococcus*, and *Anaeromyxobacter* species, respectively. Each of these ORFs is predicted to encode an inner membrane protein with a size similar to that of LdsA, although none has been assigned a function.

The *ldsB* gene is also predicted to encode a small inner membrane protein with eight transmembrane domains. Similar to the case for *ldsA*, the *L. pneumophila* strains Lens and Paris contain highly conserved homologs to *ldsB* (lpl1431 and lpp1552, respectively). Most gram-negative bacteria, including *C. burnetii*, do not possess an LdsB homolog. The exception may be *Burkholderia pseudomallei*, which is predicted to encode a protein with limited homology to LdsB (BLAST score of ~1e−08 to LdsB). This *B. pseudomallei* hypothetical protein has been annotated as encoding a membrane-associated phospholipid phosphatase, although we could not detect this similarity and LdsB does not appear to resemble a phosphatase. In summary, LdsA and LdsB can be viewed as novel proteins that likely do not have true homologs outside of the *L. pneumophila* family.

Confirmation of suppressor phenotypes. To confirm that the Δ*dotL* lethality suppressor phenotype selected for in our screen was specifically linked to the transposon insertions we

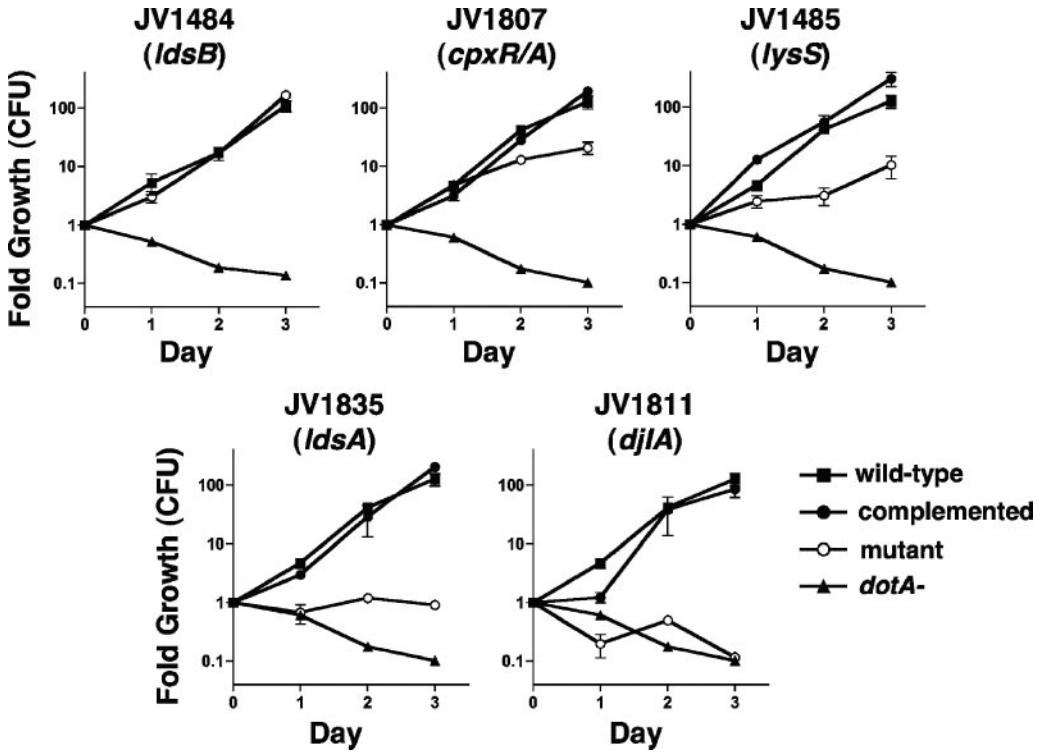

FIG. 3. Intracellular growth of the suppressor mutants in bone marrow-derived macrophages from A/J mice. Each graph contains the same data for a control strain that can grow in macrophages (JV1139, squares) and a *dot* mutant that is unable to grow inside macrophages (JV1141, triangles). Also shown are growth data for each suppressor mutant containing the vector pJB908 (open circles) or the corresponding complementing clone (filled circles). JV1484 is a *ldsB* mutant, JV1807 is a *cpxR cpxA* mutant, JV1485 is a *yitW lysS* mutant, JV1835 is an *ldsA* mutant, and JV1811 is a *djlA* mutant. Complementation of the JV1484 mutant was not performed, since the strain grew similarly to a wild-type strain. Fold growth was calculated as the number of CFU recovered each day divided by the number of CFU recovered immediately after infection (day 0). Each time point represents the mean and standard deviation of CFU recovered from triplicate wells.

identified, the transposon and surrounding DNA were transformed into an unresolved *dotL*/Δ*dotL*::Cm^r merodiploid by using natural competence (44). The resulting strains were then assayed for their ability to suppress loss of *dotL* by resolving the strains on selective media as described above. In each case, resolution of merodiploids to the Δ*dotL*::Cm^r locus was compared to that of a *dotA* mutant merodiploid. As shown in Fig. 2, each of the seven insertions was able to suppress Δ*dotL* lethality, with recovery rates ranging from 18% to 101% (normalized to a *dotA* mutant set at 100%). Although the percent recovery of Δ*dotL* mutants varied, we observed that the insertions with lower recovery rates of Δ*dotL* mutants also exhibited a corresponding diminished effect on Dot/Icm-related traits (see below). Significantly, no resolution events to the Δ*dotL*::Cm^r locus were recovered from the wild-type merodiploid (0/300 colonies scored). Thus, each newly created insertion mutant appears able to suppress loss of *dotL*, demonstrating linkage of this phenotype to the identified insertion.

The Δ*dotL* suppressor mutants have varied effects on intracellular replication. To determine the mechanism of Δ*dotL* lethality suppression, we assayed the effects of the insertion mutations on the virulence properties of *L. pneumophila*. Since the suppressor mutations were isolated in a strain that lacked *dotL*, they were first transformed into a wild-type strain of *L. pneumophila* by natural competence (44). These strains were then grown in vitro to confirm that each resembled the wild-type strain in growth rate, colony morphology, and motility (data not shown). The mutants were then assayed for their ability to replicate within macrophages derived from the bone marrow of A/J mice (47). In these cells, the wild-type strain Lp02 was able to multiply approximately 125-fold over the course of the assay, whereas a *dotA* mutant was unable to replicate (Fig. 3).

The seven insertion mutants exhibited a range of intracellular growth phenotypes, varying from wild-type growth to complete attenuation of virulence (Fig. 3). For example, the *ldsB* mutant (JV1484) grew robustly and was indistinguishable from the wild-type strain Lp02 (Fig. 3). In contrast, the *cpxR* mutant (JV1807) and the *yitW* mutant (JV1485) displayed partial defects in intracellular growth, with each having grown approximately 10- to 20-fold less than the wild-type strain at day 3. A strain containing an insertion in *ldsA*, JV1835, exhibited a strong growth defect, resulting in no observable increase in CFU over the course of the assay. Notably, this mutant did not display a decrease in intracellular CFU, whereas the CFU of the *dotA* mutant decreased approximately 10-fold by day 3. The other *ldsA* mutant, JV1837, showed a similar intracellular growth phenotype (data not shown). Finally, the *djlA* mutant (JV1811) was the most attenuated in that it was unable to replicate, lost viability, and had a phenotype resembling that of a *dotA* mutant (Fig. 3). The other *djlA* mutant, JV1817, exhibited the same level of attenuation (data not shown). Thus, the

non-*dot*/*icm* Δ*dotL* lethality suppressors vary significantly in their intracellular replication defects, and we observed a positive correlation between the severity of their attenuation and the level of recovery of Δ*dotL* mutants.

The growth defects of the *ldsA* and *djlA* insertions could be fully restored by introduction of the corresponding gene on a plasmid (pJB3411 for *ldsA*$^+$ or pJB3230 for *djlA*$^+$) (Fig. 3). Since *cpxR* is in an operon with *cpxA*, and the genes each encode an element of a two-component regulator whose components function together, we assayed only complementation of the *cpxR* insertion by introduction of a plasmid encoding the *cpxR-cpxA* operon. The *cpxR-cpxA* operon plasmid pJB3243 was able to fully restore growth to the *cpxR* insertion strain JV1807 (Fig. 3).

Likewise, *yitW* is found in an operon with a second gene, *lysS*. Therefore, the growth defect of the *yitW* insertion could be due to inactivation of *yitW*, of the downstream gene *lysS*, or of both genes. Similar to the case for the *cpxR* insertion, addition of a plasmid (pJB3197) containing both genes to the *yitW* insertion strain (JV1485) was able to fully restore growth (data not shown), suggesting that both genes might be involved. However, JV1485 could also be fully complemented by introduction of just the *lysS* gene (pJB3198) (Fig. 3), leading to the conclusion that the phenotype of the *yitW* transposon insertion must be due to polar effects on expression of *lysS*. Complementation of the *ldsB* mutant (JV1484) was not performed, as this strain exhibited no intracellular growth defect. As a result, we can conclude that *ldsB* is dispensable for intracellular replication, the *cpxR-cpxA* operon and *lysS* are partially required, and *ldsA* and *djlA* are necessary. The requirement for *djlA* closely resembled that of most *dot*/*icm* genes.

The *cpxR* mutation, but not the other suppressor mutations, affects expression of the Dot/Icm apparatus proteins. Based on the observation that inactivation of various *dot*/*icm* genes is able to suppress the lethality associated with loss of *dotL* (6), we hypothesized that these non-*dot*/*icm* suppressors might affect the expression and/or the stability of components of the Dot/Icm type IV secretion machinery. For example, it is well established for other bacterial species that *cpxR*/*cpxA* mutations can affect the expression of many proteins (34, 37). To determine if any of the Δ*dotL* suppressor mutations affected Dot/Icm protein levels, we performed Western blot analysis on whole-cell lysates by using antibodies to 20 Dot/Icm proteins and 2 secreted substrates. Six representative Dot/Icm proteins and the two secreted substrates, RalF and SdeC, are shown in Fig. 4A. An antibody that recognized the constitutively expressed housekeeping protein ICDH was included as a control. None of the suppressor mutations significantly affected expression of the majority of the Dot/Icm apparatus proteins or of the secreted substrates in either the wild-type or Δ*dotL* strain background in either the exponential or stationary phase of growth (Fig. 4A and data not shown).

However, reduced amounts of two Dot/Icm proteins, IcmV and IcmW, were observed in stationary-phase cultures of one suppressor, the *cpxR* mutant (Fig. 4B). This effect was more pronounced in exponentially growing cultures of the *cpxR* mutant (Fig. 4C). Diminished levels of DotA, encoded downstream of *icmV*, were also observed in the *cpxR* mutant (data not shown). Notably, IcmV levels appeared to be more affected than DotA or IcmW levels. Based on our previous observation

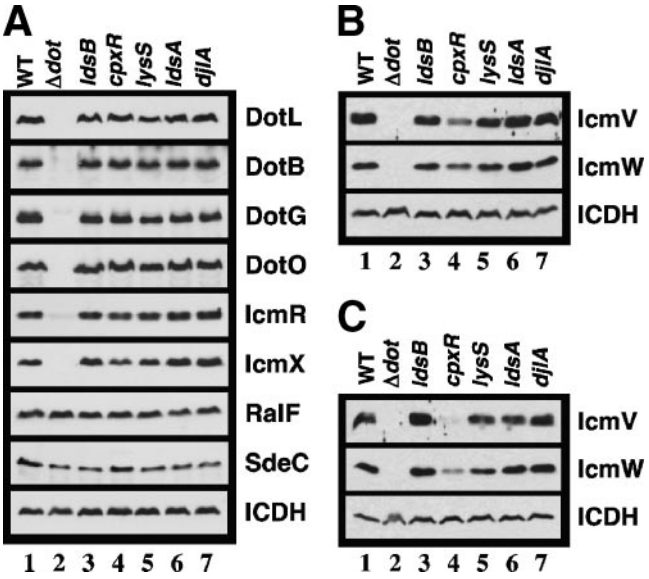

FIG. 4. Only the *cpxR* suppressor mutation affects the steady-state levels of the Dot/Icm secretion apparatus proteins. The following strains were assayed: Lp02 (wild-type [WT] *L. pneumophila*) in lane 1, JV4044 (a strain lacking all 26 *dot*/*icm* genes) in lane 2, JV1484 (an *ldsB* mutant) in lane 3, JV1807 (a *cpxR cpxA* mutant) in lane 4, JV1485 (a *yitW lysS* mutant) in lane 5, JV1835 (an *ldsA* mutant) in lane 6, and JV1811 (a *djlA* mutant) in lane 7. The seven strains were grown in liquid AYE, and lysates were analyzed by Western blotting using antibodies specific to the proteins listed to the right of each blot. (A) Representative proteins not affected by the suppressor mutations, using lysates generated from stationary-phase cultures (optical density at 600 nm of approximately 3.1). Shown are Western blots using antibodies recognizing six representative components of the Dot/Icm complex (DotL, DotB, DotG, DotO, IcmR, and IcmX), two secreted substrates (RalF and SdeC), and the constitutively expressed housekeeping protein ICDH. (B) Dot/Icm proteins affected by the *cpxR*::Kan^r mutation, assaying lysates generated from early-stationary-phase cultures (optical density at 600 nm of approximately 3.1). (C) Dot/Icm proteins affected by the *cpxR*::Kan^r mutation, assaying lysates generated from exponential-phase cultures (optical density at 600 nm of approximately 1.5). In both panels B and C, antibodies recognizing IcmV, IcmW, and ICDH were used.

that disruption of *icmV* or *dotA*, but not *icmW*, suppresses Δ*dotL* lethality (6), we hypothesized that decreased expression of IcmV and DotA was the mechanism of Δ*dotL* lethality suppression of the *cpxR* mutant. To test this, we transformed a plasmid containing the *icmV dotA* operon under a constitutive promoter or the corresponding vector into the *cpxR*::Kan^r Δ*dotL* mutant. Although transformants were obtained with the vector, no transformants were recovered when the *icmV dotA* plasmid was transformed into this strain (data not shown). This result was dependent on inactivation of both *dotL* and *cpxR*, as transformants could be obtained in the *cpxR*::Kan^r mutant background. Thus, the *cpxR*::Kan^r mutant appears to suppress Δ*dotL* lethality through decreased levels of IcmV and DotA. However, the virulence defects of the remaining suppressor mutants do not appear to be due to altered expression or stability of the Dot/Icm T4SS but may instead be due to the improper assembly or activation of the secretion apparatus.

***The* Δ*dotL* suppressor mutants have varied effects on plasmid mobilization.** Having eliminated the most likely explanation for the Δ*dotL* suppressor phenotype, we employed a num-

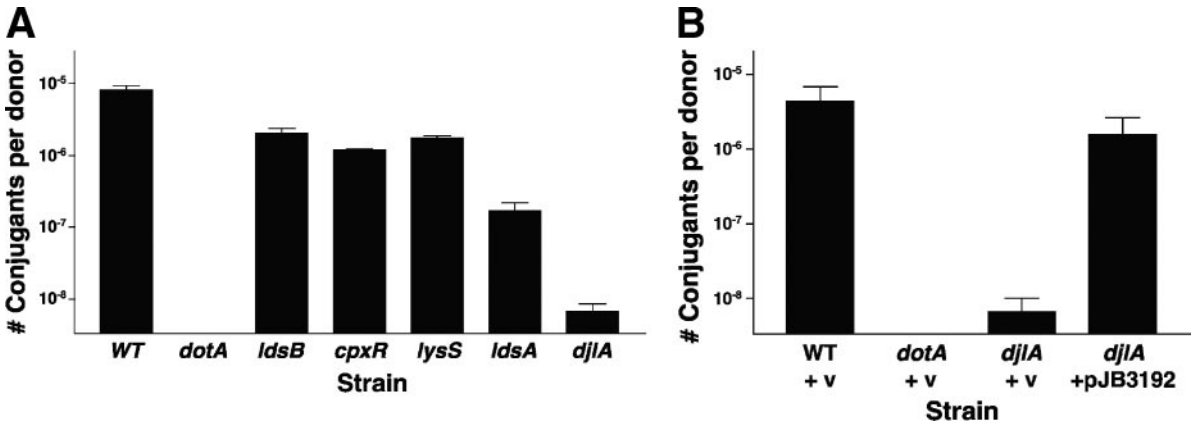

FIG. 5. Ability of Δ*dotL* suppressors to mobilize a plasmid. Strains were assayed for the ability to mobilize an RSF1010 plasmid from one bacterium to another via the Dot/Icm secretion system. Strains containing the RSF1010 plasmid pKB5 were mated with *E. coli* strain ER1821, and transconjugants were selected on LB plates containing ampicillin. (A) Strains used were JV4157 (Lp02 plus pKB5), JV4158 (Lp03 plus pKB5), JV4163 (*ldsB* plus pKB5), JV4165 (*cpxR cpxA* plus pKB5), JV4164 (*yitW lysS* plus pKB5), JV4172 (*ldsA* plus pKB5), and JV4166 (*djlA* plus pKB5). (B) The conjugation defect of the *djlA* mutant can be complemented by the presence of the wild-type *djlA* gene. Strains JV4157 (wild type [WT] plus pKB5), JV4158 (*dotA* plus pKB5), JV4166 (*djlA* plus pKB5), and JV4313 (*djlA* plus *djlA*-complementing clone) were assayed for the ability to mobilize RSF1010 as described above. Data shown are the average number of transconjugants per *L. pneumophila* donor ± the standard deviation obtained from three reactions. v, vector.

ber of Dot/Icm-dependent assays to test the Δ*dotL* suppressor mutants for Dot/Icm T4SS functionality. The first assay we used is based on the ability of the *L. pneumophila* Dot/Icm T4SS to transfer a plasmid from one bacterium to another (39, 49). A wild-type *L. pneumophila* strain, JV4157, mobilized pKB5 with an efficiency of 8.1×10^{-6} transconjugants per donor, whereas a *dotA* mutant (JV4158) was completely deficient for plasmid transfer (limit of detection = 1×10^{-9}) (Fig. 5A).

The *ldsB* (JV4163), *cpxR cpxA* (JV4165), and *lysS* (JV4164) mutants were each able to conjugate pKB5 by the Dot/Icm system but with slightly lower efficiency than wild-type cells (2.0×10^{-6}, 1.2×10^{-6}, and 1.7×10^{-6} transconjugants per donor, respectively). Consistent with its more severe intracellular growth defect, the *ldsA* mutant (JV4172) was 48-fold less efficient than the wild type at plasmid transfer (1.7×10^{-7} transconjugants per donor). The *djlA* mutant (JV4166), which was severely attenuated for intracellular growth, was almost completely defective for plasmid transfer (6.7×10^{-9} transconjugants per donor). Although we had shown complementation of the intracellular growth defect of our *djlA* mutant (Fig. 3), we tested whether the conjugation defect of the *djlA* mutant could also be complemented. As expected, the plasmid mobilization defect of the *djlA* mutant could be restored to wild-type levels when a *djlA*-expressing plasmid, pJB3192, was present, confirming that the phenotype of the *djlA* mutant is specifically due to the transposon insertion (Fig. 5B). In summary, three of the suppressors exhibited only subtle defects in plasmid transfer, the *ldsA* mutant exhibited an intermediate defect, and the *djlA* mutant was severely defective.

The Δ*dotL* suppressor mutants have varied effects on contact-dependent cytotoxicity. As an additional measure of Dot/Icm T4SS functionality, we assayed the ability of the Δ*dotL* suppressor mutants to cause rapid cytotoxicity of host macrophages. This form of cytotoxicity, also termed "contact-dependent cytotoxicity," is observed at high MOI and results in host cell death consistent with the insertion of pores in the host cell membrane (23). Rapid contact-dependent cytotoxicity requires the majority of the Dot/Icm proteins, excluding the type IV adaptors IcmS and IcmW, and therefore accurately reflects the presence of an assembled and functional T4SS (12, 23).

To assay cytotoxicity, *L. pneumophila* strains were pelleted onto mouse bone marrow-derived macrophages at a range of MOI. After incubation for 1 hour, the infected macrophages were stained with acridine orange and ethidium bromide in order to determine their intactness. Live, intact cells stain green, whereas permeabilized cells stain red due to the uptake of ethidium bromide (23). Representative images of the cytotoxic effects of a wild-type strain, a *dotA* mutant, and the Δ*dotL* suppressor mutant strains are shown in Fig. 6A to H. Uninfected cells are green, as they are impermeable to ethidium bromide (Fig. 6A). Infection using a wild-type strain at an MOI of 50 bacteria/macrophage resulted in the majority of the macrophages being permeabilized (Fig. 6B). Infection using a *dotA* mutant at an identical MOI caused no cytotoxicity (Fig. 6C). Macrophages infected with the *ldsB* mutant (Fig. 6D), the *cpxR cpxA* mutant (Fig. 6E), and the *lysS* mutant (Fig. 6F) closely resembled those infected with wild-type cells (Fig. 6B). In contrast, the *ldsA* mutant (Fig. 6G) and the *djlA* mutant (Fig. 6H) were significantly less cytotoxic but did not appear to be as defective as the *dotA* mutant (compare Fig. 6G and H with Fig. 6C).

To examine this phenotype in more detail, the infections were repeated using a range of MOI and cytotoxicity was quantitated (Fig. 6I). As before, the *ldsB* mutant (JV1484), the *cpxR cpxA* mutant (JV1807), and the *lysS* mutant (JV1485) closely resembled the wild-type strain (Lp02) at each MOI. The *ldsA* mutant appeared equally cytotoxic as the wild-type strain when assayed using the highest MOI (500), where both permeabilized nearly 100% of the macrophages. However, it was clearly attenuated when examined using an intermediate MOI (Fig. 6I). Quantitation of the *djlA* mutant cytotoxicity

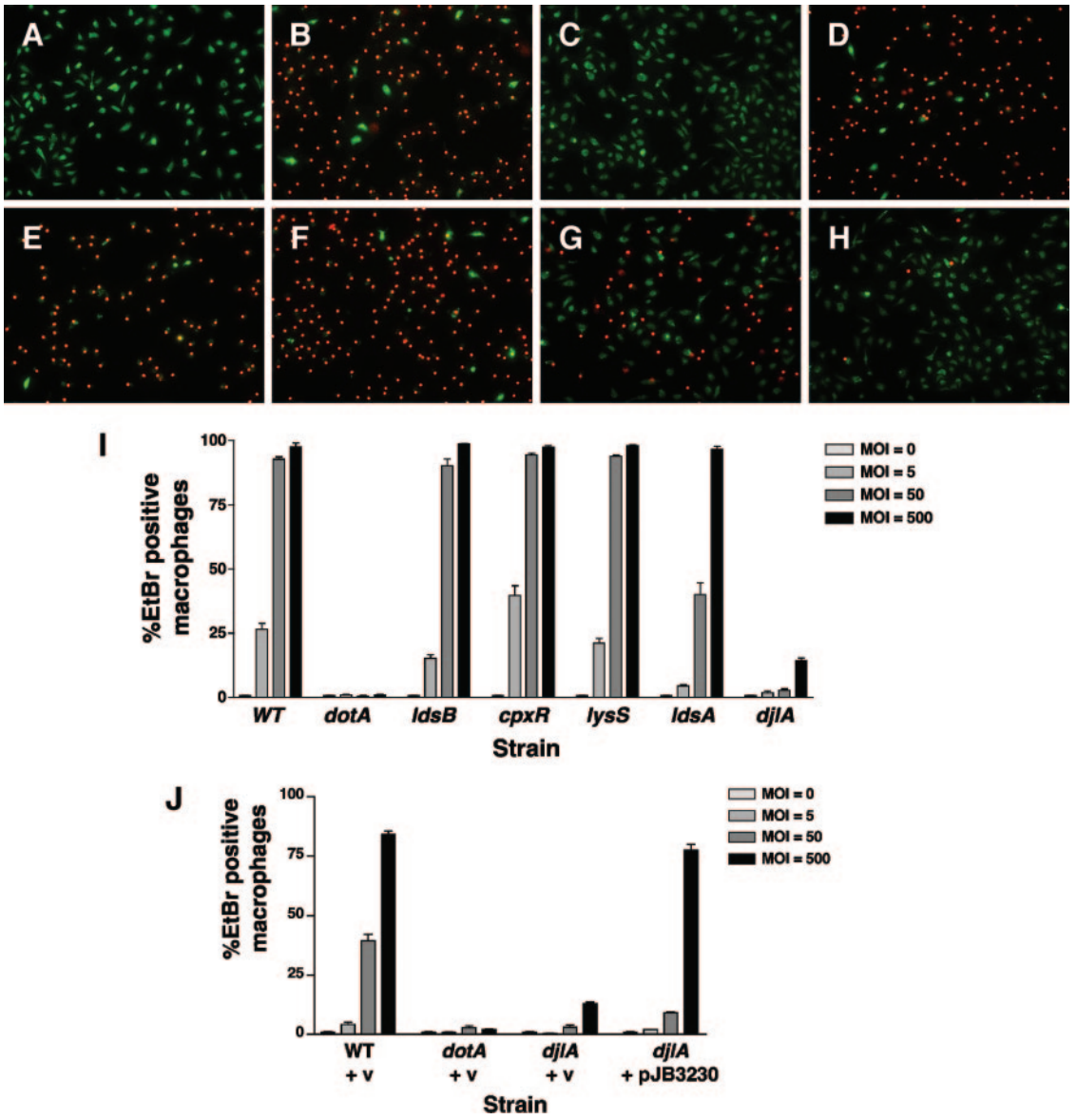

FIG. 6. Contact-dependent cytotoxicity of the Δ*dotL* suppressors. Cytotoxic effects of *L. pneumophila* strains on mouse bone marrow-derived macrophages were examined using vital stains. Bacteria were incubated with mouse bone marrow-derived macrophages for 1 h. Cytotoxicity was assayed by staining with ethidium bromide and acridine orange. Live cells appear green, whereas permeabilized cells appear green with red nuclei. Representative images for infections done using an MOI of 50 are shown for the following strains: (A) uninfected macrophages, (B) Lp02 (wild type), (C) Lp03 (*dotA*), (D) JV1484 (*ldsB*), (E) JV1807 (*cpxR cpxA*), (F) JV1485 (*yitW lysS*)" (G) JV1835 (*ldsA*), and (H) JV1811 (*djlA*). (I) Quantitation of cytotoxicity, using four different MOI (0, 5, 50, or 500). Results are indicated as the percentage of ethidium bromide (EtBr)-positive-staining macrophages divided by the total number of macrophages observed per field. The averages and standard deviations for four randomly selected fields are shown for each strain at each MOI indicated. (J) Complementation of the cytotoxicity defect of the *djlA* mutant. Strains JV1139 (wild type [WT] plus pJB908), JV1141 (*dotA* plus pJB908), JV4077 (*djlA* plus pJB908), and JV4078 (*djlA* plus *djlA*-complementing clone) were assayed for contact-dependent cytotoxicity as described for panel I. v, vector.

revealed that although it was severely attenuated at lower MOI, it retained some activity (14% cytotoxicity) when assayed using the highest MOI. This is strikingly in contrast to the *dotA* mutant, which was not cytotoxic at any MOI tested (Fig. 6I). As before, the defect in cytotoxicity of the *djlA* mutant could be complemented when *djlA* was supplied in *trans* on a plasmid (Fig. 6J). These results demonstrate that the Dot/Icm secretion complex functions normally in the *ldsB*, *lysS*, and *cpxR cpxA* mutants. However, the T4SS is not fully functional in the *ldsA* and *djlA* mutants, although both do retain some residual activity.

The *ldsA* and *djlA* mutants are defective in altering the endocytic pathway of host cells. Since the *ldsA* and *djlA* mutants were the only suppressors that exhibited severe defects in *dot*/*icm*-related phenotypes, we examined the intracellular growth defect of these mutants further. It is possible that these mutants are impaired for growth within macrophages simply because they replicate at lower rates inside host cells. Alter-

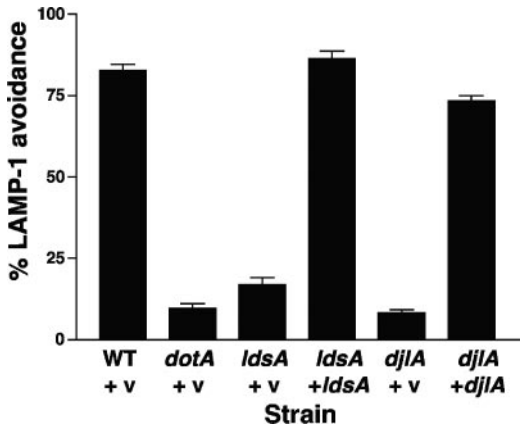

FIG. 7. The *ldsA* mutant and the *djlA* mutant are defective in altering the host cell endocytic pathway. Colocalization of the endocytic marker LAMP-1 with intracellular bacteria was assayed by immunofluorescence microscopy. Strains JV1139 (wild type [WT] plus pJB908), JV1141 (*dotA* plus pJB908), JV4358 (*ldsA* plus pJB908), JV4359 (*ldsA* plus *ldsA*-complementing clone), JV4077 (*djlA* plus pJB908), and JV4078 (*djlA* plus *djlA*-complementing clone) were used to infect mouse bone marrow-derived macrophages for 1 hour prior to fixation and staining for intracellular versus extracellular bacteria and for LAMP-1. Avoidance of LAMP-1 by intracellular *L. pneumophila* is shown as the average and standard deviation of three counts of 100 bacteria. v, vector.

natively, they could lack a function that is necessary for intracellular replication. For example, they could be unable to alter intracellular trafficking of the host cell, a process that absolutely requires a functional Dot/Icm type IV secretion system. To assess *L. pneumophila* avoidance of the endocytic pathway, we quantitated colocalization of *Legionella*-containing phagosomes with the endocytic marker LAMP-1 (48). As shown in Fig. 7, the majority of wild-type *L. pneumophila* bacteria did not colocalize with LAMP-1, whereas only 10% of *dotA* mutants were LAMP-1 negative. Both the *ldsA* mutant and the *djlA* mutant resembled the *dotA* mutant in that they were severely defective in altering the endocytic pathway. The *ldsA* mutant was slightly less attenuated than the *dotA* mutant for avoiding the endocytic marker LAMP-1 (17% LAMP-1 negative), while the *djlA* mutant was indistinguishable from the *dotA* mutant (8% LAMP-1 negative). As observed with intracellular growth, the targeting defect of each of the mutants was fully restored by introduction of complementing clones (86% and 73% LAMP-1 avoidance, respectively). Because the severity of the intracellular growth defect correlated with the defect in targeting for both mutants, it seems likely that the observed defects in intracellular growth are caused solely by the inability to alter the host endocytic pathway.

The *ldsA* and *djlA* mutants are defective in the secretion of the Dot/Icm substrate SdeC. Alteration of the host endocytic pathway by the Dot/Icm T4SS is mediated by the export of protein substrates into the cytoplasm of the host cell (4, 13, 14, 26, 30, 32). Export of a representative substrate, SdeC, can be visualized by immunofluorescence staining of infected macrophages with an antibody raised against SdeC (4). Similarly to other *L. pneumophila* T4SS substrates, SdeC is secreted into the host cytoplasm, adjacent to the poles of the bacterium, where it remains associated with the phagosome (4).

To assay SdeC export by the *ldsA* and *djlA* mutants, the strains were added to mouse bone marrow-derived macrophages, and infections were allowed to proceed for 30 min prior to fixation and staining. DAPI (blue) was used to stain macrophage and bacterial DNAs, whereas the SdeC antibody was decorated with a goat anti-rabbit antibody conjugated to Oregon green. Representative images of each strain are shown

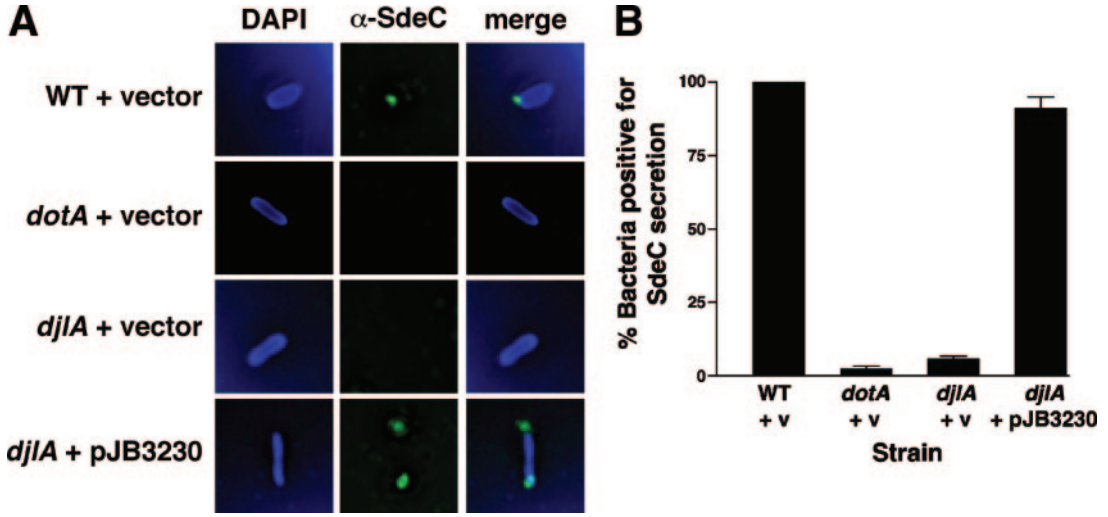

FIG. 8. The *djlA* mutant is defective in export of substrates of the Dot/Icm secretion system. Dot/Icm-mediated secretion of SdeC was assayed by immunofluorescence microscopy. Mouse bone marrow-derived macrophages were infected with various *L. pneumophila* strains for 30 minutes prior to fixation and staining with DAPI (to detect DNA) and a polyclonal serum that detects SdeC. (A) Representative immunofluorescence images of macrophages infected with strains JV1139 (wild type [WT] plus pJB908), JV1141 (*dotA* plus pJB908), JV4077 (*djlA* plus pJB908), and JV4078 (*djlA* plus *djlA*-complementing clone). DAPI staining for DNA is shown in the left panels, SdeC staining is shown in the center panels, and merged images are shown in the right panels. (B) The results in panel A were quantitated by scoring intracellular *L. pneumophila* cells for the presence of SdeC on the surface of the phagosome. The results were normalized to the amount of SdeC secretion observed with wild-type *L. pneumophila* set at 100%. Shown are the averages and standard deviations of three counts of 100 intracellular bacteria. v, vector.

in Fig. 8A, with quantitation provided in Fig. 8B. As described previously (4), wild-type cells (JV1139) frequently exhibit foci of SdeC staining at one or both poles of the phagosome, whereas *dotA* mutants (JV1141) do not. Consistent with the defect in altering the endocytic pathway as observed by LAMP-1 colocalization, the *djlA* mutant (JV4077) was almost completely defective for SdeC secretion (Fig. 8A and B). The *ldsA* mutant was also defective for SdeC export, although not to the same extent (data not shown). Secretion of SdeC was fully restored in each mutant by the introduction of the corresponding complementing clone (Fig. 8 and data not shown). Thus, the intracellular growth defect of the Δ*dotL* suppressor mutants *ldsA* and *djlA* appears to be due to a failure to properly export T4SS substrates and therefore a failure to alter the endocytic pathway of the host cell.

DISCUSSION

We report here the characterization of five genes that were isolated in a screen for mini-Tn*10* insertions able to suppress the lethality caused by loss of *dotL*. The insertions were in *cpxR*, *djlA*, *yitW*, and two novel genes, which we have named *ldsA* and *ldsB* (*l*ethality of Δ*dotL* *s*uppressor). Each of these suppressor mutations conferred the ability of *L. pneumophila* to survive in the absence of *dotL*. When assayed in a *dotL*$^+$ background, these mutants exhibited differential effects on Dot/Icm-dependent assays. These results support the hypothesis that there are multiple means to suppress Δ*dotL* lethality, including by decreasing expression of *dot*/*icm* genes and by diminishing the assembly and/or activity of the *L. pneumophila* Dot/Icm secretion system.

The CpxRA (*c*onjugative *p*lasmid e*x*pression) stress response system is a two-component regulatory system induced in response to extracytoplasmic signals generated in the cell envelope, principally misfolded proteins in the periplasm (34, 37). Once these signals are detected, the system is responsible for activating the expression of a number of factors required for responding to cell envelope stress (35). In addition to a role in F plasmid conjugation (28), the Cpx system has been implicated in several virulence mechanisms, including invasion (*Salmonella enterica*), transcriptional regulation (*Shigella sonnei*), P-pilus biogenesis (uropathogenic *E. coli*), and assembly of type IV bundle-forming pili (enteropathogenic *E. coli*) (reviewed in reference 34).

Based on our model of Δ*dotL* toxicity and the important role of the Cpx system in responding to periplasmic stress in other organisms, it is not surprising that this system is involved in the response to loss of *dotL*. We hypothesized that the *cpx* mutation could affect expression of *dot*/*icm* genes, thereby suppressing Δ*dotL* lethality due to lowered levels of Dot/Icm proteins. This hypothesis was supported by a previous study, using β-galactosidase reporters, that showed decreased transcription of *icmR*, *icmV*, and *icmW* in a *cpxR* mutant (16). Although we did not observe that IcmR protein levels were significantly affected by a *cpxR* insertion (Fig. 4A), we did observe an effect on DotA, IcmV, and IcmW. Interestingly, the *cpxR*::Kan^r mutant had only a minor effect on the levels of IcmX (Fig. 4A), a protein encoded downstream of *icmW*. By assaying the effects of overexpression of IcmV and DotA, we were able to demonstrate that decreased levels of IcmV and DotA were solely responsible for suppression of Δ*dotL* lethality in the *cpxR*::Kan^r mutant. This is consistent with our previous observation that disruption of *icmV* or *dotA*, but not *icmW*, suppresses Δ*dotL* lethality (6). Surprisingly, although decreased IcmV and DotA levels were sufficient to suppress loss of *dotL* in the *cpxR*::Kan^r mutant, it was still able to conjugate and remained cytotoxic. Thus, the Δ*dotL* lethality phenotype is more sensitive to decreased levels of Dot/Icm proteins than other Dot/Icm-associated traits.

The remaining non-*dot*/*icm* suppressors described here likely represent mutations that indirectly affect the activity of the *L. pneumophila* T4SS or mutations that do not affect the Dot/Icm complex itself but allow the strain to survive in the absence of *dotL*. These mutations can be subdivided into three phenotypic classes: (i) mutations that affect virulence by disrupting the assembly and/or the activation of the Dot/Icm system (e.g., *ldsA* and *djlA*), (ii) mutations that negatively affect the ability of the strain to grow in macrophages but do not appear to grossly affect the assembly and function of the Dot/Icm complex (e.g., *lysS*), and (iii) mutations that do not affect any of the virulence-related assays (e.g., *ldsB*). Before examining the overall implications of these suppressors, we will discuss each class separately.

The first class of mutants reported here includes the *ldsA* and *djlA* mutants. Each of these mutants was much more attenuated in intracellular growth than the other suppressor mutants, and the *djlA* mutant was completely defective for growth in macrophages. Further analysis revealed that the reason for the attenuation of these mutants is that they likely affect the assembly and/or activity of the Dot/Icm apparatus. Although these mutants still make wild-type levels of Dot/Icm proteins and substrates, they are less efficient than the wild type at avoiding phagosome-lysosome fusion and exhibit decreased Dot/Icm-mediated plasmid transfer and contact-dependent cytotoxicity. Finally, both mutants are severely attenuated for the secretion of a T4SS substrate, SdeC, into the cytoplasm of macrophages. Thus, as predicted, our Δ*dotL* lethality suppressor screen did identify factors involved in the assembly or activity of the Dot/Icm apparatus.

The *ldsA* gene encodes a polytopic inner membrane protein that does not contain any distinguishing motifs, making prediction of its molecular function difficult. Based on the protein's putative membrane localization and the Dot/Icm-related defects associated with loss of LdsA, it is formally possible that LdsA is an additional Dot/Icm protein. However, we do not favor this possibility since LdsA lacks homology to a component of any known type IV secretion system and is encoded distantly from the two Dot/Icm pathogenicity islands (49).

In contrast to the *ldsA* mutant, it is easier to propose an explanation for the *djlA* mutant. DjlA (for *D*na*J*-*l*ike protein) is an inner membrane-anchored homolog of DnaJ with its J domain located in the cytoplasm (11). Similarly to DnaJ, *E. coli* DjlA is known to interact with the DnaK (Hsp70) chaperone and stimulate its ATPase activity (50). DjlA has been proposed to function as a chaperone for the assembly and/or activity of membrane proteins and may play a role as a sensor of envelope stress (21). The involvement of a stress response system in the suppression of Δ*dotL* lethality is consistent with our model that the absence of DotL leads to the accumulation of a toxic subcomplex in the envelope of cells. Based on the proposed

activities of *E. coli* DjlA, the highly conserved *L. pneumophila* homolog is likely to be involved in sensing misfolded proteins in the membrane and assisting in their proper folding. Therefore, *L. pneumophila* may require DjlA to properly fold and assemble components of the Dot/Icm complex. Loss of *djlA* would therefore phenocopy *dot*/*icm* mutations and suppress Δ*dotL* lethality by a similar mechanism. Additionally, *djlA* has also been implicated as being required for the intracellular growth of another *Legionella* species, *Legionella dumoffii* (33). Because our *djlA* mutant still appears to synthesize components of the Dot/Icm apparatus and yet is severely defective for all Dot/Icm-dependent traits, it likely serves as a key factor in the assembly/quality control of the *L. pneumophila* T4SS. To our knowledge, DjlA is the first such factor identified.

The second class of mutations reported here includes an insertion in *yitW* that affects the expression of the lysyl-tRNA synthetase gene *lysS*. This mutation caused subtle defects in intracellular growth in mouse bone marrow-derived macrophages. However, the mutant did not display a significant defect in plasmid transfer or contact-dependent cytotoxicity, traits requiring a functional Dot/Icm secretion apparatus. LysS is an aminoacyl-tRNA synthetase that catalyzes the formation of Lys-tRNA^{Lys}, which is then used to insert lysine into proteins (31). It was surprising to obtain an insertion that appeared to down-regulate the expression of *lysS*, since most tRNA synthetases are essential. However, it is possible that the *yitW*::Kan^{r} allele exhibited only an incomplete polar effect on *lysS*, thereby partially down-regulating its expression. Alternatively, *lysS* may not be essential because *Legionella* possesses a homolog of *lysU*, which encodes a secondary, inducible form of lysyl-tRNA synthetase in other bacterial species (31). Although the molecular mechanism of suppression by inactivation of *lysS* remains cryptic, the Lys-tRNA^{Lys} molecule has been implicated in additional roles, including functioning as a signaling molecule (31). It is thus possible that the mechanism of *lysS* suppression of Δ*dotL* lethality is indirect and may be unlinked to its role in protein synthesis.

The insertion in *ldsB* was the only mutant in the third class identified in this screen whose inactivation suppressed Δ*dotL* lethality but did not affect the virulence of a *dotL*$^{+}$ strain of *L. pneumophila*. LdsB is predicted to be a novel polytopic, inner membrane protein that does not possess any apparent protein motifs, thus precluding an obvious prediction of function. Although we do not understand the molecular mechanism of *ldsB* suppression of Δ*dotL* lethality, the ability of this mutant to replicate normally in macrophages suggests that the suppression may be mediated by an indirect mechanism of action (see below). Nevertheless, the identification of *ldsB* is important for two reasons. First, since a Δ*dotL* strain is not viable in the Lp02 background, it was previously only possible to examine this strain lacking *dotL* in the presence of another *dot*/*icm* mutation, e.g., Δ*dotL* Δ*dotA*, thus severely complicating the analysis of the Δ*dotL* phenotype. As a result of this discovery, it is now possible to examine the *dot*/*icm* phenotypes of Lp02 lacking *dotL* in the Δ*dotL* Δ*ldsB* strain. Second, the existence of a Δ*dotL* lethality suppressor that does not affect the intracellular replication of *L. pneumophila* provides credence to our proposal that the JR32 Δ*dotL* strain is viable because the JR32 strain background contains a suppressor mutation (6).

Based on our initial observation that *dot*/*icm* mutations could suppress loss of *dotL*, we proposed that an *L. pneumophila* Δ*dotL* strain is not viable because it accumulates a toxic substrate in the cell envelope. This poisonous structure could be a misfolded Dot/Icm subcomplex, similar to pilin subunits that accumulate in the absence of the P-pilus chaperone PilD (25). Alternatively, the toxic substrate might be a normally assembled Dot/Icm subcomplex that functions improperly in the absence of DotL, perhaps as an unregulated secretion pore that leads to a loss of homeostasis. The former model implies that accumulation of a subcomplex, likely to consist of a few Dot/Icm proteins, is responsible for the toxicity. If this were the case, inactivation of only a few *dot*/*icm* genes should suppress Δ*dotL* lethality. Instead, inactivation of almost any *dot*/*icm* gene suppresses loss of *dotL*, which is more consistent with the toxicity being due to an improperly functioning Dot/Icm complex.

Taken in this context, inactivation of factors that are required for the proper assembly or activity of the Dot/Icm complex, such as DjlA, should also suppress Δ*dotL* lethality. However, the existence of suppressors that replicate in macrophages and have only subtle or no effects on the Dot/Icm complex in a *dotL*$^{+}$ background is perplexing. We propose two possible explanations for this conundrum. First, the number of functional Dot/Icm complexes required for intracellular growth may be significantly less than the number of toxic Δ*dotL* complexes required for cell death. In this case, a mutation that decreases the number of functional complexes may be able to suppress Δ*dotL* lethality but have no effect on intracellular growth in a wild-type background. Alternatively, one could imagine a type of mutation that induces a response capable of allowing a Δ*dotL* strain to live that is not needed for the normal assembly/activity of the complex. For example, inactivation of a repressor of a periplasmic protease might allow the elimination of a toxic Dot/Icm subcomplex but yet not be required in a wild-type cell. In either case, further examination of the Δ*dotL* lethality suppressors should reveal insights into both the normal assembly/activity of the T4SS and envelope stress response systems in *L. pneumophila*.

ACKNOWLEDGMENTS

We thank James Kirby for the generous gift of pJK211-2 and Petra Levin and Jessica Sexton for critical reading of the manuscript.

This work was funded by NIH grant AI48052 to J.P.V.

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