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Jessica A. Sexton
Washington University School of Medicine in St. Louis

Jennifer L. Miller
Washington University School of Medicine in St. Louis

Aki Yoneda
Washington University School of Medicine in St. Louis

Thomas E. Kehl-Fie
Washington University School of Medicine in St. Louis

Joseph P. Vogel
Washington University School of Medicine in St. Louis

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Infection and Immunity

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Legionella pneumophila DotU and IcmF Are Required for Stability of the Dot/Icm Complex

Jessica A. Sexton,[1] Jennifer L. Miller,[1] Aki Yoneda,[1] Thomas E. Kehl-Fie,[2] and Joseph P. Vogel[1]*

Department of Molecular Microbiology[1] and Department of Pediatrics,[2] Washington University School of Medicine, St. Louis, Missouri

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***Legionella pneumophila* utilizes a type IV secretion system (T4SS) encoded by 26 *dot/icm* genes to replicate inside host cells and cause disease. In contrast to all other *L. pneumophila dot/icm* genes, *dotU* and *icmF* have homologs in a wide variety of gram-negative bacteria, none of which possess a T4SS. Instead, *dotU* and *icmF* orthologs are linked to a locus encoding a conserved cluster of proteins designated IcmF-associated homologous proteins, which has been proposed to constitute a novel cell surface structure. We show here that *dotU* is partially required for *L. pneumophila* intracellular growth, similar to the known requirement for *icmF*. In addition, we show that *dotU* and *icmF* are necessary for optimal plasmid transfer and sodium sensitivity, two additional phenotypes associated with a functional Dot/Icm complex. We found that these effects are due to the destabilization of the T4SS at the transition into the stationary phase, the point at which *L. pneumophila* becomes virulent. Specifically, three Dot proteins (DotH, DotG, and DotF) exhibit decreased stability in a Δ*dotU* Δ*icmF* strain. Furthermore, overexpression of just one of these proteins, DotH, is sufficient to suppress the intracellular growth defect of the Δ*dotU* Δ*icmF* mutant. This suggests a model where the DotU and IcmF proteins serve to prevent DotH degradation and therefore function to stabilize the *L. pneumophila* T4SS. Due to their wide distribution among bacterial species and their genetic linkage to known or predicted cell surface structures, we propose that this function in complex stabilization may be broadly conserved.**

Legionella pneumophila is a gram-negative bacterium that is the causative agent of a form of pneumonia known as Legionnaires' disease (16). In the environment, this pathogen is an intracellular parasite of freshwater amoebae (39). When in contact with humans, it can cause disease by replicating inside alveolar macrophages (20). *L. pneumophila* is able to survive and replicate inside these normally bactericidal phagocytic cells by altering their endocytic pathway to create a novel niche, the replicative phagosome, where it can grow (19, 36). Later during infection, the replicative phagosome can fuse with the lysosome prior to lysis of the host cell and release of bacteria (35).

L. pneumophila alteration of the endocytic pathway is central to its ability to cause disease and is mediated by a large number of genes called *dot* (defect in organelle trafficking) or *icm* (intracellular multiplication) that encode a type IV secretion system (T4SS) (29, 37). This family of secretion systems comprises both classical plasmid transfer systems and adapted conjugation systems used by a wide range of bacterial pathogens. Most T4SS have significant homology to the components of the *Agrobacterium tumefaciens* VirB complex, which exports a number of virulence factors into plant cells (6, 10). By comparison, homologs of the *L. pneumophila dot/icm* genes are only found in a single other pathogenic T4SS, that of *Coxiella burnetii*, and on the IncI plasmids ColIb-P9 and R64 (23, 32, 34).

* Corresponding author. Mailing address: Department of Molecular Microbiology, Washington University, Campus Box 8230, 660 S. Euclid Ave., St. Louis, MO 63110. Phone: (314) 747-1029. Fax: (314) 362-3203. E-mail: jvogel@borcim.wustl.edu.

In contrast to the other *dot/icm* genes, the *icmF* gene has at least 27 homologs present in the finished and unfinished microbial genome database (9). Organisms that contain *icmF* homologs represent diverse phylogeny, most notably the alpha-, beta-, and gammaproteobacteriaceae. They include plant pathogens such as *A. tumefaciens*, *Rhizobium leguminosarum*, and *Xanthomonas axonopodis* as well as the animal pathogens *Yersinia pestis*, *Escherichia coli* O157:H7, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Salmonella enterica* (9). The only *icmF* homolog characterized to date is the *V. cholerae* gene VCA0120, which was initially described as a gene induced during *V. cholerae* replication in rabbit ileal loops (8). Inactivation of VCA0120 had pleiotropic effects including reduced motility, increased adherence to human intestinal epithelial cells, and enhanced conjugation as a recipient. It was speculated that these phenotypes may be the result of a change in a hypothetical cell surface structure (7, 9).

The *L. pneumophila icmF* gene was originally identified in a screen for Tn*903*dII*lacZ* insertion mutants defective in the ability to kill HL-60-derived macrophages (27, 28). In contrast to most of the other *dot/icm* genes, *icmF* is only partially required for *L. pneumophila* replication in human macrophages, though it is fully required for replication inside the more restrictive host *Acanthamoeba castellanii* (27, 31). Finally, a strain lacking *icmF* functions slightly better as a conjugal recipient of an RSF1010 plasmid when compared to a wild-type *L. pneumophila* strain similar to what occurs with the *V. cholerae icmF* mutant (7, 30). The *L. pneumophila icmF* gene is located at one end of *dot/icm* region II (27, 37) and can be found downstream of a gene previously designated *dotU* (34). In this paper, we characterize the role of *icmF* and *dotU* in the intracellular

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference or source
E. coli		
DH5α::λ*pir*	DH5α(λ*pir*) *tet*::Mu	22
ER1821	*E. coli* F^- *glnV44 e14-(McrA-) endA1 thi-1* Δ(*mcrC-mrr*)*114*::IS*10*	New England Biolabs (Beverly, Mass.)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^r)	Stratagene (La Jolla, Calif.)
L. pneumophila		
Lp02	*L. pneumophila* Philadelphia-1 Sm^r *rpsL hsdR thyA*	1
Lp03	Lp02 *dotA* mutant	1
JV1116	Lp02 Δ*dotU*	This study
JV4015	Lp02 Δ*dotU* (internal)	This study
JV1184	JV1116 + pJB908	This study
JV1183	JV1116 + pJB1180	This study
JV1188	JV1116 + pJB1186	This study
JV1179	Lp02 Δ*icmF*	This study
JV1192	JV1179 + pJB908	This study
JV1194	JV1179 + pJB1186	This study
JV1181	Lp02 Δ*dotU* Δ*icmF*	This study
JV1196	JV1181 + pJB908	This study
JV1199	JV1181 + pJB1191	This study
JV2572	JV1181 + pJB1555	This study
JV2573	JV1181 + pJB1554	This study
JV2574	JV1181 + pJB2121	This study
JV2575	JV1181 + pJB2132	This study
Plasmids		
pKB5	RSF1010 cloning vector *thyA*$^+$, *bla*, *mob*	1
pJB908	pKB5 Δ*oriT*	33
pJB1180	pJB908, *dotU*$^+$	This study
pJB1186	pJB908, *icmF*$^+$	This study
pJB1191	pJB908, *dotU*$^+$ *icmF*$^+$	This study
pJB1555	pJB908, *dotH*$^+$	This study
pJB1554	pJB908, *dotG*$^+$	This study
pJB2121	pJB908, *dotF*$^+$	This study
pJB2132	pJB908, *dotH*$^+$ *dotG*$^+$ *dotF*$^+$	This study
pSR47S	R6K suicide vector (Kan^r, *sacB*)	25
pJB1149	pSR47S with *dotU* flanking regions	This study
pJB1720	pSR47S with *dotU* internal regions	This study
pJB1156	pSR47S with *icmF* flanking regions	This study
pJB1168	pSR47S with *dotU icmF* flanking regions	This study
pQE30	Expression vector for His-tagged proteins	Qiagen
pJB1537	pQE30 with *dotU*	This study
pJB1544	pQE30 with *icmF*	This study

replication of *L. pneumophila* and report that they are essential for the stability of the Dot/Icm complex.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains are listed in Table 1. The wild-type *L. pneumophila* strain Lp02 (*thyA hsdR rpsL*) is a derivative of the serogroup 1 clinical isolate Philadelphia-1 (1). All *L. pneumophila* strains were cultured with *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffered yeast extract as described previously (12, 17). Lp02 and Lp02 derivatives were cultured on media supplemented with 100 μg of thymidine/ml as needed. *E. coli* strains XL1-Blue and DH5α::λ*pir* were used for cloning. Strain XL1-Blue was used for expression of His-tagged fusion proteins.

Strain and plasmid construction. The suicide plasmids pJB1149, pJB1720, pJB1156, and pJB1168, used to create the Δ*dotU* strains JV1116 and JV4015, the Δ*icmF* strain JV1179, and the Δ*dotU* Δ*icmF* strain JV1181, were constructed as follows. Five hundred-base pair regions flanking the relevant open reading frames or internal to the *dotU* gene were PCR amplified from Lp02 chromosomal DNA with primers engineered to have SalI, BamHI, or EagI restriction sites. The resulting products were cut with SalI/BamHI or BamHI/EagI, cloned in a three-piece ligation into the SalI/NotI sites of the suicide vector pSR47S (25), and confirmed by sequencing. Next, each plasmid was transformed into the wild-type *L. pneumophila* strain Lp02 where it integrated into the chromosome via homologous recombination to generate a merodiploid strain. Merodiploids were resolved by using a counterselection based on sucrose sensitivity, and resulting colonies were screened for the absence of the relevant gene via PCR, thus generating strains JV1116, JV4015, JV1179, and JV1181. Two different Δ*dotU* strains (JV1116 and JV4015) were constructed because Δ*dotU* in strain JV1116 appeared to have a slight polar effect on IcmF expression. Though JV1116 can be fully complemented for intracellular growth by *dotU*, indicating that levels of IcmF in this strain are sufficient, we chose to use a second Δ*dotU* strain (JV4015) for IcmF localization experiments, since it had no polar effect on IcmF expression.

The cloning vector pJB908 (33) was used to create *dotU*, *icmF*, *dotU icmF*, *dotH*, *dotG*, *dotF*, and *dotHGF* complementing clones. To construct plasmids pJB1180 and pJB1186, the *dotU* or *icmF* open reading frames were PCR amplified from Lp02 chromosomal DNA with primers containing KpnI/BamHI or XbaI/SalI restriction sites, cloned into the KpnI/BamHI or XbaI/SalI sites of pJB908, and confirmed by sequencing. The *dotU icmF* double complementing clone pJB1191 was constructed by inserting the pJB1186 XbaI/SalI (*icmF*) fragment into the XbaI/SalI sites of pJB1180, thus placing the *icmF* open reading frame downstream of *dotU*. The *dotH*, *dotG*, and *dotF* genes were each PCR amplified from Lp02 chromosomal DNA with primers containing KpnI/BamHI, BamHI/XbaI, or XbaI/SalI restriction sites, respectively. Products were cloned

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TABLE 2. *L. pneumophila* DotU and IcmF homologs exist in a wide range of other bacteria

Homolog no.	Organism	IcmF locus no.	No. of IcmF amino acids	E value (% identity)	DotU locus no.	No. of DotU amino acids	E value (% identity)
1	*V. cholerae*	VCA120	1,181	2e−35 (19)	VCA115	257	1e−09 (23)
2	*E. coli* O157:H7 VT2 Sakai	Z0250	1,144	7e−32 (19)	Z0255	253	2e−07 (23)
3	*Rhizobium*	ImpL	1,158	1e−28 (21)	ImpK	510	5e−05 (24)
4	*Salmonella* serovar *Typhimurium* LT2	STM0285 (SciS)	1,289	6e−15 (20)	SciP	434	6e−05 (24)
5	*A. tumefaciens*	AGR_L_1062	1,159	3e−31 (19)	AGR_L_1060	506	6e−05 (23)
6	*P. aeruginosa*	PA0077	1,101	4e−40 (20)	PA0078	449	3e−11 (29)
7	*P. aeruginosa*	PA1669	1,175	3e−44 (19)	PA1668	289	6e−11 (28)
8	*P. aeruginosa*	PA2361	1,271	ND (13)[a]	PA2362	252	ND (17)
9	*Y. pestis* CO92	YPO3603	1,177	4e−36 (21)	YPO3598	255	3e−03 (19)
10	*Y. pestis*	YPO0515	1,150	1e−22 (19)	YPO0514	536	8e−05 (27)
11	*Y. pestis*	YPO2724	1,275	2e−11 (18)	None	NA[b]	NA
12	*Y. pestis*	YPO1482	1,140	3e−05 (22)	None	NA	NA
13	*X. axonopodis* pv citri strain 306	XAC4119	1,176	3e−24 (19)	XAC4120	451	3e−14 (30)

[a] ND, not detectable by BLAST search.
[b] NA, not applicable.

into pJB908, confirmed by sequencing, and named pJB1555, pJB1554, and pJB2121. The *dotH dotG dotF* triple complementing clone pJB2132 was constructed by first cloning the XbaI/SalI (*dotF*) fragment from pJB2121 into pJB1555 and then adding the pJB1554 BamHI/XbaI (*dotG*) fragment, so that the final construct contained the *dotH*, *dotG*, and *dotF* open reading frames in that order.

For DotU and IcmF protein purification and generation of antibodies, the *dotU* and *icmF* open reading frames were PCR amplified with primers containing BamHI or BamHI/SalI restriction sites, cloned on a BamHI (*dotU*) or a BamHI/SalI (*icmF*) fragment into the His_6 expression vector pQE30 (Qiagen, Valencia, Calif.), and confirmed by sequencing. The plasmids were named pJB1537 (His-DotU) and pJB1544 (His-IcmF).

Cell fractionation. One hundred optical density at 600 nm (OD_{600}) units of bacterial cells were grown to mid-exponential phase, harvested, and stored at −20°C until needed. For preparation of membrane fractions, cells were resuspended in 2.5 ml of cold 20 mM Tris (pH 8.0), 5 mM EDTA. Lysozyme was added to 100 μg/ml, and cells were incubated on ice for 1 h and then lysed by sonication on ice (applied in 30-s bursts for a total of 4 min). The lysate was cleared by 10 min of centrifugation at 10,000 × *g* at 4°C, and a sample (representing the total protein fraction) was taken. The lysate was then subjected to ultracentrifugation for 1 h at 100,000 × *g* at 4°C to pellet the membrane fractions. The supernatant (soluble protein fraction) was removed and transferred to a new tube while the membrane pellet (total membrane fraction) was washed in cold 20 mM Tris (pH 8.0). Both fractions were centrifuged for 30 min at 100,000 × *g* at 4°C, the membrane pellet was resuspended in the original volume, and samples were taken (33).

Intracellular growth assays. The human monocytic cell line U937 was passaged, and cells were differentiated as described previously (26). Mouse bone marrow macrophages (BMM) were prepared from female A/J mice as described previously (5). In growth assays not requiring isopropyl-β-D-thiogalactopyranoside (IPTG) induction of gene expression, approximately 10^9 *L. pneumophila* cells were harvested from a 2-day 37°C patch, resuspended in sterile deionized water, and diluted 1:4,000 (for U937 cells) or 1:1,000 (for BMM) in RPMI 1640 (BioWhittaker, Walkersville, Md.) tissue culture media prior to infection. In growth assays where IPTG induction was used, *L. pneumophila* cells were grown in broth to exponential phase, back diluted into media containing 100 μM IPTG for overexpression of *dotH*, *dotG*, or *dotF*, and grown to early stationary phase. Approximately 10^9 bacteria were pelleted, washed one time in sterile deionized water, resuspended in 1 ml of sterile deionized water, and then diluted 1:4,000 into RPMI. In all cases, 0.5 ml of an *L. pneumophila* cell suspension was added to a monolayer of differentiated U937 cells (1×10^6 per well) or mouse BMM (5×10^5 per well) in 24-well dishes and the mixture was coincubated for 1 h at 37°C in 5% CO_2. Medium containing extracellular bacteria was then aspirated, and cell monolayers were washed two times with 0.5 ml of RPMI (containing 100 μM IPTG, where needed). Cell monolayers were maintained at 37°C in 5% CO_2 for 3 days, and each day, bacteria were recovered and plated to determine the total number of CFU in a given well. Because *L. pneumophila* cannot replicate in RPMI, daily quantitation of CFU allows an accurate representation of bacterial intracellular growth over time.

Antibody generation and affinity purification. His-DotU and His-IcmF were expressed by using plasmids pJB1537 and pJB1544, purified on nickel-nitrilotriacetic acid-agarose (Qiagen), and injected into rabbits (Cocalico, Inc., Reamstown, Pa.) for generation of polyclonal antibodies. DotL antibodies were generated similarly (J. P. Vogel et al., unpublished data). DotB antibodies were raised as described previously; DotB, DotU, and IcmF antibodies were affinity purified as described previously (33). DotF, DotG, DotH, DotI, DotN, and DotO antibodies were provided by Ralph Isberg. Isocitrate dehydrogenase (ICDH) antibodies were provided by Linc Sonenshein.

Conjugation and sodium sensitivity assays. Conjugation assays were performed as described previously (37). Briefly, $\sim 1 \times 10^9$ *L. pneumophila* donor cells containing plasmid pKB5 were grown to stationary phase, mixed with $\sim 1 \times 10^{10}$ *E. coli* recipient cells (strain ER1821), and applied to a 45-mm-pore-size hemagglutinin filter (Millipore, Bedford, Mass.) on a prewarmed agar plate. After a 2-h incubation at 37°C, cells were resuspended in sterile deionized water and plated on selective media to quantitate the total number of donors and recipients in the reaction mixture. Sodium sensitivity was determined by plating dilutions of stationary-phase *L. pneumophila* cells on charcoal yeast extract thymidine plates with or without 0.65% mM NaCl, as previously described (4, 38).

RESULTS

***dotU* and *icmF* are conserved among diverse species.** *L. pneumophila* DotU (GenBank accession no. AAQ10306) and IcmF (GenBank accession no. T18341) have orthologs in a wide range of gram-negative bacterial species, with more than 27 orthologs for each protein now reported in GenBank. Notably, in every organism where an *icmF* ortholog is found, a *dotU* ortholog also exists. The IcmF orthologs share 19 to 24% identity over their entire length and are typically of a size similar to that of *L. pneumophila* IcmF (973 amino acids) (Table 2). The same is true for the corresponding DotU orthologs, which are close in size to that of *L. pneumophila* DotU (261 amino acids). In some cases, however, DotU orthologs are slightly larger; an example is the *R. leguminosarum* ImpK protein, which contains an amino-terminal domain homologous to DotU and a carboxy-terminal domain homologous to OmpA. In addition, some species contain multiple copies of DotU and IcmF (Table 2) (15).

The *L. pneumophila dotU* and *icmF* genes are located at one end of *dot*/*icm* region II, which encodes structural components of the T4SS (29, 37) (Fig. 1). In contrast, the *dotU* and *icmF* homologs in other organisms can be found in a conserved

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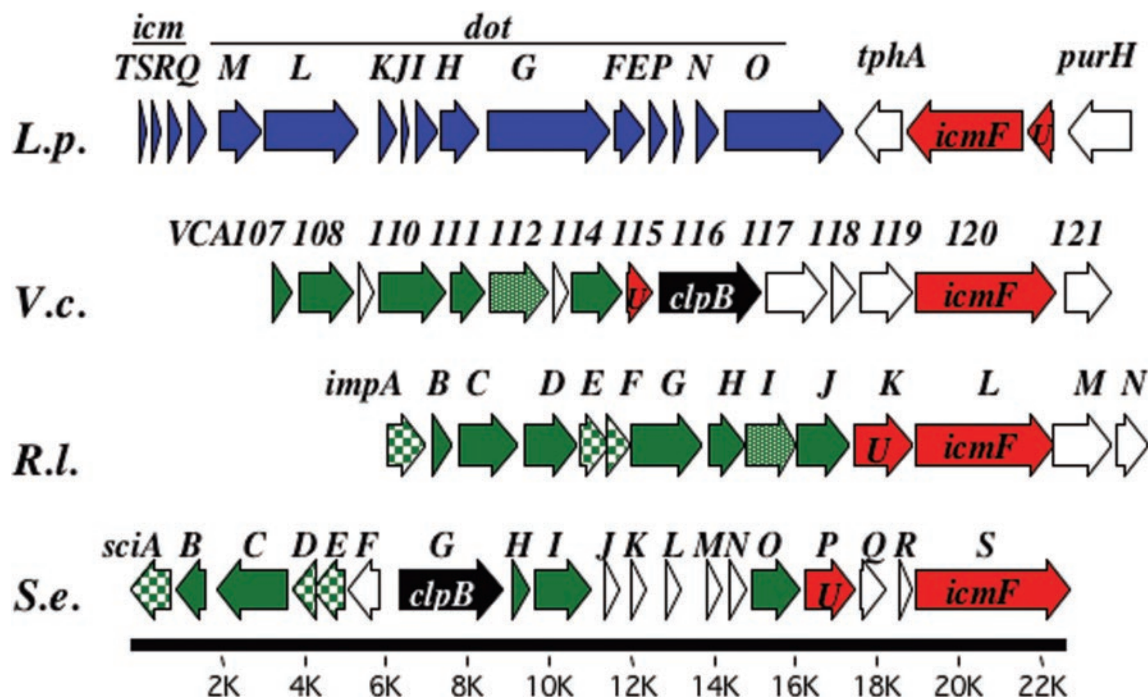

FIG. 1. IcmF and DotU orthologs are found in IAHP gene clusters. The *L. pneumophila* (*L.p.*) *dotU* and *icmF* genes (shown by red arrows) are located adjacent to a large number of *dot/icm* genes (shown by blue arrows) and are immediately flanked by genes with no role in type IV secretion (shown by white arrows). The IAHPs from *V. cholerae* (*V.c.*), *R. leguminosarum* (*R.l.*), and *S. enterica* (*S.e.*) all contain orthologs to *dotU* and *icmF* (shown by red arrows). The IAHP clusters contain a conserved core set of proteins (shown by solid green arrows) and less well conserved proteins that are found only in a subset of IAHP loci (indicated by stippled or checkered green arrows). Finally, most of these loci also contain a *clpB* homolog (shown by a black arrow).

cluster of genes that encode proteins known as IcmF-associated homologous proteins (IAHPs) (9). Three such loci have been characterized to date, and these are found in *V. cholerae*, *R. leguminosarum*, and *S. enterica* (Fig. 1) (2, 7, 15). Each locus contains approximately a dozen proteins with various degrees of conservation, and some IAHP clusters also contain a ClpB homolog (15). The majority of these proteins are predicted to localize to the membrane and likely constitute a macromolecular complex of unknown function.

DotU and IcmF are partially required for intracellular growth. To test whether *dotU*, like *icmF*, plays a role in *L. pneumophila* replication, we compared the growth of the wild-type strain Lp02 with a strain containing an in-frame deletion of *dotU* (JV1116) in U937 macrophages. The wild-type strain grew more than 1,000-fold in 3 days. In contrast, the Δ*dotU* strain was partially defective in replication, with 10- to 100-fold-fewer CFU than strain Lp02. This is in contrast to most *dot/icm* mutant strains, including the *dotA* null mutant strain Lp03, which are completely defective for replication (Fig. 2A). JV1116 (Δ*dotU*) intracellular growth was restored to wild-type levels by a *dotU* complementing clone and could not be restored with an *icmF* complementing clone (Fig. 2A). This indicated that the Δ*dotU* mutant JV1116 can be fully complemented for intracellular growth and that the intracellular growth defect we observed was not due to polarity on the downstream *icmF* gene.

To compare the *L. pneumophila* requirement for *dotU* and *icmF* in U937 cells, we tested an Δ*icmF* strain (JV1179) and found that it also has a partial growth defect in this cell line. This phenotype could be fully complemented by expression of *icmF* from a plasmid (Fig. 2B). Thus, the *dotU* and *icmF* mutants have similar intracellular growth phenotypes. In addition, a *dotU icmF* double deletion strain (JV1181) likewise has a partial growth defect in U937s, indicating that the DotU and IcmF proteins may work together to perform a common function (Fig. 2C).

The intracellular replication phenotypes of the Δ*dotU*, Δ*icmF*, and Δ*dotU* Δ*icmF* strains were also characterized in mouse BMM, a more stringent host (21). The growth defect observed for the single mutants was more severe in mouse BMM than in U937s (Fig. 3A and B). However, the mutants were still able to replicate at low levels compared to the replication-deficient *dotA* null mutant Lp03. Finally, the *dotU icmF* double deletion strain appeared similar to the single mutants, confirming the observation in U937 cells (Fig. 3C).

DotU and IcmF localize to the *L. pneumophila* membrane. Examination of the DotU and IcmF predicted protein sequences reveals few hints as to their potential functions. IcmF is a large protein that contains a potential nucleotide binding motif (GXXXXGKS/T) (40). In addition, it appears to possess several transmembrane domains, consistent with localization to the bacterial inner membrane. DotU is a smaller protein that does not contain any obvious functional motifs and that is also predicted to localize to the membrane. To confirm DotU and IcmF intracellular localization, *L. pneumophila* extracts were fractionated into cytoplasm and membrane samples and subjected to Western blots with polyclonal DotU and IcmF antibodies. Although IcmF protein was readily detected in cell

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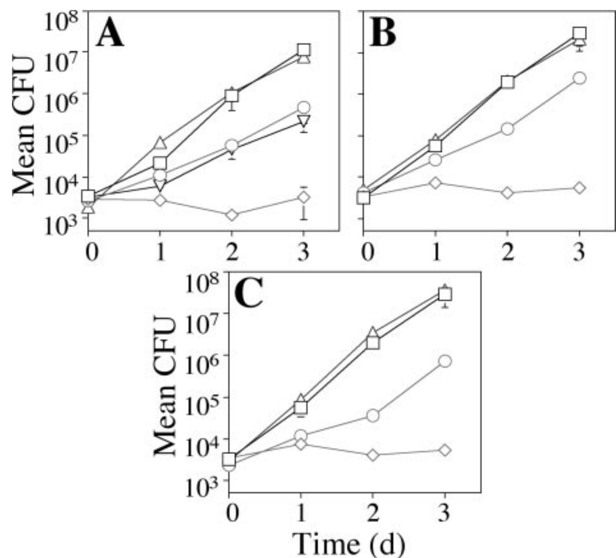

FIG. 2. Intracellular growth of *dotU* and *icmF* mutants in U937 monocytes. (A) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*dotU* strain JV1116 containing either the empty vector pJB908 (circles), the *dotU* complementing clone pJB1180 (triangles), or the *icmF* complementing clone pJB1186 (inverted triangles) were assayed for growth in U937 cells. Mean numbers of CFU are plotted as a function of time. (B) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*icmF* strain JV1179 containing either the empty vector pJB908 (circles) or the *icmF* complementing clone pJB1186 (triangles) were assayed for growth in U937 cells. (C) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*dotU* Δ*icmF* strain JV1181 containing either the empty vector pJB908 (circles) or the *dotU icmF* complementing clone pJB1191 (triangles) were assayed for growth in U937 cells. Assays were done in triplicate. Error bars indicate standard deviations of the means. d, day.

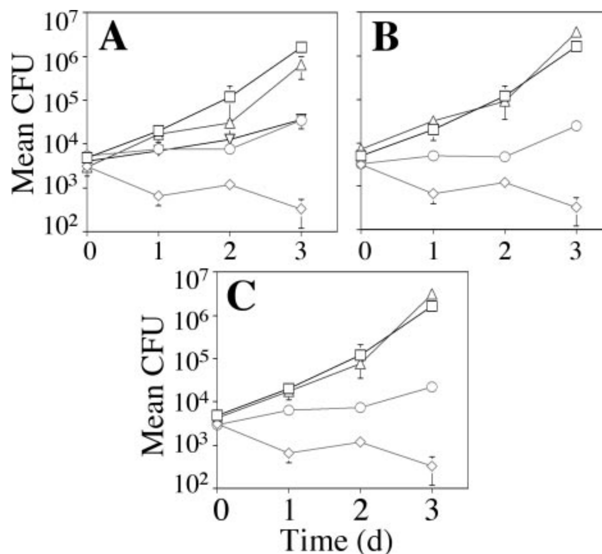

FIG. 3. Intracellular growth of *dotU* and *icmF* mutants in mouse BMM. (A) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*dotU* strain JV1116 containing either the empty vector pJB908 (circles), the *dotU* complementing clone pJB1180 (triangles), or the *icmF* complementing clone pJB1186 (inverted triangles) were assayed for growth in BMM. Mean numbers of CFU are plotted as a function of time. (B) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*icmF* strain JV1179 containing either the empty vector pJB908 (circles) or the *icmF* complementing clone pJB1186 (triangles) were assayed for growth in BMM. (C) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*dotU* Δ*icmF* strain JV1181 containing either the empty vector pJB908 (circles) or the *dotU icmF* complementing clone pJB1191 (triangles) were assayed for growth in BMM. Assays were done in triplicate. Error bars indicate standard deviations of the means. d, day.

extracts from an early-stationary-phase culture, we failed to detect any DotU (data not shown). Subsequent analysis of whole-cell samples taken at various stages of *L. pneumophila* growth revealed that DotU is present in the exponential phase but not in the stationary phase, consistent with rapid processing or degradation upon entry into stationary phase (Fig. 4). Similarly, IcmF is also absent in the late stages of *L. pneumophila* growth. In contrast to DotU, however, complete disappearance of full-length IcmF does not appear to occur until late in stationary phase and happens gradually (Fig. 4). This type of growth-phase-related fluctuation in protein levels is atypical for most Dot/Icm proteins, which appear to be constitutively expressed (Vogel et al., unpublished) (Fig. 4, DotL; see also Fig. 7, DotB).

Based on the above-described result, cell fractions generated from exponential cells were used to determine the intracellular localization of DotU and IcmF. As predicted, both DotU and IcmF were found to be present almost entirely in the membrane fraction (Fig. 5, lanes 1 to 3). Levels and membrane localization of IcmF were not affected by the absence of *dotU* (Fig. 5, lanes 7 to 9), confirming that this Δ*dotU* strain was not polar on the downstream *icmF* gene. In contrast, the loss of *icmF* had a strong effect on DotU protein levels (Fig. 5, lanes 4 to 6). This result is consistent with a DotU/IcmF protein-

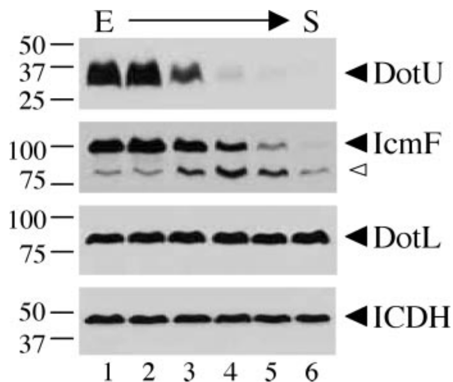

FIG. 4. DotU and IcmF disappear at different points in stationary-phase *L. pneumophila*. Equivalent OD_{600} units of cells from *L. pneumophila* wild-type strain Lp02 were taken at various time points during growth in broth, from early exponential (E) through late stationary (S) phase. Lanes 1 through 6 correspond to culture OD_{600}s of 2.5, 2.8, 3.1, 3.2, 3.4, and 3.4, respectively. Cells became motile between OD_{600}s of 2.8 and 3.1, just prior to entering the stationary phase. Cell lysates were used for DotU, IcmF, DotL, or ICDH Western blots. Solid arrowheads point to bands that correspond to full-length proteins while the open arrowhead indicates a smaller reactive species likely to represent processed or partially degraded IcmF protein. The molecular masses of relevant markers (in kilodaltons) are shown on the left.

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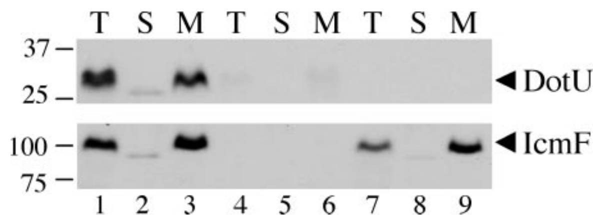

FIG. 5. Subcellular localization of DotU and IcmF. (A) Total protein (T, lanes 1, 4, and 7), total soluble protein (S, lanes 2, 5, and 8), and total membrane protein (M, lanes 3, 6, and 9) fractions were taken from equivalent OD_{600} units of three *L. pneumophila* strains and were subjected to DotU and IcmF Western blots. Strains included the wild-type Lp02 strain (lanes 1 to 3), the Δ*icmF* strain JV1179 (lanes 4 to 6), and the Δ*dotU* strain JV4015 (lanes 7 to 9). The molecular masses of relevant markers (in kilodaltons) are shown on the left. Results are representative of those of several experiments.

protein interaction, as interacting proteins often stabilize each other (13, 18).

The *dotU* and *icmF* genes are partially required for additional *dot/icm*-associated phenotypes. The observation that *dotU* and *icmF*-like genes are not normally associated with T4SS but are instead found in diverse organisms that lack T4SS genes (Table 2) (2, 9, 15) suggests that they are not likely to be core components of the *L. pneumophila* T4SS. Nevertheless, Δ*dotU* Δ*icmF* mutants were incapable of wild-type intracellular growth, a process that requires a functional Dot/Icm complex. This phenotype was not due to a general defect in replication, as these strains could grow normally outside of host cells in AYET broth (data not shown). To determine whether the intracellular growth defect of the *dotU* and *icmF* mutants was due to an effect on the T4SS, we examined these mutant strains for additional *dot/icm*-dependent phenotypes. First, the Δ*dotU*, Δ*icmF*, and Δ*dotU* Δ*icmF* strains were tested for the ability to transfer an RSF1010 plasmid to an *E. coli* recipient. While the wild-type strain Lp02 could transfer a plasmid at a frequency of $\sim 5 \times 10^{-6}$ recipients per donor cell, strains JV1116 (Δ*dotU*), JV1179 (Δ*icmF*), and JV1181 (Δ*dotU* Δ*icmF*) did so with a 10-fold-reduced frequency. Strain Lp03 (*dotA* null mutant) was incapable of transferring a plasmid above the limit of detection, $\sim 1 \times 10^{-9}$ (Fig. 6A). Thus, *dotU* and *icmF* appear to be partially required for Dot/Icm-mediated plasmid transfer.

Next, the *dotU* and *icmF* mutants were characterized for sodium sensitivity. Wild-type *L. pneumophila* is normally highly sodium sensitive, whereas mutations in the Dot/Icm machinery confer sodium resistance. The plating efficiencies of strains JV1116 (Δ*dotU*), JV1179 (Δ*icmF*), and JV1181 (Δ*dotU* Δ*icmF*) on medium with sodium were found to be 10- to 100-fold reduced compared to the *dotA* null mutant strain Lp03 but approximately 100-fold greater than the wild-type strain Lp02 (Fig. 6B), indicating a partial role for DotU and IcmF in sodium resistance. Both the sodium sensitivity and plasmid transfer deficiencies of strain JV1181 (Δ*dotU* Δ*icmF*) could be fully complemented (data not shown). Because the *dotU* and *icmF* mutant strains are partially defective in intracellular growth, plasmid transfer, and sodium sensitivity, we predicted that the DotU and IcmF proteins may directly affect the Dot/Icm complex.

Mutations in *dotU* and *icmF* affect stability of the DotH, DotG, and DotF proteins. To check for effects of DotU and IcmF on the Dot/Icm complex, we performed Western blots against Lp02 and JV1181 (Δ*dotU* Δ*icmF*) whole-cell extracts with a panel of Dot/Icm antibodies. We found that the DotH, DotG, and DotF proteins were affected for stability in the *dotU icmF* mutant strain compared to wild-type strain Lp02 (Fig. 7). Altered protein levels were only apparent in whole-cell extracts taken from late-exponential- or stationary-phase cultures, reminiscent of the DotU processing previously observed. The fact that DotHGF destabilization correlates with DotU processing suggests that the latter event may be required for the normal activity of DotU.

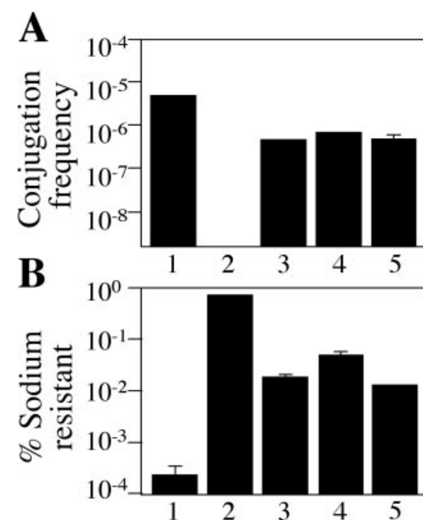

FIG. 6. Conjugation and salt resistance phenotypes of *dotU* and *icmF* mutants. (A) The following *L. pneumophila* strains were assayed for the ability to transfer an RSF1010 plasmid to *E. coli* recipient cells: wild-type strain Lp02 (column 1), *dotA* mutant strain Lp03 (column 2), Δ*dotU* strain JV1116 (column 3), Δ*icmF* strain JV1179 (column 4), and Δ*dotU* Δ*icmF* strain JV1181 (column 5). The conjugation frequency was calculated as the number of *E. coli* recipients per *L. pneumophila* donor cell. Assays were done in triplicate. Error bars indicate the standard deviations of the means. (B) The plating efficiency on 0.65% NaCl was determined for strains used in panel A, and is shown here as the percentage of NaCl-resistant CFU in a cell population. The strains were wild-type Lp02 (column 1), *dotA* mutant Lp03 (column 2), the Δ*dotU* strain JV1116 (column 3), the Δ*icmF* strain JV1179 (column 4), and the Δ*dotU* Δ*icmF* strain JV1181 (column 5). Assays were done in triplicate. Error bars indicate standard deviations of the means.

The DotG protein was the most profoundly altered by the absence of *dotU* and *icmF* and could only be found as an ~75-kDa breakdown product in the late stages of in vitro growth. While levels of full-length DotF were not markedly reduced in the Δ*dotU* Δ*icmF* strain, an ~25-kDa breakdown product not seen in the wild-type strain appeared in the late stages of growth (Fig. 7). In contrast, levels of full-length DotH protein were reduced in the late stages of JV1181 (Δ*dotU* Δ*icmF*) growth compared to those of Lp02, but this did not correlate with a visible increase in breakdown product (Fig. 7). All observed stability effects were identical in the Δ*dotU*, Δ*icmF*, and Δ*dotU* Δ*icmF* strains and could be complemented (data not shown). Notably, these effects were specific to a subset of Dot/Icm proteins, as the majority were not destabilized in the Δ*dotU* Δ*icmF* strain; specifically, levels of DotB,

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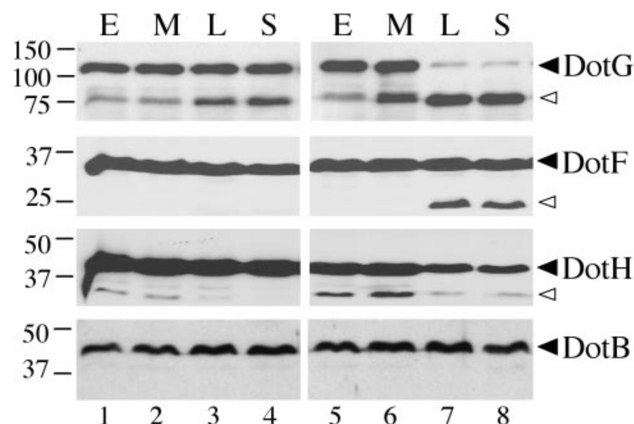

FIG. 7. DotG, DotF, and DotH protein levels are altered in a Δ*dotU* Δ*icmF* strain. Equivalent OD_{600} units of cells from the *L. pneumophila* wild-type strain Lp02 (lanes 1 to 4) or the Δ*dotU* Δ*icmF* strain JV1181 (lanes 5 to 8) were taken at the early (E), mid-exponential (M), late exponential (L), and stationary (S) phases of growth and used as total protein samples for DotG, DotF, or DotH Western blots. The same samples were used for a DotB Western blot as a negative control. The molecular masses of relevant markers (in kilodaltons) are shown on the left. Solid arrowheads point to bands that correspond to full-length proteins while open arrowheads indicate smaller reactive species likely to represent processed or partially degraded proteins. Results are representative of those of several experiments.

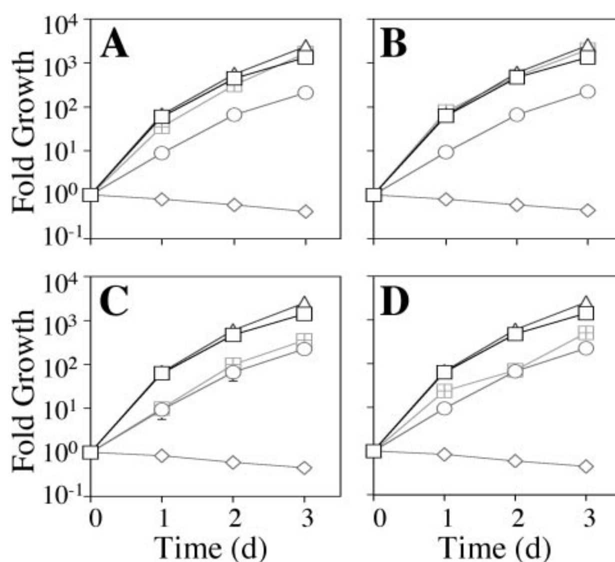

FIG. 8. Suppression of Δ*dotU* Δ*icmF* intracellular growth defect by overexpression of DotH. (A) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*dotU* Δ*icmF* strain JV1181 containing either the empty vector pJB908 (circles), the *dotU icmF* complementing clone pJB1191 (triangles), or the *dotHGF* complementing clone pJB2132 (hatched squares) were assayed for growth in U937 cells. Mean numbers of CFU are plotted as a function of time. (B) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*dotU* Δ*icmF* strain JV1181 containing either the empty vector pJB908 (circles), the *dotU icmF* complementing clone pJB1191 (triangles), or the *dotH* complementing clone pJB1555 (hatched squares) were assayed for growth in U937 cells. (C) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*dotU* Δ*icmF* strain JV1181 containing either the empty vector pJB908 (circles), the *dotU icmF* complementing clone pJB1191 (triangles), or the *dotG* complementing clone pJB1554 (hatched squares) were assayed for growth in U937 cells. (D) The wild-type *L. pneumophila* strain Lp02 (squares), the *dotA* mutant Lp03 (diamonds), and the Δ*dotU* Δ*icmF* strain JV1181 containing either the empty vector pJB908 (circles), the *dotU icmF* complementing clone pJB1191 (triangles), or the *dotF* complementing clone pJB2121 (hatched squares) were assayed for growth in U937 cells. Growth curves were determined in the presence of 100 μM IPTG. Assays were done in triplicate. Error bars indicate standard deviations of the means. d, day.

DotI, DotL, DotN, and DotO were not altered. Thus, DotU and IcmF affect the stability of a subset of Dot/Icm proteins.

Overexpression of *dotH* suppresses the intracellular growth defect of a *dotU icmF* mutant. To determine whether the *dot/icm* phenotypes of the Δ*dotU* Δ*icmF* strain were caused only by the reduced levels of DotH, DotG, and DotF, we expressed the corresponding genes in strain JV1181 (Δ*dotU* Δ*icmF*) and assayed intracellular growth. We found that simultaneous overexpression of *dotHGF* was sufficient to eliminate the Δ*dotU* Δ*icmF* intracellular growth defect in U937 cells (Fig. 8A). Furthermore, overexpression of just the *dotH* gene alone was able to suppress the growth defect, whereas this effect was not seen with just the *dotF* or *dotG* complementing clones (Fig. 8B to D).

One explanation for this is that DotU and IcmF directly affect only DotH stability, and the decreased levels of DotG and DotF result indirectly from lowered levels of DotH. Therefore, we tested whether overexpression of *dotH* in the Δ*dotU* Δ*icmF* strain could restore wild-type levels of DotG and DotF. We compared DotH, DotG, and DotF protein levels in the wild-type strain Lp02, the Δ*dotU* Δ*icmF* strain, or the Δ*dotU* Δ*icmF* strain containing the *dotH* complementing clone pJB1555, each exposed to a range of IPTG concentrations. Expression of *dotH* with all concentrations of IPTG resulted in overproduction of DotH (Fig. 9, compare lanes 9 to 12 with lanes 5 to 8). Moreover, expression of *dotH* restored wild-type levels of DotG and eliminated the apparent degradation of DotF. These findings suggest the DotU and IcmF proteins work together to stabilize the Dot/Icm complex by maintaining proper levels of functional DotH, which is required for the stability of DotG and DotF.

DISCUSSION

We have shown here that the *dotU* gene, adjacent to *icmF* on the *L. pneumophila* chromosome, is required for the optimal intracellular growth of this organism. Loss of either *dotU* alone, *icmF* alone, or both genes causes a similar impairment in intracellular replication of *L. pneumophila*. We have observed that several of the Dot/Icm proteins, DotH, DotG, and DotF, appear to be significantly destabilized in a Δ*dotU* Δ*icmF* mutant strain. This destabilization can be suppressed by overproduction of a single gene, *dotH*, indicating that the DotH protein is likely a key factor in the stability of a putative DotHGF subcomplex. Furthermore, overexpression of *dotH* was sufficient to abolish the intracellular growth defect of the Δ*dotU* Δ*icmF* mutant strain, suggesting that destabilization of DotH is the critical factor inhibiting intracellular replication in the Δ*dotU* Δ*icmF* background. Since DotH, DotG, and DotF are components of the Dot/Icm secretion machinery and since

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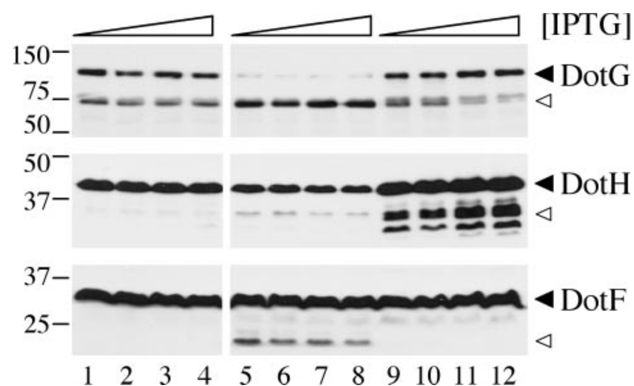

FIG. 9. Suppression of Δ*dotU* Δ*icmF* protein aberrations by overexpression of DotH. *L. pneumophila* wild-type strain Lp02 (lanes 1 to 4) or Δ*dotU* Δ*icmF* strain JV1181 containing either the empty vector pJB908 (lanes 5 to 8) or the *dotH* complementing clone pJB1555 (lanes 8 to 12) was grown in the presence of 0, 10, 100, or 1,000 μM IPTG to stationary phase. Whole-cell samples (equivalent OD_{600} units) were taken and subjected to DotH, DotG, or DotF Western blots. The molecular masses of relevant markers (in kilodaltons) are shown on the left. Arrowheads are as described in the legend to Fig. 7.

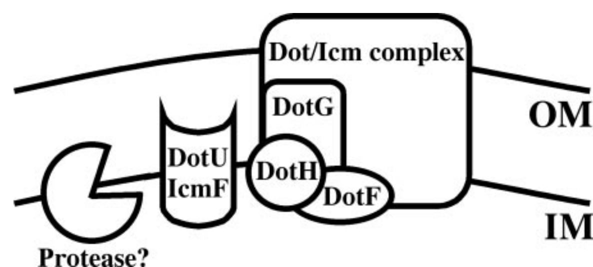

FIG. 10. Model of how DotU and IcmF prevent destabilization of the Dot/Icm complex. The DotU and IcmF proteins localize to the inner membrane where they likely work together to shield one or more components of the Dot/Icm complex from degradation by an as yet unidentified protease. In the absence of DotU/IcmF, the subcomplex DotH, DotG, and DotF is selectively targeted for proteolysis.

this machinery is known to be of critical importance for alteration of the endocytic pathway, it is reasonable to conclude that the Δ*dotU* Δ*icmF* intracellular growth defect likely results from mistargeting of *L. pneumophila* cells early in infection.

The *icmF* gene was originally identified in a screen for *L. pneumophila* mutants defective in the ability to kill HL-60-derived macrophages (27, 28). *dotU*, immediately upstream of *icmF*, was first identified via a transposon insertion that impaired the ability of *L. pneumophila* to cause disease in a guinea pig model of infection (11). However, since the insertion was not complemented, it remained possible that the phenotype was simply due to a polar effect on the downstream *icmF* gene (11). In this report, we have generated a nonpolar Δ*dotU* strain that displays a partial intracellular growth defect similar to an Δ*icmF* strain. The Δ*dotU* Δ*icmF* double mutant has a phenotype identical to that of either single mutant, which suggests that the two proteins may function together. This notion is supported by several facts. First, the two genes appear to be carried on a single operon, a trait often indicative of genes that perform a similar activity. Second, the proteins colocalize to the *L. pneumophila* membrane. Finally, loss of the IcmF protein affects the stability of DotU, a trait common among proteins that interact (13, 18).

It was a formal possibility that the intracellular growth defect seen in *dotU icmF* mutants was independent of the T4SS. However, several lines of evidence exist to the contrary. First, the Δ*dotU* Δ*icmF* mutant is partially resistant to low levels of sodium added to the media, a trait shared with other *dot*/*icm* mutants. Second, the Δ*dotU* Δ*icmF* mutant is partially defective for transfer of a plasmid, a second trait shared with the other *dot*/*icm* mutants. Finally, the Δ*dotU* Δ*icmF* mutant is able to suppress the lethality caused by the loss of the *dotL* gene (Vogel et al., unpublished). In certain strains of *L. pneumophila*, loss of the DotL protein has been observed to be a lethal event that can be suppressed by inactivation of the majority of the *dot*/*icm* genes. Thus, suppression of Δ*dotL* lethality is an additional *dot*/*icm* phenotype (Vogel et al., unpublished). Taken together, these data suggest that while DotU and IcmF are not likely to be core components of the Dot/Icm complex, they somehow modify it.

Consistent with this, we observed degradation of at least three Dot/Icm proteins in the Δ*dotU* Δ*icmF* strain background: DotF, DotG, and DotH. Although DotH is predicted to localize to the inner membrane by sequence analysis, it has been shown to be extruded on the *L. pneumophila* cell surface during infection (41). DotF has been shown to interact with several Dot/Icm secreted substrates by a two-hybrid system (24). DotG has limited similarity to the *A. tumefaciens* VirB10 protein, a core component of the VirB T4SS, in which it forms a subcomplex with VirB9 and VirB11 (14). Based on the homology of DotG to VirB10 and the data presented here, it is possible that DotF, DotG, and DotH form a similar subcomplex in *L. pneumophila*.

Overexpression of *dotH* alone was sufficient to suppress the destabilization of the Dot/Icm complex due to loss of *dotU* and *icmF*, resulting in restored levels of DotG and DotF and elimination of the Δ*dotU* Δ*icmF* intracellular growth defect. This suggests that DotH may be the key component of a proposed DotHGF subcomplex. Analysis of Δ*dotH*, Δ*dotG*, and Δ*dotF* strains is in keeping with this. Levels of DotH protein are unaffected by mutations in *dotG* or *dotF* while levels of DotF are mildly affected by mutations in *dotH* but not *dotG*, and finally, levels of DotG are drastically affected by mutations in either *dotH* or *dotF* (Vogel et al., unpublished). Therefore, DotU and IcmF may act directly on DotH, the disruption of which would then affect the DotG and DotF proteins.

The loss of *dotU*/*icmF* results in degradation of components of the Dot/Icm complex at the transition from exponential phase into stationary phase. Based on this growth phase specificity, it is apparent that *dotU* and *icmF* are critical to *L. pneumophila* precisely at the time when it becomes virulent (3). Curiously, DotU itself also appears to be processed and/or degraded at the transition into the virulent state, suggesting that this event may be important in DotU and/or IcmF function.

Data shown here are consistent with a role for *L. pneumophila* DotU and IcmF as accessory factors to the type IV secretion machinery, protecting it from degradation (Fig. 10). One possibility is that they assist in the assembly of a functional Dot/Icm complex and that in their absence the complex mis-

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assembles, leading to partial proteolysis. Alternatively, DotU and IcmF could be critical components for maintaining the assembled complex in a stable and active form, thus protecting it from destabilization and proteolysis in the stationary phase. A third possibility is that these proteins function to regulate the Dot/Icm complex, controlling a switch from an inactive to an active T4SS in response to growth phase; DotU degradation and processing could be the key event in this process. Finally, these proteins could be involved in recycling of the *L. pneumophila* T4SS.

One of the most intriguing features of DotU and IcmF is their high degree of conservation among diverse bacterial species. In many organisms, *dotU* and *icmF* orthologs are found in IAHP loci, which have been proposed to encode cell surface structures (2, 9, 15). Inactivation of the *icmF* homolog in the *V. cholerae* IAHP locus appeared to have pleiotropic effects on cell surface structures (7, 9). Deletion of the *S. enterica* IAHP locus resulted in a slightly decreased ability to invade eukaryotic cells (15). Although the precise function of these IAHP loci remains unknown, they appear to encode a cell surface organelle. In fact, the *R. leguminosarum* IAHP locus (designated *imp*), involved in nodule formation, has been shown to encode a temperature-dependent secretion system (2). Thus, similar to the case with *L. pneumophila*, the *dotU* and *icmF* orthologs are genetically linked to macromolecular membrane-spanning complexes. Whether for T4SS or IAHP clusters, DotU and IcmF proteins are likely to serve a related function as novel accessory factors that maintain the stability of membrane complexes.

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