

Membrane Chaperone SecDF Plays a Role in the Secretion of *Listeria monocytogenes* Major Virulence Factors

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***Listeria monocytogenes* is a Gram-positive human intracellular pathogen that infects diverse mammalian cells. Upon invasion, *L. monocytogenes* secretes multiple virulence factors that target host cellular processes and promote infection. It has been presumed, but was not empirically established, that the Sec translocation system is the primary mediator of this secretion. Here, we validate an important role for SecDF, a component of the Sec system, in the secretion of several critical *L. monocytogenes* virulence factors. A $\Delta secDF$ mutant is demonstrated to exhibit impaired membrane translocation of listeriolysin O (LLO), PlcA, PlcB, and ActA, factors that mediate *L. monocytogenes* phagosomal escape and spread from cell to cell. This impaired translocation was monitored by accumulation of the factors on the bacterial membrane and by reduced activity upon secretion. This defect in secretion is shown to be associated with a severe intracellular growth defect of the $\Delta secDF$ mutant in macrophages and a less virulent phenotype in mice, despite normal growth in laboratory medium. We further show that SecDF is upregulated when the bacteria reside in macrophage phagosomes and that it is necessary for efficient phagosomal escape. Taken together, these data support the premise that SecDF plays a role as a chaperone that facilitates the translocation of *L. monocytogenes* virulence factors during infection.**

A prerequisite for a pathogen to succeed is an ability to influence the host's cellular processes. In bacteria, this ability is largely mediated by secreting multiple virulence factors that target host cellular components during the course of infection. Whereas Gram-negative pathogenic bacteria are armed with several specialized secretion systems that serve this purpose (types I to VI), Gram-positive bacteria lack these systems and presumably use mainly the canonical Sec translocation system (1). *Listeria monocytogenes* is a Gram-positive intracellular bacterial pathogen that invades and grows within diverse mammalian cells (2). It is the causative agent of the food-borne disease listeriosis in humans, which can result in meningitis, abortion, or septicemia. During infection, *L. monocytogenes* invades host cells by inducing its own internalization into a vacuole (or via phagocytosis into a phagosome), from which it rapidly escapes by producing and secreting the pore-forming hemolysin toxin listeriolysin O (LLO; encoded by the *hly* gene) (3–6). LLO, together with two additional secreted phospholipases (PLC), PlcA, a phosphoinositol-PLC (PI-PLC), and PlcB, a phosphatidylcholine-PLC (PC-PLC), perforate the phagosomal membrane and enable the bacteria to reach the host cell's cytosol (7, 8). In the cytosol, *L. monocytogenes* replicates and secretes the surface protein, ActA, that recruits the host cell's actin polymerization machinery to propel the bacteria around the cell and to facilitate spread from cell to cell without causing lysis (9, 10). While these virulence factors are essential for *L. monocytogenes* pathogenesis, currently not much is known about the mechanisms that mediate their secretion during infection.

Most *L. monocytogenes* secretory proteins are assumed to be secreted via the Sec translocation system based on signal peptide predictions and proteomic studies (11–15). The Sec system is found in both Gram-positive and Gram-negative bacteria and serves as the predominant protein translocation system for integral membrane and secretory proteins. As such, most of the components of the Sec system are highly conserved (also beyond the prokaryotic kingdom) and are essential for growth (16). The Sec

system has been studied primarily in *Escherichia coli* and was shown to comprise a protein-conducting channel (the translocon) and several accessory proteins. The translocon is composed of three integral membrane proteins, SecYEG, which together conduct the translocation of proteins across and into the cytoplasmic membrane (17). Secretory proteins (preproteins) are targeted to the membrane posttranslationally by the SecB chaperone, which stabilizes them in an unfolded conformation. Once localized to the membrane, SecB directs the preproteins to the translocon motor protein, SecA, which threads the unfolded preproteins through the translocon channel using ATP hydrolysis. Across the membrane, at the translocon exit site, the SecD-SecF/YajC complex pulls out the preproteins, completing their translocation (18, 19). Another component of the Sec system is the insertase YidC, which is thought to facilitate mainly the insertion of inner membrane proteins (20). Although the Sec components are generally conserved among bacteria, there are several differences between the Sec systems of Gram-negative and Gram-positive bacteria. Typically, Gram-positive bacteria lack the SecB chaperone, some species encode additional copies of *sec* genes, such as *secA*, *secY*, and *yidC* (*secA2*, *secY2*, and *yidC2*), and the SecD-SecF complex is fused into a single protein encoded by a single gene (21, 22). In addition, some Gram-positive species encode a chaperone termed PrsA, which is located extracellularly and facilitates the folding of secretory proteins upon their secretion (23). Specifically, *L. mono-*

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cytogenes encodes an additional *secA* gene (*secA2*, but not *secY2*), two *yidC* genes (*yidC1* and *yidC2*), two *prsA* genes (*prsA1* and *prsA2*), and a fused *secDF* gene. SecA2 was shown to facilitate the secretion of several autolytic enzymes, cell wall proteins, and a superoxide dismutase, termed MnSOD, all of which contribute to *L. monocytogenes* *in vivo* infection (13, 24, 25). PrsA2 was shown to promote the folding and activity of LLO and PC-PLC upon secretion (26–29); however, the role of SecDF in *L. monocytogenes* has not been characterized.

L. monocytogenes SecDF is predicted to contain 12 transmembrane segments and two large extracellular loops, which is topologically similar to the *E. coli* SecDF complex (22). The initial observation that *E. coli* SecD and SecF are the only Sec components harboring periplasmic domains led to the suggestion that they are involved in later stages of protein export (30). Further studies have implicated SecD and SecF in playing a role in facilitating efficient translocation of preproteins. However, they are not completely necessary for protein export *in vivo* and *in vitro* (31–33). This ambiguity concerning the function of SecDF in the protein secretion process was only recently resolved with the publication of the *Thermus thermophilus* SecDF crystal structure (19). This study demonstrated that SecDF functions as a membrane-integrated chaperone that binds the emerging preprotein and tugs it out of the translocon in a highly precise manner, using proton-motive force as an energy source (19, 34). Nevertheless, the essentiality of SecDF in protein secretion remains mysterious. For example, in *E. coli* the *secDF* mutant is characterized by a severe growth defect in rich laboratory medium at 30 and 37°C, yet it grows normally in glucose minimal defined medium at 37°C (31). In *Bacillus subtilis* and *Staphylococcus aureus*, *secDF* deletion mutants exhibit a cold-sensitive growth phenotype (at 15°C) but grow normally at 37°C (22, 35). Notably, the cold sensitivity of the *B. subtilis* *secDF* mutant is exacerbated when the bacteria overexpress a secretory protein, yet this phenotype disappears if the bacteria are grown at 37°C (22). These observations highlight the flexibility in the requirement for SecDF during protein export with different bacteria and even under different growth conditions. In this study, we show that *L. monocytogenes* SecDF is required for *in vivo* bacterial infection, yet it is dispensable for growth in laboratory-rich medium (at both 15 and 37°C). We further demonstrate that SecDF plays a role in membrane translocation of several critical virulence factors; thus, it contributes to the *L. monocytogenes* infection process.

MATERIALS AND METHODS

Strains, cells, growth conditions, and reagents. *L. monocytogenes* strains used in this study are shown in Table 2. *E. coli* XL-1 Blue strain was used for vector propagation (Stratagen), and *E. coli* strain SM-10 (53) was used as a donor when delivering plasmids to *L. monocytogenes* by conjugation. *E. coli* strains were grown in Luria-Bertani (LB; BD) media at 37°C, and *L. monocytogenes* strains were grown in brain heart infusion (BHI; BD) rich media at 37°C. For induction of the virulence genes, bacteria were grown in LB morpholinopropanesulfonic acid (MOPS)-glucose 1-phosphate (LB-Glu-1P) medium (44). Anti-LLO antibody was purchased from Abcam, Inc. (ab43018). Anti-PlcA antibody was a gift from Helen Marquis (Cornell), and anti-PlcB antibody was a gift from Howard Goldfine (University of Pennsylvania) and Daniel Portnoy (Berkeley). HisProbe-horse-radish peroxidase (HRP) conjugated for ActA-6His detection was purchased from Thermo Scientific (15165). Anti-GroEL antibody was a gift from Abdussalam Azem (Tel-Aviv University). Peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Jack-

son ImmunoResearch Laboratories, Inc. (catalog no. 111-035-003 and 115-035-003, respectively). For infection experiments, *L. monocytogenes* bacteria were grown overnight in BHI at 30°C without agitation. Bone marrow-derived (BMD) macrophages were isolated from C57BL/6 (6- to 8-week-old) female mice (Harlan Laboratories, Ltd., Israel) and cultured as described previously (3). The ISRE-L929 cell line was a gift from Bruce Beutler (University of Texas Southwestern Medical Center) (38).

Bacterial genetic screen. Bacterial genetic screening was performed as described before (36). Briefly, a total of 5,000 Mariner-Tn mutants were grown in BHI medium in 96-well plates overnight at 30°C. BMD macrophages from C57BL/6 mice were plated in 96-well plates; 4×10^4 macrophages per well were infected with 2×10^6 bacterial cells. Thirty minutes postinfection, macrophages were washed and gentamicin was added (50 µg/ml) to kill extracellular bacteria. At 6 h postinfection (h p.i.), macrophage culture supernatants were taken and frozen at -80°C . The amount of beta interferon (IFN- β) in the culture supernatants was detected using the type I interferon reporter cell line (ISRE-L929) (38). Reporter cells were grown in 96-well plates and incubated with a $10\times$ dilution of the macrophage culture supernatants for 4 h. Cells were lysed using Glo lysis buffer (Promega), and luciferase activity was detected using the Beetlejuice D-luciferine kit (PJK) and measured with a luminescence plate reader (Biotek Synergy HT).

***L. monocytogenes* intracellular growth in macrophages.** *L. monocytogenes* intracellular growth curves were determined as described in reference 54. Briefly, 2×10^6 cells were seeded on a 60-mm petri dish with glass coverslips. About 8×10^6 *L. monocytogenes* bacteria were used to infect the BMD macrophages. At 30 min postinfection, the macrophage monolayers were washed and fresh medium was added. At 1 h p.i., gentamicin was added to a final concentration of 5 µg/ml to limit the growth of extracellular bacteria. At each time point, three coverslips were taken and transferred into 2 ml of sterile water to release the intracellular bacteria, and then serial dilutions were plated on BHI agar plates. CFU were counted after 24 h of incubation at 37°C. Error bars represent the standard errors from the triplicate samples. All experiments were repeated at least three times. To analyze the bacterial gene expression of intracellular *L. monocytogenes*, 25×10^6 BMD macrophages were seeded on 150-mm petri dishes and were infected with 1×10^8 bacteria. At 2.5 and 6 h p.i., the macrophages were lysed in 20 ml of ice-cold water, and then the released bacteria were collected on 0.45-µm hemagglutinin (HA) filters (HAWP04700; Millipore). Bacterial RNA was purified as described below for gene expression analysis.

Construction of *L. monocytogenes* gene deletion mutants and Histagged ActA. Upstream and downstream regions of the selected genes were amplified using Phusion DNA polymerase and cloned into the pKSV7oriT vector (55) (see Table S1 in the supplemental material for primers used in this study). Cloned plasmids were sequenced and then conjugated to *L. monocytogenes* using *E. coli* SM-10. *L. monocytogenes* conjugants were grown at 30°C with chloramphenicol (Cm) and then transferred to 41°C and grown for another 2 days on BHI plates with Cm (20 µg/ml) to stabilize the plasmid in the bacterial chromosome. To cure the integrated plasmids and generate a clean in-frame deletion, bacteria were passed several times in fresh BHI medium without Cm at 30°C. Bacteria were then seeded on BHI plates, and deletion mutants were identified by screening for Cm-sensitive bacteria. Finally, the deletion genotype was validated using PCR. Since the *yidC1* complete gene deletion vector was found to be unstable in *E. coli* XL-1 Blue, the $\Delta yidC1$ mutant was generated by removing the ribosome binding site at the 5' untranslated region (UTR) and the first 378 coding nucleotides of the *yidC1* gene (LMRG_00831). To construct a chromosomal ActA-6His translational fusion, a DNA sequence encoding 6 histidines was fused in-frame at the end of the *actA* gene (LMRG_02626).

Gene expression analysis. The transcription levels of bacterial genes and macrophage genes were analyzed using quantitative real time-PCR analysis (RT-qPCR). RNA was purified from bacteria grown exponentially in BHI and from intracellularly grown bacteria at 2.5 and 6 h p.i.

using standard phenol-chloroform extraction methods. RNA of intracellularly grown bacteria was amplified using the MessageAmp II (Ambion) bacterial amplification kit according to the manufacturer's instructions. When analyzing the expression of macrophage genes, RNA of infected macrophages was extracted using TRIzol reagent according to standard protocols. In all cases, 1 µg of RNA was reverse transcribed to cDNA using a high-capacity reverse transcription kit (Applied Biosystems). RT-qPCR was performed on 10 ng of cDNA using SYBR green with the StepOnePlus RT-PCR system (Applied Biosystems) (see Table S1 in the supplemental material for RT-qPCR primers). The relative expression of bacterial genes was determined by comparing their transcription levels to that of the bacterial 16S rRNA as a reference. The transcription levels of macrophage cytokine genes were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. Statistical analysis was performed using StepOneV2.1 software. All experiments were performed on at least three independent occasions ($n = 3$). Error bars represent a 95% confidence interval, i.e., the value is expected to fall within the bar range in 95% of repeat experiments. When the error bars of two samples do not overlap, the significance of the difference (P value) is $\ll 0.01$.

Immunofluorescence microscopy and phagosomal escape assays.

The percentage of bacteria that escaped from macrophage phagosomes was determined by two immunofluorescence assays as described previously (42, 43). In the first assay, BMD macrophages were infected with wild-type (WT) *L. monocytogenes* or $\Delta secDF$ mutant bacteria that constitutively express the mCherry fluorescent protein (cloned on the integrative pPL2 plasmid) to visualize all intracellular bacteria. At 3 h p.i. the BMD macrophages were washed three times with KHM buffer (110 mM potassium acetate, 20 mM HEPES, 2 mM $MgCl_2$, pH 7.3), and the plasma membrane was selectively permeabilized by treating the cells with 50 µg/ml digitonin (Sigma) in KHM buffer for 1 min at room temperature. Cells were then washed immediately with KHM buffer, and fluorescein-conjugated anti-*L. monocytogenes* antibody (Meridian) was delivered to the macrophage cytosol for 10 min at 37°C to label accessible cytosolic bacteria. BMD macrophages were then washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde-PBS, and permeabilized with Triton X-100. Coverslips were mounted with Vectashield mounting media containing 4',6-diamidino-2-phenylindole (DAPI). Images were taken using a Nikon Eclipse Ti microscope. In the second assay, BMD macrophages were infected with WT *L. monocytogenes* or $\Delta secDF$ mutant bacteria and then fixed at 3 h p.i. with 4% paraformaldehyde-PBS. The fixed cells were permeabilized with Triton X-100 and incubated with rhodamine phalloidin (Biotium) for actin labeling and with fluorescein-conjugated anti-*L. monocytogenes* antibody (Meridian) for bacterial labeling. Coverslips were mounted with Vectashield mounting media containing DAPI. Images were taken using a Zeiss LSM 510-META confocal microscope.

Mouse infections. *L. monocytogenes* bacteria were grown in BHI medium at 30°C overnight. Bacterial cultures were washed twice in Ringer's lactate solution and counted ($\sim 2 \times 10^9$ bacteria per ml). C57BL/6 (6- to 8-week-old) female mice (Harlan Laboratories, Ltd., Israel) were infected via tail vein injections with 4×10^4 bacteria in 200 µl of PBS. Animals were observed daily for any signs of illnesses and were euthanized 72 h postinfection. Spleens and livers were harvested and homogenized in 0.2% saponin, and the numbers of viable bacteria in each organ were determined by plating serial dilutions of homogenates onto BHI agar plates. The experiment involved 6 mice in each group and was repeated twice, yielding similar results.

Analysis of LLO, PlcA, PlcB, and ActA secretion and activity. *L. monocytogenes* bacteria were grown in LB-Glu-1P medium at 37°C to an optical density (OD) of 0.4 to 0.7 for 5 to 8 h or overnight (12 h), and supernatants were separated by centrifugation. For Western blot analysis, bacteria from 20 ml culture were lysed by 1 h of incubation with mutanolysin (M9901; Sigma) at 37°C, followed by sonication. Membrane proteins were precipitated by centrifugation at 50,000 rpm for 10 min (4°C) and solubilized by 1% SDS. Cytosolic and total secreted proteins (super-

natants) were precipitated by trichloroacetic acid (TCA). Total protein content in each sample was quantified by the Lowry method. Ten µg of total protein was separated on 12.5% SDS-PAGE and probed by a standard immune-blotting technique. The hemolytic activity assay was performed as described previously (43): bacterial supernatants were treated with 5 mM dithiothreitol (DTT), serially diluted in PBS, and incubated with 0.5% sheep blood red cell suspension (NovaMed) following hemolysis at 540 nm. The PI-PLC activity assay was adapted from Geoffroy et al. (46): 1 ml of sodium-cholate (58 mM), $CaCl_2$ (10 mM), and 0.036 g phosphatidyl-inositol (P6636; Sigma) were mixed with 7 ml NaCl (0.15 M). One hundred µl of the assay solution was then mixed with 100 µl of bacterial supernatants and incubated in a plate reader at 37°C for 10 h, following turbidity assessment at 510 nm. PC-PLC activity was assayed using the EnzChek direct phospholipase C assay kit (Molecular Probes) according to the manufacturer's instructions. The fluorescent signal was recorded following overnight incubation. For activity assays on indicative agar plates, 1 µl of overnight bacterial culture was spotted on blood, agar listeria according to Ottaviani and Agosti (ALOA) or egg yolk plates, which were prepared as described previously (45) and incubated for 24 to 48 h at 37°C.

RESULTS

A genetic screen designed to identify genes that contribute to *L. monocytogenes* intracellular growth. In a genetic screen designed to identify genes that contribute to *L. monocytogenes* intracellular growth in macrophages, we identified the *secDF* gene. Briefly, we used a previously described screening approach that relies on detecting beta interferon (IFN-β) in the supernatants of *L. monocytogenes*-infected cells (36). IFN-β is a cytokine of the type I interferon innate immune response that is robustly expressed and secreted by mammalian cells upon *L. monocytogenes* infection. The expression level of IFN-β was shown to correlate with the localization of the bacteria in the cell cytosol and with their ability to further replicate within this niche (37). Based on these observations, in this screen we used IFN-β as a reporter for *L. monocytogenes* intracellular growth in macrophages. An *L. monocytogenes* *Himar-mariner1* transposon mutant library was used to infect BMD macrophages, and the levels of IFN-β secreted to the culture supernatants were detected using an ISRE-type I interferon reporter cell line, which expresses luciferase in response to type I interferons (28, 36, 38). Approximately 5,000 *L. monocytogenes* mutants were screened in a 96-well format, revealing 20 mutants to be low inducers of IFN-β compared to wild-type (WT) bacteria. Among the mutants, we identified transposon insertions within genes related to the flagella, the *prfA* gene, and genes encoding transporters, as well as metabolic and regulatory factors (Table 1). As expected, mutants in the flagella or in the *prfA* gene, the master regulator of the virulence genes, indeed induce less IFN-β production, since they are impaired in mammalian cell invasion and in intracellular replication, respectively (39, 40). Among the remaining mutants, we identified a mutant in the *secDF* gene that was particularly impaired in its ability to induce IFN-β; hence, it was chosen for further characterization.

The $\Delta secDF$ mutant is defective in intracellular growth in macrophages yet grows normally in a rich laboratory medium. To validate the observed phenotype of the *secDF::Tn* mutant, we completely deleted the *secDF* gene in the *L. monocytogenes* 10403S strain ($\Delta secDF$) (Table 2). First, the ability of the $\Delta secDF$ mutant to grow in the laboratory rich medium BHI at both 37 and 15°C was examined. Interestingly, we found that unlike the phenotype observed in *B. subtilis* and *S. aureus*, the *L. monocytogenes* $\Delta secDF$ mutant grew similarly to WT bacteria at both temperatures (Fig.

TABLE 1 Identification of *L. monocytogenes* mutants that exhibit low IFN- β response during macrophage infection

<i>L. monocytogenes</i> 10403S gene	<i>L. monocytogenes</i> EGDe gene identifier	Description	INF- β fold of induction mutant/WT bacteria
LMRG_02622.6	<i>lmo0200</i>	PrfA main virulence regulator of <i>L. monocytogenes</i>	0.159
LMRG_00367.6	<i>lmo0679</i>	Flagellar biosynthesis protein FlhB	0.383
LMRG_00376.6	<i>lmo0688</i>	Similar to glycosyl-transferase, group 2 family protein	0.720
LMRG_00380.6	<i>lmo0692</i>	Two-component sensor histidine kinase CheA	0.486
LMRG_00386.6	<i>lmo0697</i>	Similar to flagellar hook protein FlgE	0.282
LMRG_00395.6	<i>lmo0706</i>	Similar to flagellar hook-associated protein 3, FlgL	0.445
LMRG_0397.6	<i>lmo0708</i>	Similar to flagellar biosynthesis protein FliS	0.561
LMRG_00402.6	<i>lmo0713</i>	Flagellar basal-body M-ring protein FliS	0.594
LMRG_00413.6	<i>lmo0724</i>	Similar to <i>B. subtilis</i> YvpB protein	0.742
LMRG_02270.6	<i>lmo0847</i>	Glutamine transporter	0.493
~20bp upstream LMRG_00534.6	~20bp upstream <i>lmo1072</i>	Highly similar to pyruvate carboxylase	0.331
LMRG_01443.6	<i>lmo1527</i>	Similar to protein-export membrane protein SecDF	0.354
LMRG_02761.6	<i>lmo1687</i>	Hypothetical protein	0.725
LMRG_02767.6	<i>lmo1693</i>	Regulatory protein RecX	0.830
LMRG_01002.6	<i>lmo1855</i>	D-alanyl-D-alanine carboxypeptidase	0.814
LMRG_01003.6	<i>lmo1856</i>	Purine nucleoside phosphorylase	0.818
LMRG_01264.6	<i>lmo2110</i>	Similar to mannose-6 phosphate isomerase	0.707
LMRG_1717.6	<i>lmo2531</i>	ATP synthase F1 alpha subunit	0.417
LMRG_01981.6	<i>lmo2715</i>	ABC transporter CydDC cysteine exporter CydC	0.840
LMRG_01787.6 and LMRG_01360.6	<i>lmo2461</i> and <i>lmo1606</i>	RNA polymerase sigma-54 factor (sigma-L) and DNA segregation ATPase FtsK/SpoIIIE	0.506

1A and B). Of note, the $\Delta secDF$ mutant did not exhibit a rough colony phenotype or filamentous cells, as has been shown for other Sec-related mutants of *L. monocytogenes* (24, 41). The induction of IFN- β and two additional proinflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α), next was measured upon infection of macrophages with $\Delta secDF$ mutant versus WT bacteria (at a multiplicity of infection [MOI] of 1) using real-time quantitative PCR (RT-qPCR) analysis at 6 h p.i. As shown in Fig. 1C, the transcription levels of the three cytokines were greatly reduced in the $\Delta secDF$ -infected cells compared to the cells infected with WT bacteria. To test whether the reduced cytokine response is a result of impaired intracellular growth of the $\Delta secDF$ mutant, we monitored its replication in BMD macrophages. Notably, we found that the $\Delta secDF$ mutant is severely defective in intracellular growth compared to WT bacteria and a

$\Delta secDF$ -complemented strain, harboring a copy of the *secDF* gene on an integrative plasmid (pLIV2-*secDF*) (Fig. 1D). Taken together, these results demonstrated that SecDF is dispensable during extracellular growth under the tested laboratory conditions, but it is required early during *L. monocytogenes* intracellular growth in macrophages.

The *secDF* gene is upregulated when bacteria reside in macrophage phagosomes and contributes to phagosomal escape. Since the $\Delta secDF$ mutant did not grow intracellularly in macrophages, we asked whether it is impaired in the ability to escape the macrophage phagosomes or to spread from cell to cell, critical steps in *L. monocytogenes* infection. To this end, we performed two fluorescence microscopy visualization assays, which are used to assess bacterial phagosomal escape and binding of host actin filaments (42, 43). The first fluorescence assay relies on the selective

TABLE 2 List of strains and mutants used in this study

Name	LMRG gene/ <i>L. monocytogenes</i> EGDe gene identifier	Description and/or source
WT		<i>L. monocytogenes</i> 10403S, parental strain used in this study; Daniel A. Portnoy, laboratory stock
Δhly	LMRG_02076/ <i>lmo0202</i>	Deletion of <i>hly</i> gene encoding LLO DP-L2161; 6
$\Delta plcAB$	LMRG_02627/ <i>lmo0205</i> LMRG_02623/ <i>lmo0201</i>	Deletion of <i>plcA</i> and <i>plcB</i> genes DP-L1936; 8
$\Delta secDF$	LMRG_01443/ <i>lmo1527</i>	Deletion of <i>secDF</i> gene, AH-L101; this study
$\Delta secDF$ + pLIV2- <i>secDF</i>		$\Delta secDF$ mutant harboring the integrative plasmid pLIV2 encoding the <i>secDF</i> gene under inducible promoter, AH-L102; this study
$\Delta yidC1$	LMRG_00831/ <i>lmo1379</i>	Deletion of <i>yidC1</i> gene, AH-L103; this study
$\Delta secA2$	LMRG_00265/ <i>lmo0583</i>	Deletion of <i>secA2</i> gene, DP-L4342; 24
WT <i>actA</i> -6his-tag	LMRG_02626/ <i>lmo0204</i>	<i>L. monocytogenes</i> 10403S, encoding 6 histidines at the 3' end of <i>actA</i> gene (chromosomally), AH-L104; this study
WT + pPL2- <i>mCherry</i>		<i>L. monocytogenes</i> 10403S harboring the integrative plasmid pPL2 carrying <i>mCherry</i> gene under a constitutive promoter; this study
$\Delta secDF$ + pPL2- <i>mCherry</i>		$\Delta secDF$ mutant harboring the integrative plasmid pPL2 carrying <i>mCherry</i> gene under a constitutive promoter; this study

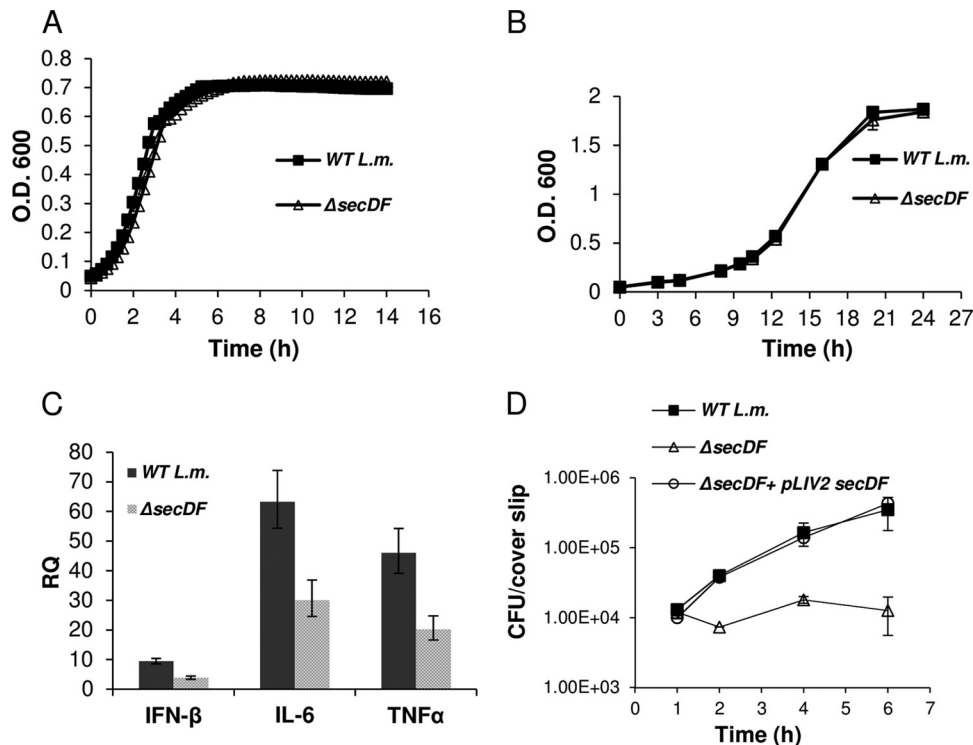


FIG 1 $\Delta secDF$ mutant shows restricted intracellular growth and a decreased cytokine response upon macrophage infection. (A) Growth curves of WT *L. monocytogenes* and the $\Delta secDF$ mutant grown in BHI laboratory medium at 37°C. Representative growth curves are shown. Error bars represent the standard deviations from triplicate trials and are hidden by the symbols. Growth curves were performed in 3 biological repeats ($n = 3$). (B) Growth curves of WT *L. monocytogenes* and $\Delta secDF$ mutant grown in BHI laboratory medium at 15°C. Data shown represent the means from 3 biological repeats ($n = 3$). Error bars represent the standard deviations. O.D. 600, optical density at 600 nm. (C) RT-qPCR analysis of the IFN- β , IL-6, and TNF- α transcription levels in BMD macrophages infected with WT *L. monocytogenes* or the $\Delta secDF$ mutant (MOI of 1) at 6 h p.i. Transcription levels are represented as the relative quantity (RQ) relative to the levels in uninfected cells. The data represent 3 biological repeats ($n = 3$). Error bars represent a 95% confidence interval, i.e., $P \ll 0.01$. (D) Intracellular growth curves of the WT *L. monocytogenes*, $\Delta secDF$ mutant, and $\Delta secDF$ complemented strain harboring pLIV2-*secDF* plasmid grown in BMD macrophages. Error bars represent the standard deviations from triplicate trials. The data represent 3 independent biological repeats ($n = 3$).

permeabilization of the plasma membrane, which allows the detection of cytosolic bacteria, and not of phagosomally trapped bacteria, using antibodies. Briefly, BMD macrophages were infected with mCherry-expressing $\Delta secDF$ mutant or WT bacteria (Table 2) and gently treated with digitonin at 3 h p.i. to selectively permeabilize the plasma membrane. Fluorescein-conjugated anti-listerial antibody then was added to label accessible cytosolic bacteria. As shown in Fig. 2A, at 3 h p.i. most of the WT bacteria were labeled with mCherry and fluorescein in the macrophage cytosol (green/yellow), whereas more of the $\Delta secDF$ bacteria were labeled solely with mCherry (red), indicating that the $\Delta secDF$ bacteria are impaired in phagosomal escape. Quantifying the percentage of bacteria labeled with mCherry and fluorescein as opposed to mCherry alone confirmed that the proportions of bacteria that escaped the phagosomes were 80 and 50% for WT and $\Delta secDF$ bacteria, respectively (Fig. 2A). The second fluorescence assay relies on the observation that *L. monocytogenes* recruits host actin filaments in the cytosol, whereas bacteria in phagosomes do not. Briefly, BMD macrophages were infected with $\Delta secDF$ mutant or WT bacteria, fixed at 3 h p.i., and permeabilized with Triton X-100 (permeabilizing both plasma and phagosomal membranes). Cells were stained with rhodamine phalloidin (red) for host actin labeling, with fluorescein-conjugated anti-listerial antibody (green) for bacterial visualization and DAPI (blue) for host nuclear staining. As shown in Fig. 2B, at 3 h p.i. most of the WT bacteria were

associated with actin filaments in the macrophage cytosol (red/yellow), whereas the majority of the $\Delta secDF$ mutant bacteria were labeled solely with fluorescein (green), suggesting that most of the $\Delta secDF$ mutant bacteria are still located in the phagosomes and/or impaired in actin filament recruitment. Quantifying the percentage of bacteria labeled with rhodamine phalloidin and fluorescein as opposed to fluorescein alone confirmed that 80% of WT bacteria versus 36% of $\Delta secDF$ mutant bacteria were engaged with actin filaments at 3 h p.i. (Fig. 2B). Together, these assays indicated that the $\Delta secDF$ mutant is mostly impaired in phagosomal escape and perhaps in actin binding as well.

To gain further insight into the importance of SecDF during infection, we analyzed the transcription levels of the *secDF* gene and additional genes of the Sec system in both intracellularly grown (WT) and phagosomally trapped (Δhly mutant) bacteria and compared them to levels for WT bacteria grown extracellularly in BHI medium. We observed that the transcription levels of the Sec system genes, e.g., *secY*, *secG*, *secDF*, *secA1*, *secA2*, *yajC*, *yidC1*, and *yidC2*, generally were lower during intracellular growth (6 h p.i.) than during exponential growth in BHI medium (Fig. 2C). Exceptional in this regard was the *secA2* gene, which showed similar transcription levels under both conditions (Fig. 2C). Notably, this transcription profile was largely altered when the bacteria were located in phagosomes (i.e., in the Δhly mutant). Here, we found that *secDF*, *secA2*, and *yidC1* in particular were

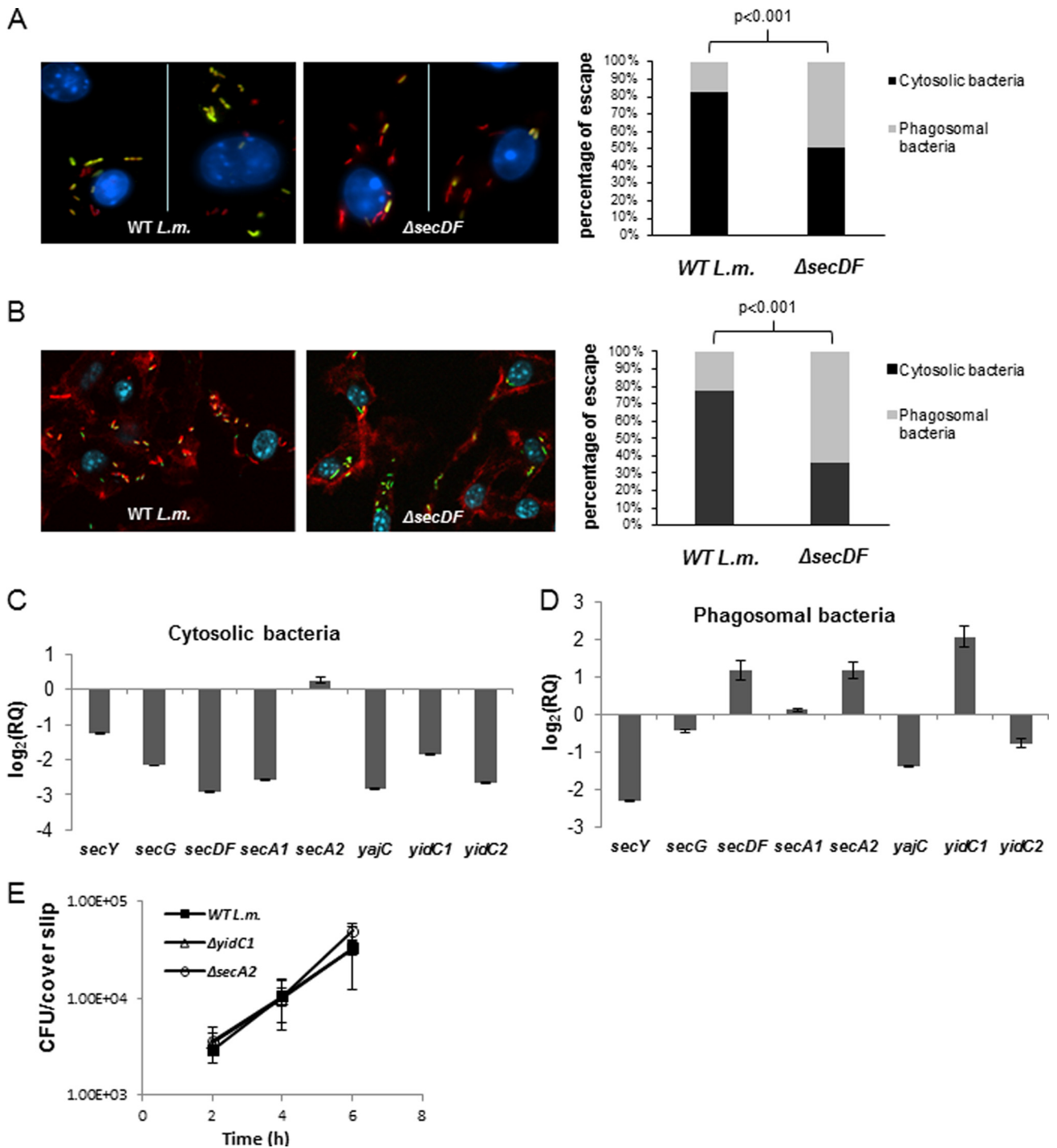


FIG 2 SecDF contributes to phagosomal escape. (A, left) Confocal fluorescence microscope images of digitonin-treated BMD macrophages infected with mCherry expressing WT *L. monocytogenes* or Δ secDF bacteria at 3 h p.i. Cytosolic bacteria were labeled with fluorescein-conjugated anti-*Listeria* antibody (green), and macrophage nuclei were labeled with DAPI (blue). (Right) percentage of bacteria that escaped macrophage phagosomes at 3 h p.i. as determined by a microscopic fluorescence assay based on selective digitonin membrane permeabilization. The results are representative of 10 microscopic images from 2 independent biological repeats for each strain (in each repeat between 300 and 400 bacteria were counted). The *P* value was calculated using a chi-square test. (B, left) Confocal fluorescence microscope images of BMD macrophages infected with WT *L. monocytogenes* or Δ secDF mutant bacteria at 3 h p.i. Bacteria were labeled with fluorescein-conjugated anti-*Listeria* antibody (green), macrophage nuclei were labeled with DAPI (blue), and macrophage actin filaments were labeled with rhodamine phalloidin (red). (Right) Percentage of bacteria that escaped macrophage phagosomes at 3 h p.i. as determined by a microscopic fluorescence assay based on actin labeling. The results are representative of 10 microscopic images from 3 independent biological repeats for each strain (in each image, between 50 and 100 bacteria were counted). The *P* value was calculated using a chi-square test. (C) RT-qPCR transcription analysis of different sec-related genes in intracellularly grown (cytosolic) bacteria (WT *L. monocytogenes*) in macrophages at 6 h p.i. Transcription levels were measured as relative quantity (RQ), i.e., relative to their levels in bacteria grown exponentially in BHI laboratory medium. Data are presented as \log_2 values of the RQ. The data represent 3 biological repeats ($n = 3$). Error bars represent a 95% confidence interval. (D) RT-qPCR transcription analysis of different sec-related genes in phagosomally trapped bacteria (Δ hly mutant) in macrophages at 2.5 h p.i. Transcription levels were measured as the RQ relative to their levels in bacteria grown exponentially in BHI laboratory medium. Data are presented as \log_2 values of RQ. The data represent 3 biological repeats ($n = 3$). Error bars represent a 95% confidence interval, i.e., $P \ll 0.01$. (E) Intracellular growth curves of WT *L. monocytogenes* and the Δ yidC1 and Δ secA2 mutants in BMD macrophages. Error bars represent the standard deviations from triplicate trials. The data represent 3 independent biological repeats ($n = 3$).

transcribed at a high level inside the phagosomes compared to their levels during extracellular bacterial growth in BHI medium (Fig. 2D). To assess whether SecA2 and YidC1 also contribute to *L. monocytogenes* intracellular growth like SecDF, $\Delta secA2$ and $\Delta yidC1$ mutants (Table 2) were grown in BMD macrophages and compared to WT bacteria. Remarkably, we found that although these components were highly induced inside the phagosome they were dispensable, since the $\Delta secA2$ and $\Delta yidC1$ mutants grew like WT bacteria in the macrophages (Fig. 2E). Taken together, these observations suggested a role for SecDF early within the phagosome compartment, possibly in the secretion of virulence factors that contribute to *L. monocytogenes* phagosomal escape and intracellular growth.

SecDF contributes to the proper translocation and activity of LLO, PlcA, PlcB, and ActA. To determine whether SecDF contributes to membrane translocation of the critical virulence factors that mediate phagosomal escape and spread from cell to cell, i.e., LLO, PlcA, PlcB, and ActA, we compared the subcellular distribution of these proteins within $\Delta secDF$ and WT bacteria. Briefly, $\Delta secDF$ mutant and WT bacteria were grown in LB-MOPS-glucose 1P charcoal medium (LB-Glu-1P), which is known to activate the production and secretion of *L. monocytogenes* virulence factors (44). This activation specifically occurs when the bacteria switch from utilizing glucose to glucose 1-phosphate. At different time points during growth, bacteria were harvested and the bacterial membranes, cytosol, and supernatants fractionated and subjected to semiquantitative Western blot analysis using specific antibodies to LLO, PlcA, PlcB, 6His-tagged ActA, and GroEL, with the latter serving as a control protein. Notably, under these growth conditions the $\Delta secDF$ mutant grew better than WT bacteria in a reproducible manner (Fig. 3A). Initially, the distribution of LLO preproteins was examined after 5.5 and 7 h (Fig. 3A, arrows), at which time the production of virulence factors is known to occur (44). As shown in Fig. 3B, at both time points LLO preproteins were observed to accumulate in the cytosol and on the membrane of the $\Delta secDF$ mutant, unlike WT bacteria, whereas the levels of GroEL remained constant. Notably, despite the observed accumulation of LLO preproteins, the levels of secreted LLO in the supernatants of $\Delta secDF$ mutant and WT bacteria were comparable. Similar results were observed with PlcA, PlcB, and ActA preproteins (Fig. 3C and D). This accumulation of preproteins on the membrane suggests that in the absence of SecDF their translocation is delayed, presumably within the translocons, leading to the observed accumulation in the cytosol. Accumulation of preprotein precursors on the membrane could be expected to stimulate stress responses that suspend growth. However, GroEL levels were comparable in WT and $\Delta secDF$ mutant bacteria, as assessed by Western blot analysis (Fig. 3B to D), indicating the absence of a stress response, since GroEL is a chaperone typically upregulated under protein-related stresses. Currently it remains unclear why the $\Delta secDF$ mutant grows better than WT bacteria under these conditions. The possibility that the bacteria form chains or long filaments that can affect OD measurements was excluded.

Since the Western blot analysis demonstrated similar levels of secreted proteins in the supernatants of $\Delta secDF$ mutant and WT bacteria, we examined the possibility that the activity of the secreted proteins is impaired in the $\Delta secDF$ mutant. To this end, assays for LLO, PlcA, and PlcB activity were performed using indicative agar plates. $\Delta secDF$ and WT bacteria were spotted on

blood, ALOA, and egg yolk plates, where a zone of hemolysis or opacity is indicative of LLO, PlcA, and PlcB activity, respectively, upon secretion to the agar medium. As controls, the Δhly (LLO⁻) and $\Delta plcAB$ mutants were used. As shown in Fig. 4A, the $\Delta secDF$ mutant exhibited decreased LLO activity, as manifested by a reduced hemolysis zone surrounding the bacterial patch on the blood plate compared to the zone observed with WT bacteria (25% reduction in diameter). Similarly, it also exhibited reduced PlcA and PlcB phospholipase activity (lecithinase activity) on the ALOA and egg yolk plates, as manifested by the smaller white precipitate zones (30% reduction in diameter) (45). These results confirmed that the virulence factors secreted by the $\Delta secDF$ mutant indeed exhibit less activity. To measure directly the activity of LLO, PlcA, and PlcB in the supernatants of $\Delta secDF$ mutant and WT bacteria grown in LB-Glu-1P medium, quantitative activity assays were performed for each of the proteins. Supernatants were collected and subjected to a hemolysis assay (43), a phosphatidylinositol assay (46), and a phospholipase C assay (Molecular Probes) that measures LLO, PlcA, and PlcB activity, respectively. Similar to what we observed on the indicative agar plates, the different assays evidenced that LLO, PlcA, and PlcB secreted by the $\Delta secDF$ mutant exhibit lower activity levels (Fig. 4B to D). Given that $\Delta secDF$ mutant bacteria secrete normal levels of LLO, PlcA, and PlcB to the supernatant and yet a substantial portion of the secreted proteins is nonactive (~30%), we conclude that SecDF plays an important chaperone role, promoting the translocation of the virulence factors in a way that facilitates their secretion in a fully active state.

SecDF contributes to *L. monocytogenes* virulence in mice. Finally, we examined the contribution of SecDF to *L. monocytogenes* virulence in mice. C57BL/6 young female mice were injected intravenously with 4×10^4 of $\Delta secDF$ mutant or WT bacteria, and bacterial counts in the spleens and livers of the infected mice were analyzed at 72 h p.i. (6 mice in each group). As shown in Fig. 5, the $\Delta secDF$ mutant colonized the livers and spleens of the infected mice to a lesser extent, exhibiting an ~30-fold decrease in CFU recovered from both organs compared to WT bacteria. These results clearly demonstrate that SecDF is necessary to promote *L. monocytogenes* virulence.

DISCUSSION

In this study, we demonstrate that the *L. monocytogenes* SecDF protein is not required for growth, as reported for other Gram-positive strains. Nevertheless, we reveal that it possesses an auxiliary role in facilitating the translocation of several virulence factors, LLO, PlcA, PlcB, and ActA, which are key to *L. monocytogenes* phagosomal escape and spread in mammalian cells. In the absence of SecDF, precursors of these preproteins accumulate on the bacterial membrane and within the cytosol, in line with impairment in their translocation process. Although reduced secretion levels were not detected, roughly 30% of the secreted proteins were found to be inactive. In line with these findings, the $\Delta secDF$ mutant exhibited a reduced ability to escape macrophage phagosomes and grew less efficiently intracellularly *in vitro* and *in vivo* in mice. This study not only highlights SecDF as an important determinant of *L. monocytogenes* virulence but also provides important insights into the role of SecDF in protein secretion.

Until recently, the role of SecDF in protein export was not well defined. While it was found to be essential for protein secretion *in*

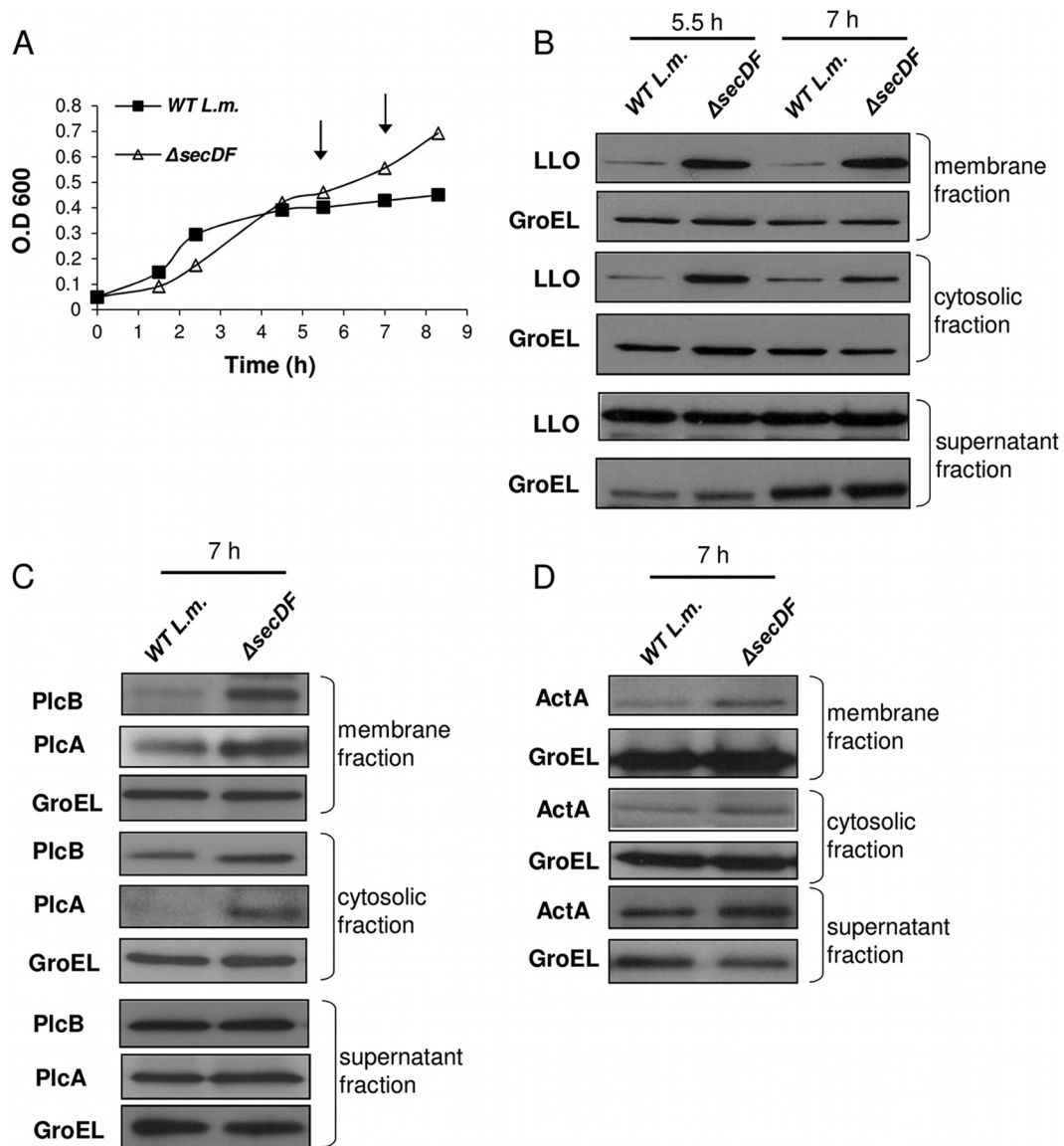


FIG 3 SecDF promotes LLO, PlcA, PlcB, and ActA membrane translocation. (A) Growth curves of WT *L. monocytogenes* and the $\Delta secDF$ mutant in LB-Glu-1P media. Data are representative of 5 biological experiments ($n = 5$). (B) Western blot analysis of LLO preproteins' subcellular distribution within the membrane, cytosol, and supernatant fractions of WT *L. monocytogenes* and the $\Delta secDF$ mutant grown in LB-Glu-1P media using anti-LLO antibodies. GroEL was used as a control protein. The data are representative of three biological repeats ($n = 3$). (C) Western blot analysis of PlcA and PlcB preproteins' subcellular distribution in the membrane, cytosol, and supernatant fractions of WT *L. monocytogenes* and $\Delta secDF$ mutant grown in LB-Glu-1P media using specific antibodies. GroEL was used as a control protein. The data are representative of three biological repeats ($n = 3$). (D) Western blot analysis of 6His-tagged ActA preprotein subcellular distribution in the membrane, cytosol, and supernatant fractions of WT *L. monocytogenes* and the $\Delta secDF$ mutant grown in LB-Glu-1P media using anti-His tag antibodies. GroEL was used as a control protein. The data are representative of three biological repeats ($n = 3$).

in vivo (in *E. coli*), it was largely dispensable during *in vitro* Sec-reconstituted translocation experiments (31). Recent crystal structures of SecDF and *in vitro* structure-function studies have provided novel insights concerning the role of this protein as a membrane chaperone. These studies demonstrated that during translocation the emerging preprotein is captured by the extracellular P1 domain of SecDF, which then rotates and moves 75 Å apart, to further pull out another ~25 amino acids of the unfolded preprotein (19). This action, presumed to occur repetitively until the full protein is translocated, was hypothesized to enhance the translocation process by preventing a backward movement of the

preprotein. In the present study, we provide *in vivo* observations to support this premise. The accumulation of virulence factor preproteins on the membrane (and in the cytosol) clearly reflects improper translocation and inability to cross the membrane efficiently, perhaps due to sliding backwards through the translocon channel. Such a forward and backward movement, in the absence of SecDF, could be expected to interfere with proper folding of the secreted proteins and explain the observed reduction in the factors' activities. In summary, our results support the premise that SecDF is an accessory chaperone that facilitates the translocation and proper folding of secretory proteins.

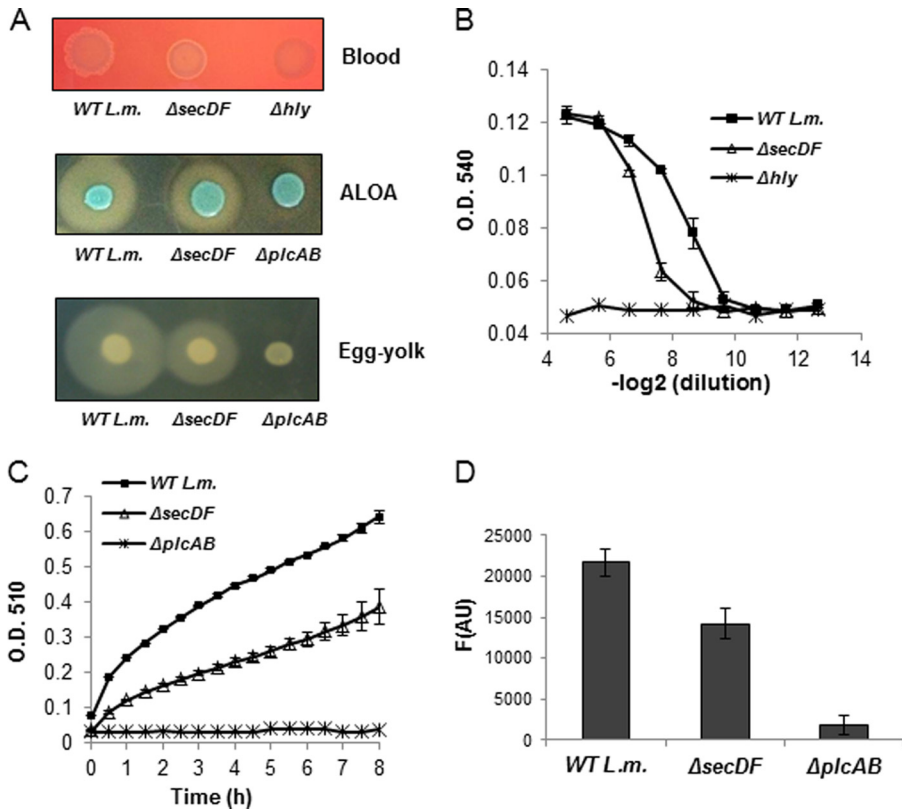


FIG 4 $\Delta secDF$ mutant exhibits low activity of LLO, PlcA, and PlcB. (A) Activity assays of LLO, PlcA, and PlcB in WT *L. monocytogenes* and the $\Delta secDF$ mutant grown on blood, ALOA, and egg yolk indicative agar plates, respectively. The halo (clear or opaque) zone around the bacterial patch is proportional to the LLO, PlcA, and PlcB activity. The pictures shown are representative of 3 independent experiments ($n = 3$). (B) LLO activity hemolysis assay based on lysis of sheep red blood cells. LLO activity was measured in the supernatants of WT *L. monocytogenes* and the $\Delta secDF$ mutant grown overnight in LB-Glu-1P media. The Δhly mutant was used as a control. The data are representative of three biological repeats ($n = 3$). The error bars represent standard deviations from triplicate trials. (C) PI-PLC (PlcA) activity assay, measuring reaction turbidity (adapted from Geoffroy et al. [46]). PI-PLC activity was measured in supernatants of WT *L. monocytogenes* and $\Delta secDF$ mutant grown overnight in LB-Glu-1P media. The $\Delta plcAB$ mutant was used as a control. The data are representative of three biological repeats ($n = 3$). Error bars represent the standard deviations from triplicate trials. (D) A measurement of PC-PLC (PlcB) activity using the EnzChek direct phospholipase C assay kit (Molecular Probes). PC-PLC activity was measured in supernatants of WT *L. monocytogenes* and the $\Delta secDF$ mutant grown overnight in LB-Glu-1P media. The $\Delta plcAB$ mutant was used as a control. The data are representative of three biological repeats ($n = 3$). Error bars represent the standard deviations from triplicate trials.

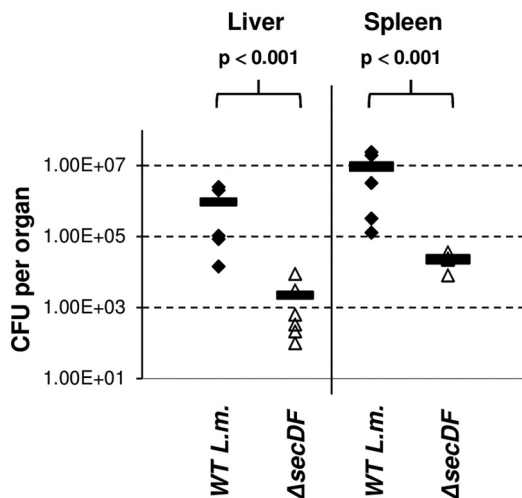


FIG 5 $\Delta secDF$ mutant is less virulent in mice. Intravenous infection of C57BL/6 female mice with WT *L. monocytogenes* and $\Delta secDF$ mutant bacteria. Bacterial CFU were enumerated at 72 h p.i. from livers and spleens taken from 6 infected mice per group. Horizontal bars represent the means. The P value was calculated using a t test.

In this study, we identified a requirement for SecDF only during *L. monocytogenes* growth in mammalian cells. This finding is in accordance with prior observations that SecDF is particularly important under conditions of hypersecretion (22). Indeed, during infection *L. monocytogenes* produces and secretes many secretory and surface-anchored proteins that mediate invasion, phagosomal escape, intracellular replication, and spread from cell to cell (15, 40). Notably, most of these virulence factors are coregulated by the master virulence activator, PrfA, which, in response to various host signals, such as temperature and availability of nutrients, becomes activated and coordinates upregulated expression of the virulence factors (44, 47, 48). It is generally thought that *L. monocytogenes* initiates the secretion of most virulence factors during phagocytosis, which could explain the upregulation of SecDF within the phagosome. Although in this report we demonstrated the effect of *secDF* deletion mainly on factors that mediate phagosomal escape, we predict that the secretion of additional virulence factors is also affected, including factors that promote intracellular growth. Our observation that the $\Delta secDF$ mutant does not grow well intracellularly, although 30 to 50% of the bacteria succeed to escape to the macrophage cytosol, largely supports this notion.

Taken together, the data link the secretion of *L. monocytogenes* virulence factors to the Sec system and evidence the importance of this system in *L. monocytogenes* pathogenesis (24). Of note, the possibility that SecDF assembles with other secretion systems has not been ruled out.

Lastly, in this report we identified differential expression of the various Sec system components during *L. monocytogenes* growth in macrophages. Overall the core translocon (SecYEG) was moderately downregulated, while some accessory proteins were upregulated (e.g., SecDF and SecA2). These observations suggest that the bacteria rearrange translocon composition and/or stoichiometry in order to adapt to the hypersecretion state during infection. In this regard it was shown recently that the translocon stoichiometry varies upon translocation of different precursors, for example, outer membrane versus periplasmic protein (49). In light of these findings and the numerous reports that the SecYEG translocon can be found in various types of oligomers (50–52), it is tempting to speculate that bacteria modify the composition and/or the stoichiometry of the translocon apparatuses as a mechanism to cope with different secretion/translocation requirements. Such a strategy could enable *L. monocytogenes* to manage the enhanced secretion of virulence and housekeeping proteins during infection. Further study is required to characterize the Sec translocon during *L. monocytogenes* infection and the mechanisms that control its expression and assembly.

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