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The Posttranslocation Chaperone PrsA2 Contributes to Multiple Facets of *Listeria monocytogenes* Pathogenesis▽†

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***Listeria monocytogenes* is an intracellular bacterial pathogen whose virulence depends on the regulated expression of numerous secreted bacterial factors. As for other gram-positive bacteria, many proteins secreted by *L. monocytogenes* are translocated across the bacterial membrane in an unfolded state to the compartment existing between the membrane and the cell wall. This compartment presents a challenging environment for protein folding due to its high density of negative charge, high concentrations of cations, and low pH. We recently identified PrsA2 as a gene product required for *L. monocytogenes* virulence. PrsA2 was identified based on its increased secretion by strains containing a mutationally activated form of *prfA*, the key regulator of *L. monocytogenes* virulence gene expression. The *prsA2* gene product is one of at least two predicted peptidyl-prolyl *cis*/*trans*-isomerases encoded by *L. monocytogenes*; these proteins function as posttranslocation protein chaperones and/or foldases. In this study, we demonstrate that PrsA2 plays a unique and important role in *L. monocytogenes* pathogenesis by promoting the activity and stability of at least two critical secreted virulence factors: listeriolysin O (LLO) and a broad-specificity phospholipase. Loss of PrsA2 activity severely attenuated virulence in mice and impaired bacterial cell-to-cell spread in host cells. In contrast, mutants lacking *prsA1* resembled wild-type bacteria with respect to intracellular growth and cell-to-cell spread as well as virulence in mice. PrsA2 is thus distinct from PrsA1 in its unique requirement for the stability and full activity of *L. monocytogenes*-secreted factors that contribute to host infection.**

Listeria monocytogenes is a gram-positive, food-borne facultative intracellular bacterial pathogen that is capable of crossing the intestinal epithelial barrier of susceptible humans to cause serious and life-threatening infections (65). While healthy individuals primarily exhibit mild forms of disease such as gastroenteritis, immunocompromised and elderly individuals often suffer more severe forms of illness that include meningoencephalitis and septicemia (42, 71). Central to its ability to cause serious infections is the bacterium's capacity to gain access to its protected replication niche within the cytosol of infected host cells (12, 71). Bacterial mutants that fail to establish and exploit this replication niche are severely attenuated in host infection models (10, 19, 48, 50, 57, 58, 80).

A number of secreted bacterial factors that contribute to *L. monocytogenes* cell invasion, entry into the cytosol, intracellular bacterial replication, and cell-to-cell spread have been described (71). These factors include the internalins InlA and InlB, which mediate entry into nonprofessional phagocytic cells, and listeriolysin O (LLO; encoded by *hly*), which is required for bacterial escape from host cell vacuoles into the cytosol (17, 18, 20, 33, 37, 48). Two phospholipases, a phosphatidylinositol-specific phospholipase (PI-PLC; encoded by *plcA*) and a broad-specificity phospholipase (PC-PLC; encoded by *plcB*) also contribute to vacuole lysis (21, 41, 61, 70). A bacterial surface protein known as ActA is required for bacterial actin-based motility and *L. monocytogenes* spread to adjacent cells (11, 29, 30, 68). The expression of these secreted gene products is regulated by a transcriptional activator known as PrfA (16, 35, 43). PrfA exists in a low activity state in bacteria grown outside of host cells; upon bacterial entry into the cytosol of infected cells, PrfA becomes activated and induces the expression of the gene products required for intracellular bacterial replication and cell-to-cell spread (54). PrfA thus serves a critical role in regulating the expression of *L. monocytogenes* virulence gene products within the appropriate cellular location.

We recently identified a secreted protein, PrsA2, based on its abundant expression by bacteria containing *prfA* mutations that result in the constitutive expression of PrfA-dependent gene products (*prfA** mutations) (47). Insertional disruption of lmo2219, which encodes PrsA2, was found to significantly reduce *L. monocytogenes* virulence in mice (47). PrsA2 had previously been identified by Chatterjee et al. (7) and was found to contribute to *L. monocytogenes* intracellular growth and/or cell-to-cell spread in tissue culture cells. PrsA2 is one of two predicted peptidyl-prolyl *cis*/*trans*-isomerases of the Parvulin family encoded within the *L. monocytogenes* genome (the other is encoded by *prsA1*, or lmo1444); both share significant homology with the single PrsA protein produced by *Bacillus subtilis* (Fig. 1A). In *B. subtilis*, *prsA* is an essential gene that assists in the secretion, folding, and sequestration of a number of secreted proteins (69, 73–75).

In this work, we demonstrate that PrsA2 plays a unique and

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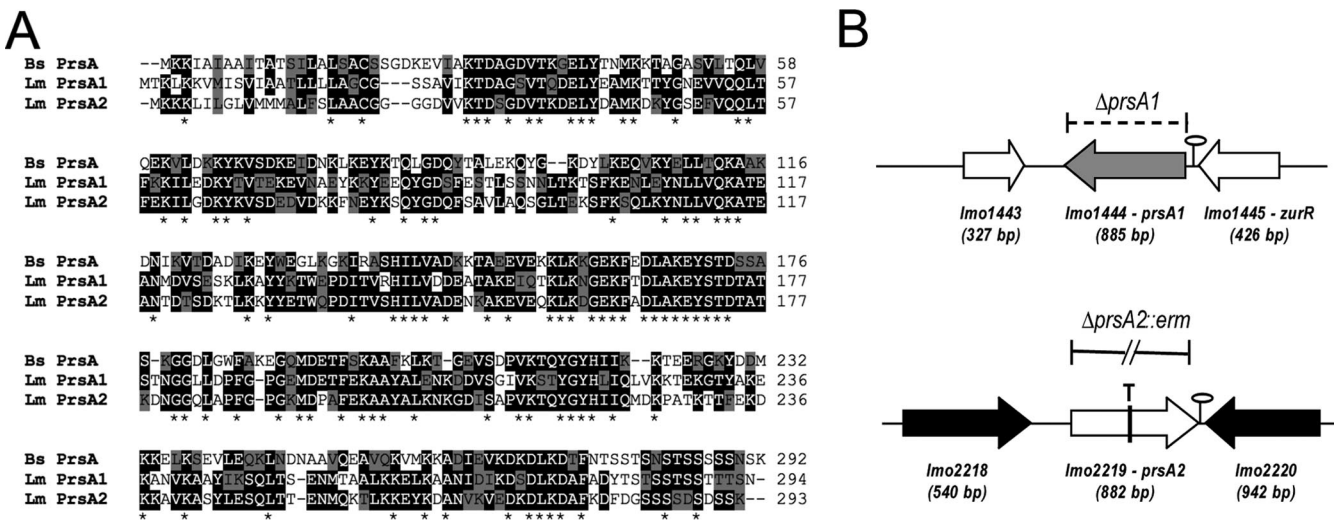

FIG. 1. Amino acid sequence alignment and construction of *prsA1* and *prsA2* loss-of-function mutants. (A) Amino acid sequence alignment of *B. subtilis* PrsA and the two PrsA homologues in *L. monocytogenes* (PrsA1 and PrsA2) using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/index.html). Amino acid residues that are identical between at least two of the three PrsA proteins are shaded in black; asterisks indicate amino acid residues that are identical in all three proteins. Residues that are similar between protein sequences are shaded in gray. (B) Schematic of the Δ*prsA1* deletion mutant, the *prsA2*::T targetron insertion mutant, and the Δ*prsA2*::*erm* mutant. The gray and white center arrows denote the open reading frames of *prsA1* and *prsA2*, respectively, while the white and black flanking arrows denote flanking gene sequences. Predicted transcriptional terminators are depicted by stem-loops. The dashed bracket indicates the deletion of the *prsA1* open reading frame, the vertical line through the lmo2219 open reading frame and T denote the intron insertion at nucleotide 457 in *prsA2*, and the solid broken line designates the Δ*prsA2*::*erm* deletion.

distinguishable role from PrsA1 by promoting the activity and stability of *L. monocytogenes*-secreted factors critical for bacterial virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains used in this study are listed in Table 1. *L. monocytogenes* 10403S containing an *actA-gus* transcriptional fusion (NF-L476) was used as the parent strain for genetic manipulations unless otherwise specified (60). *Escherichia coli* alpha select (BioLine, Boston, MA), One Shot TOP10 (Invitrogen, Carlsbad, CA), and SM10 were used as host strains for recombinant plasmids. All strains were grown in Luria broth (LB) (Invitrogen Corp., Carlsbad, CA) or brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) and supplemented with the appropriate antibiotic. Antibiotics were used at the following concentrations unless otherwise noted: ampicillin, 100 μg/ml; carbenicillin, 50 μg/ml; chloramphenicol, 10 and 7.5 μg/ml; erythromycin, 5 μg/ml; and streptomycin, 200 μg/ml. All *L. monocytogenes* strains were grown overnight at 37°C without agitation prior to in vitro and in vivo assays. The temperature-sensitive shuttle plasmid pKSV7 (62) was used for generation of *L. monocytogenes* mutants via allelic exchange, and the integration plasmid pPL2 (34) was used for genetic complementation.

Construction of the Δ*prsA1* in-frame deletion mutant. A 789-bp internal in-frame deletion was generated in lmo1444 as follows: initially two DNA fragments were generated by PCR with primer pairs Marq 161/162 and Marq 163/164 using *L. monocytogenes* 10403S chromosomal DNA as the template. (All oligonucleotides are listed in Table S1 in the supplemental material.) The two fragments were purified and used in a splicing-by-overlap extension (SOE) PCR with primer pair Marq 161/164, generating a 1,250-bp fragment encompassing the 5′ end of lmo1444 with upstream sequence and the 3′ end of the same gene with downstream sequence. The 1,250-bp fragment was cloned into the shuttle vector pKSV7 using KpnI and PstI restriction sites, generating pMC10. *L. monocytogenes* strain 10403S was electroporated with pMC10 and the Δ*prsA1* mutation was introduced into the bacterial chromosome in single copy by allelic exchange as previously described (6) to generate strain HEL-402. Chromosomal deletion of a 789-bp fragment within lmo1444 (*prsA1*) was confirmed by PCR. This deletion encompasses 90% of the structural gene.

Construction of the *prsA2* targetron and Δ*prsA2*::*erm* mutants. Repeated attempts to generate a Δ*prsA2* in-frame deletion mutant using standard approaches based on homologous recombination (as described above for Δ*prsA1*) were unsuccessful. As an alternative approach, a targeted insertional disruption of *prsA2* (lmo2219) was generated using the targetron gene knockout system (Sigma, St. Louis, MO) following the recommendations made by the supplier, with minor modifications (8, 56, 79, 83). Briefly, a retargeted stable group II intron derived from *Lactococcus lactis* was generated by PCR using oligonucleotide primers (*prsA2*-457IBS, *prsA2*-457EBS1D, and *prsA2*-457EBS2) in the temperature-sensitive shuttle vector pNL9146 (Sigma, St. Louis, MO). The plasmid containing the retargeted intron with its expression under the control of a cadmium-inducible promoter (pNF1520) was introduced into *L. monocytogenes* via electroporation. Overnight cultures of *L. monocytogenes* transformants containing the pNF1520 shuttle vector were diluted 1:20 in BHI broth and grown at 32°C to an approximate optical density at 600 nm (OD_{600}) of 0.5, at which point 10 μM cadmium chloride (Sigma, St. Louis, MO) was added to the growth medium to induce targeted insertion of the intron into *prsA2* between nucleotides 457 and 458. Approximately 20 bacterial colonies were screened for the presence of the intron using primers 457checkF and 457checkR. All strains positive for the insertional disruption were cured of the plasmid by shifting growth conditions to the nonpermissive temperature 41°C as follows: intron-containing colonies were inoculated into 2 ml of BHI broth and grown to stationary phase at 41°C, and then 2-μl aliquots were removed and diluted into 2 ml of fresh medium and once again allowed to reach stationary phase. This procedure was repeated five times. Individual colonies were then screened for erythromycin susceptibility signifying loss of plasmid-encoded antibiotic resistance. The presence of the stable insertional disruption was then reconfirmed by DNA sequencing of PCR products derived from the target region using primers 457checkF and 457checkR, and the *prsA2* targetron mutant (*prsA2*::T) was designated NF-L1456.

Based on the successful construction of the *prsA2* targetron mutation, we sought to generate additional *prsA2* mutations that lacked the majority of *prsA2* coding sequence and that could be marked with antibiotic resistance genes to facilitate transduction into new genetic backgrounds. Using the NF-L1456 *prsA2*::T mutant as the parent strain, a gene replacement mutant was constructed wherein the majority of the *prsA2* coding sequence (nucleotides 31 to 849 of 879 total, representing 93% of the coding region) was replaced with a constitutive *erm* cassette. Five-hundred-base-pair fragments upstream and downstream of *prsA2*, including the first and last 30 nucleotides of the coding sequence as well as PstI and XbaI restriction sites, respectively, were PCR amplified from *L. monocytogenes* strain 10403S genomic DNA using primer pairs 2219SOEaPst1/2219SOEbSalint and 2219SOEcSalint/2219SOEdXbaI. The two fragments were

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Genotype	Designation	Source or reference
Strains			
TOP10	*E. coli* host strain used for recombinant pPL2 plasmids		
DH5α	*E. coli* host strain for recombinant pKSV7 plasmids		
SM10	*E. coli* host strain for conjugation of pPL2 plasmids		
NF-E1254	*E. coli* BL21 Star (DE3) with pNF-E1251 for PrsA2 protein expression and purification		This work
NF-L100	*L. monocytogenes* 10403S parent strain		2
NF-L476	*L. monocytogenes* 10403S *actA-gus-plcB*		59
NF-L1167	10403S *actA-gus-neo prfA*(*L140F*)	L140F	45
DP-L2161	10403S containing in-frame deletion of *hly* gene	Δ*hly*	27
NF-L1456	NF-L476 with group II intron (targetron) insertion into lmo2219 (*prsA2*) at position 457	*prsA2*::T	This work
NF-L1475	NF-L1456 with integrated pPL2-*prsA2*.	*prsA2*::T + *prsA2*	This work
NF-L1587	NF-L1456 with *prsA2*::T replaced with a Δ*prsA2*::*erm* mutation		This work
NF-L1635	DP-L2161 (Δ*hly*) with Δ*prsA2*::*erm*	Δ*prsA2*::*erm* Δ*hly*	This work
NF-L1637	NF-L1167 [*prfA*(*L140F*) with Δ*prsA2*::*erm*]	*prfA*(*L140F*) Δ*prsA2*::*erm*	This work
NF-L1659	NF-L1637 [*prfA*(*L140F*) Δ*prsA2*::*erm*] with integrated pPL2-*prsA2* (pNF1255)	*prfA*(*L140F*) Δ*prsA2*::*erm* + pPL2-*prsA2*	This work
NF-L1651	10403S with Δ*prsA2*::*erm*	Δ*prsA2*::*erm*	This work
NF-L1656	NF-L1651 (Δ*prsA2*::*erm)* with integrated pPL2-*prsA2* (pNF1255)	Δ*prsA2*::*erm* + pPL2-*prsA2*	This work
HEL 402	10403S with *prsA1* in-frame deletion	Δ*prsA1*	This work
Plasmids			
pNF1256	pKSV7 with Δ*prsA2* SOE fragment with internal SalI restriction site		This work
pNF1264	pNF1256 with *erm* resistance cassette inserted at SalI restriction site		This work
pHY304	Plasmid pHY304 containing constitutive *erm* cassette		26
pNF1251	pET-TOPO N-His with *prsA2* open reading frame		This work
pNF1255	pPL2-*prsA2* containing *prsA2* with 636 bp upstream of start ATG and 68 bp downstream of stop TAA		This work
pNF1520	pNL9146 with targeted targetron group II intron for gene disruption of *prsA2* at nucleotide position 457		This work

purified and used in a SOE PCR with primer pair 2219SOEaPstI/2219SOEdXbaI, generating a 1,031-bp fragment encompassing the 5′ end of lmo2219 with upstream sequence and the 3′ end of the same gene with downstream sequence. The 1,031-bp fragment was cloned into the shuttle vector pKSV7 using PstI and XbaI restriction sites, generating pNF1256. (An internal SalI restriction site was included in the overlap region of the SOE reaction such that a SalI site would be present in between the upstream and downstream fragments.) A constitutively expressed *erm* resistance gene was then amplified from plasmid pHY304 (26) (a kind gift of Amanda Jones, Seattle Children's Research Hospital, Seattle, WA) using primers ermSalI5 and ermSalI3, each containing a SalI restriction site. The fragment was purified and cloned into pNF-1256 to generate pNF-1264. pNF1264 was introduced into *L. monocytogenes* strain NF-L1456 *prsA2*::T by electroporation, and transformants were isolated on BHI agar containing chloramphenicol. The Δ*prsA2*::*erm* mutation was introduced into the *L. monocytogenes* chromosome in single copy to replace the targetron insertion using allelic exchange as previously described (6). The resulting in-frame *prsA2* deletion mutant with the antibiotic resistance gene replacement was confirmed by PCR analysis and sequencing of genomic DNA and was designated NF-L1587.

For construction of a plasmid vector containing *prsA2* for complementation of all subsequent *prsA2* mutations, primers 2219pPL2SacIa and 2219pPL2SmaIb were used to amplify the entire open reading frame of *prsA2* from *L. monocytogenes* strain 10403S genomic DNA, including the putative PrfA box located 206 nucleotides upstream of the predicted ATG translational start site. The PCR-amplified product was digested with SacI and SmaI and subcloned into appropriately digested shuttle plasmid vector pPL2 to generate pNF1255. pPL2 integrates in single copy into the *L. monocytogenes* phage attachment site located within the $tRNA^{Arg}$ gene following conjugation, resulting in single-copy gene complementation within this neutral site in the *L. monocytogenes* chromosome (14, 34).

Construction of *prfA*(*L140F*) Δ*prsA1*, *prfA*(*L140F*) Δ*prsA2*::*erm*, and Δ*prsA2*::*erm* Δ*hly* double mutants. The Δ*prsA2*::*erm* mutation was transduced into strain 10403S, NF-L1167 [10403S *actA-gus-neo*, *prfA*(*L140F*)] (45), and DP-L2161 (10403S Δ*hly*) (27) using U153 bacteriophage-mediated phage transduction as previously described, with some minor modifications (78). Briefly, phage lysates (10^8 to 10^9 PFU per ml) were prepared from NF-L1587 (NF-L476 with Δ*prsA2*::*erm*) and mixed with 10^8 CFU of each of the indicated strains at a final ratio of 10^8 phage to 10^8 bacteria. $CaCl_2$ and $MgSO_4$ were then added to a final concentration of 10 mM, and the mixture was incubated at room temperature for 40 min. After 40 min, the bacterium-phage mixture was plated onto BHI plates containing 1 μg/ml erythromycin and resultant colonies were picked after 1 to 2 days and screened for the presence of the erythromycin marker by PCR. The resultant strains were designated NF-L1651 (10403S Δ*prsA2*::*erm*), NF-L1637 [*prfA*(*L140F*) Δ*prsA2*::*erm*], and NF-L1635 (Δ*prsA2*::*erm* Δ*hly*). Similarly, phage lysates were prepared from NF-L1167 [10403S *actA-gus-neo*, *prfA*(*L140F*)] in order to transduce the *prfA*(*L140F*) allele into HEL-402 (Δ*prsA1*). The transduction was carried out in a similar fashion with minor modifications. After incubation of the bacteria and phage for 40 min at room temperature, the mixture was plated onto BHI plates containing 5 μg/ml neomycin and 50 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Inalco, Milano Italy). Positive transductants that were both neomycin resistant and blue in color (indicative of constitutively active PrfA activity) were isolated and used for subsequent analysis.

Purification of PrsA2 and generation of rabbit polyclonal antiserum directed against PrsA2. Oligonucleotides listed in Table S1 in the supplemental material were used to amplify *prsA2* from *L. monocytogenes* strain 10403S genomic DNA, and the resulting PCR fragment was subcloned into pET100 using the Champion pET directional TOPO expression kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Plasmids containing *prsA2* coding sequences were transformed into *E. coli* BL21 Star (DE3) expression cells, and recombinant protein expression was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to mid-log-phase cultures for 5 h. Bacteria were recovered following centrifugation at 3,000 × *g* for 10 min at 4°C, the supernatant was discarded, and bacterial pellets were frozen at −80°C, thawed, resuspended in 25 ml phosphate-buffered saline (PBS), followed by bacterial cell disruption via French press. Protein extracts were passed over nickel columns, and recombinant PrsA2 was eluted off at pH 6.0 under denaturing conditions using 6 M guanidinium HCl, 8 M urea, 20 mM NaH_2PO_4, and 500 mM NaCl. Eluted protein fractions were concentrated using a Centricon centrifugal filter (Millipore, Billerica, MA), and

proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. Five hundred micrograms of recombinant PrsA2 protein was used to generate rabbit polyclonal antiserum through a commercial supplier (Cocalico Biologicals, Reamstown, PA).

Mouse intravenous infections. All animal procedures were IACUC approved and performed in the Biological Resources Laboratory at the University of Illinois at Chicago. Overnight cultures of bacteria grown in BHI broth at 37°C were diluted 1:20 into fresh medium and grown to an OD_{600} of approximately 0.6. One milliliter of culture corresponding to 6×10^8 CFU/ml was washed twice in PBS (0.144 g/ml KH_2PO_4, 9.00 g/ml NaCl, 0.795 g/ml Na_2HPO_4 [anhydrous]), diluted, and resuspended in PBS to a final concentration of 1×10^5 CFU/ml. Eight- to 10-week-old female ND4 Swiss Webster mice (Harlan, Madison, WI) were injected with 200 μl PBS containing 2×10^4 CFU of *L. monocytogenes* via the tail vein. Seventy-two hours postinfection, mice were sacrificed and livers and spleens were dissected. Organs were homogenized with a Tissue Master 125 homogenizer (Omni, Marietta, GA) and 10-fold serial dilutions were plated onto BHI plates containing 200 μg/ml streptomycin (all 10403S derivatives are streptomycin resistant).

Assessment of bacterial intracellular growth in tissue culture cells. The J774 and Henle 407 cell lines were maintained as previously described (4, 67). Monolayers of cells were grown on acid-washed coverslips and infected with *L. monocytogenes* strains at a multiplicity of infection (MOI) of 0.1 bacterium per 10 macrophage for J774s and 30 bacteria per epithelial cell in Henle 407s for wild-type (WT) and Δ*prsA2*::*erm* strains or 90 bacteria per epithelial cell for all strains containing an *hly* deletion (to compensate for the loss of LLO-mediated enhancement of bacterial invasion) (46). Infection was carried out for 30 min followed by washing three times with warm PBS and addition of fresh medium containing 50 μg/ml gentamicin to kill extracellular bacteria at 1 h. To measure bacterial intracellular growth, coverslips were removed at the time points indicated and host cells were lysed in 5 ml of sterile H_2O by vigorous vortexing. Dilutions of these lysates were then plated on LB agar plates for enumeration of bacterial counts per coverslip. Results were obtained from at least three independent experiments. Coverslips of infected Henle 407 cells (10.5 h postinfection) were also processed for immunofluorescence microscopy as previously described (46). Processed coverslips were then viewed on a Leica DM4000B wide-field epifluorescence microscope (Leica Microsystems, Wetzlar, Germany). Representative images of all fields viewed were captured using SlideBook image acquisition software (Intelligent Imaging Innovations, Inc., Denver, CO).

Plaque assays. Plaque assays were conducted as previously described (64). Monolayers of L2 fibroblast cells were grown on six-well tissue culture plates and infected for 1 h with *L. monocytogenes* at a multiplicity of 30 bacteria to 1 fibroblast. Gentamicin was added to single wells after infection (20 μg/ml) to kill extracellular bacteria. Plaque size was measured using a micrometer (Finescale, Orange, CA). Results were obtained from at least three independent experiments.

Hemolysin assays. Hemolytic activity was measured as previously described (5, 28). Briefly, overnight *L monocytogenes* cultures grown in BHI broth at 37°C were diluted 1:10 into fresh BHI broth and grown at 37°C with vigorous shaking for 5 h. OD_{600} readings were taken, and 1 ml of bacterial culture was spun down at 16,000 × *g* for 5 min. Supernatants were removed from bacterial pellets, and dilutions of bacterial supernatants (standardized for OD_{600}) were incubated with PBS-washed sheep red blood cells (RBCs; in Alsevers) for 30 min at 37°C (Cocalico Biologicals, Reamstown, PA). After incubation, RBCs were removed by centrifugation and hemolytic units were measured as the reciprocal of the dilutions that gave 50% lysis of RBCs as determined by visual inspection of RBC pellets after centrifugation. Results were obtained from at least three independent experiments.

Detection of PC-PLC activity. *plcB*-dependent phospholipase production was visualized using an egg yolk overlay agar plate assay (46). Chicken egg yolk was added in a 1:1 (vol/vol) ratio to PBS and vortexed to generate a suspension. Five milliliters of egg yolk suspension was then added to 100 ml of molten LB medium (45°C to 48°C), and 3 ml of egg yolk-agar suspension was overlaid onto an LB agar plate. Egg yolk agar used for *L. monocytogenes* strains containing a WT *prfA* allele was additionally supplemented with 0.2% activated charcoal (Fisher Scientific, Pittsburgh, PA) and 25 mM glucose-6-phosphate (Sigma, St Louis, MO) to enhance *prfA*-dependent expression of *plcb* in these strains. Following solidification of the medium, bacterial strains were gently streaked onto the surface of the plate and incubated at 37°C for 48 h for strains containing a WT *prfA* allele and 24 h for those containing a *prfA*(*L140F*) allele. Phospholipase activity was detected as a zone of opacity surrounding bacterial streaks. Results were obtained from at least three independent experiments.

Isolation of supernatant and bacterial cell-associated proteins. Overnight cultures of *L. monocytogenes* strains grown in BHI broth were diluted 1:20 into 30 ml of fresh BHI broth and were grown at 37°C with shaking for 5 h (approximate OD_{600} of 1.4). Twenty-five milliliters of each culture was centrifuged at 12,350 × *g* for 15 min at 4°C. Supernatants were recovered, and secreted proteins were obtained by precipitation using trichloroacetic acid (TCA) (Sigma Chemical Co., St. Louis, MO) at a final percentage of 10%; after TCA addition, culture supernatants were incubated on ice for 30 min. Precipitated protein pellets were recovered by centrifugation at 12,350 × *g* for 10 min, the supernatant was discarded, and protein pellets were allowed to air dry. Four milliliters of ice-cold acetone was used to wash pellets, which were then centrifuged again at 12,350 × *g* for 10 min. The TCA-precipitated protein pellet was then resuspended in 200 μl of 1× SDS boiling buffer (5% SDS, 5% β-mercaptoethanol, 10% glycerol, 60 mM Tris [pH 6.8]). For bacterial cell-associated protein fractions, bacterial pellets were resuspended in 200 μl of 2% SDS boiling buffer and boiled at 100°C for 5 min. This resulting cell-associated fraction was clarified by centrifugation at 10,000 × *g* for 20 min to remove the remaining insoluble material.

Western analysis. All protein samples were run on 4 to 12% gradient SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) for 1 h at 200 V. To ensure equivalent total protein was analyzed for each strain, equal volumes of the previously normalized supernatant and surface-associated proteins (OD of ~1.4) were loaded onto the gels. For visualization of total protein, gels were fixed in a 50% methanol and 7% acetic acid solution for 30 min, washed three times for 15 min each in deionized H_2O, and stained with GelCode Blue staining reagent (Thermo Scientific, Rockford, IL) for 1 h. For Western analysis, proteins were transferred to polyvinylidene difluoride membranes at 30 V (constant) for 1 h. Membranes were blocked for 1 h in PBS containing 0.05% Tween 20 (PBST) and 5% milk. Membranes were then probed with the following antibodies and described dilutions: primary polyclonal anti-PrsA2 antibody was used at a 1:2,500 dilution, primary polyclonal anti-LLO antibody (AbCam, Cambridge, MA) was used at a 1:2,500 dilution, and primary polyclonal anti-PC-PLC antibody (a kind gift from Helene Marquis, Cornell University, NY) was used at a 1:2,500 dilution. Primary antibody was added to 20 ml of PBST and incubated at room temperature with shaking for 2 h. Membranes were then washed three times for 15 min each in PBST. Secondary antibody conjugated to alkaline phosphatase was added at a 1:2,500 dilution for 1.5 h (Southern Biotech, Birmingham, AL). Detection was carried out via colorimetric development in the presence of BCIP-NBTPlus (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium) for approximately 5 min (Southern Biotech, Birmingham, AL). Images of blots were then photographed using an AlphaImager 2200 (Alpha Innotech, San Leandro, CA). Results were obtained from at least three independent experiments. Densitometry was performed on all Western blots from three independent experiments using NIH ImageJ Software (http://rsbweb.nih.gov/ij/). The area under the curve was used to calculate arbitrary units for both the WT and L140F mutant for each blot, which was then set to a value of 1.00. All other strains were then compared according to that value and are thus shown as a deviation from 1.00. The average values from three blots and standard deviations are shown.

RESULTS

Construction of *prsA1* and *prsA2* stable gene disruption mutants. *L. monocytogenes prsA1* and *prsA2* have significant amino acid sequence similarity to *B. subtilis* PrsA (43% and 45% identity and 63% and 65% similarity, respectively) and even higher amino acid identity and similarity to each other (58% identity and 75% similarity) (Fig. 1A). To explore the functional contributions of the *prsA1* and *prsA2* gene products to *L. monocytogenes* pathogenesis, we attempted to introduce in-frame deletion mutations into *prsA1* and *prsA2* within the *L. monocytogenes* chromosome by allelic exchange. Whereas the construction of the *L. monocytogenes* Δ*prsA1* strain proved straightforward and was rapidly accomplished, efforts to generate Δ*prsA2* strains were unsuccessful despite the fact that *prsA2* loss-of-function mutations via plasmid insertion had been readily generated (47). A similar failure to generate an *L. monocytogenes* Δ*prsA2* mutant was also reported by Milohanic et al. (44), although Chatterjee et al. (7) reported the successful construction of a Δ*prsA2* mutant in EGDe. As an alternative approach for the generation of a stable *prsA2* mutant, the

commercially available targetron gene knockout system was used to insert a *Lactococcus lactis* group II intron into *prsA2* at nucleotide position 457 (*prsA2*::T) (Fig. 1B). The *L. monocytogenes* Δ*prsA1* and *prsA2*::T mutant strains thus generated exhibited normal patterns of growth in broth culture and on solid media (see Fig. S1A in the supplemental material). Subsequent use of the *prsA2*::T mutant as the starting strain for allelic exchange enabled the construction of an in-frame *prsA2* deletion mutant containing an erythromycin resistance cassette (NF-L1587). This strain was constructed so as to facilitate transduction of the antibiotic resistance-marked Δ*prsA2* mutation into other *L. monocytogenes* genetic backgrounds. Although *prsA2* mutants were indistinguishable from WT strains for growth in broth culture, it was observed that the mutants exhibited a subtle fitness defect in mixed cultures such that after several cycles of bacterial growth and dilution, the WT strain dominated the cultures (see Fig. S1B in the supplemental material). The fitness defect of *prsA2* mutant strains in mixed cultures thus accounts for the difficulties encountered by our lab and the labs of other investigators in the isolation of in-frame deletion mutants of *prsA2* when using the repeated cycles of bacterial outgrowth required for allelic exchange.

The *prsA2* strain is unique from the *prsA1* strain in its requirement for *L. monocytogenes* virulence in mice. Δ*prsA1* and Δ*prsA2*::*erm* mutants were assessed for virulence in mice following intravenous inoculation. Bacteria (2×10^4 CFU) were injected into the tail vein of Swiss mice, and after 72 h, bacterial burdens were determined in both livers and spleens. In contrast to mice infected with either the WT strain or the Δ*prsA1* mutant, mice infected with the Δ*prsA2*::*erm* mutant appeared healthy at 3 days postinfection, with no evident signs of illness (data not shown). Enumeration of bacterial CFU from the livers and spleens of infected animals resulted in the recovery of approximately 5 to 6 logs fewer bacteria for the Δ*prsA2*::*erm* mutant compared to mice infected with the WT parent strain or the Δ*prsA1* mutant (Fig. 2). The virulence defect of the Δ*prsA2*::*erm* mutant was fully complemented by the introduction of *prsA2* in single copy on the integrative plasmid vector pPL2 (Fig. 2), thus indicating that the Δ*prsA2*::*erm* virulence defect was the result of the loss of the functional *prsA2* gene product. These data indicate that *prsA2*, but not *prsA1*, is essential for *L. monocytogenes* pathogenesis in vivo.

***prsA2* mutants exhibit altered patterns of intracellular growth and cell-to-cell spread in tissue culture cells.** The severe virulence defect associated with the loss of *prsA2* prompted examination of the mutant for intracellular growth in tissue culture cell lines. Although uptake of a Δ*prsA2*::*erm* mutant was reduced in J774 mouse macrophage-like cells, mutant growth closely resembled that of the WT and the Δ*prsA1* mutant, with comparable rates of bacterial replication up to 9 h postinfection (Fig. 3A). Inspection of infected cell monolayers by microscopy indicated that the Δ*prsA2*::*erm* mutant strain was capable of intracellular replication, actin-based motility, and spread to adjacent cells (data not shown). At 9 h postinfection, the number of mutant bacteria was seen to decline; this phenotype was consistently observed in multiple independent experiments, whereas WT and Δ*prsA1* numbers increased or remained constant up to the 11-h time point (Fig. 3A). Consistent with reduced bacterial growth at late time points of infection in J774 cells, the Δ*prsA2*::*erm* mutant was observed to be defective for intracellular growth and cell-to-cell spread in monolayers of infected mouse L2 fibroblasts (Fig. 3B). In this cell line, *L. monocytogenes* host cell invasion, intracellular growth, and cell-to-cell spread are readily visible after infection of the monolayers via the formation of small, distinct zones of cell clearing (plaques) that arise as a result of cell death following bacterial replication and spread (64). Plaques formed by the Δ*prsA2*::*erm* mutant were approximately 20% of the size of those formed by the WT *L. monocytogenes* strain, the Δ*prsA1* mutant, or the complemented *prsA2* mutant (Fig. 3B). A similar plaque formation defect was reported for the EDGe Δ*prsA2* mutant generated by Chatterjee et al. (7). The Δ*prsA2*::*erm* mutant strain is therefore capable of host cell entry, intracellular growth, and cell-to-cell spread; however, it is impaired for these activities in comparison to WT *L. monocytogenes.*

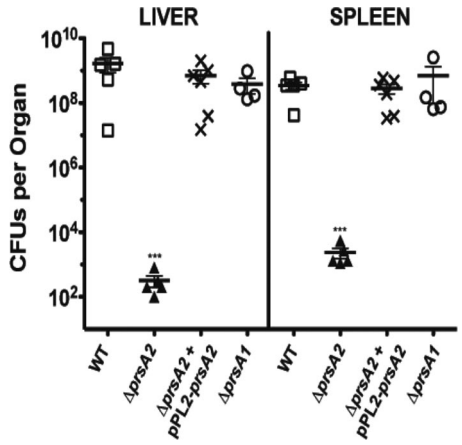

FIG. 2. Δ*prsA2*::*erm* mutants are severely attenuated for virulence in mice. Mice were intravenously infected with 2×10^4 CFU of the WT or Δ*prsA2*::*erm*, Δ*prsA2*::*erm* + pPL2-*prsA2*, or Δ*prsA1* mutant strain, and the bacterial burden in livers and spleens was determined at 72 h postinfection. The scatter plot shows CFU obtained from the livers and spleens of five individual mice; the data are representative of three independent experiments. Solid lines and brackets represent the mean and standard error of the mean, respectively, for the data points in each group. ***, statistically significant value ($P < 0.0001$) for Δ*prsA2*::*erm* in both the liver and the spleen compared to WT using a one-way analysis of variance with Dunnett's posttest (GraphPad V.5.0A). Differences between WT and the Δ*prsA2*::*erm* + pPL2 *prsA2* mutant or the WT and the Δ*prsA1* mutant were not statistically significant ($P > 0.05$).

PrsA2 is required for optimal secreted LLO and PC-PLC activity. In *B.* subtilis, PrsA has been shown to contribute to the proper folding of secreted proteins at the membrane-cell wall interface (24, 31, 49). To explore the potential role of *L. monocytogenes* PrsA2 as a posttranslocational molecular chaperone, we examined the activity of two secreted *L. monocytogenes* gene products critical for bacterial virulence, LLO and PC-PLC (9, 21, 48, 80). Measurement of LLO-associated hemolytic activity in supernatants derived from Δ*prsA2*::*erm* strains indicated an approximate twofold decrease in activity in comparison to WT and Δ*prsA1* strains, based on the lysis of sheep RBCs (33 ± 2.5 U for the Δ*prsA2*::*erm* mutant versus 60 ± 3.7 U for the parent strain and 65 ± 5.0 U for Δ*prsA1*) (Fig. 4A). The introduction of pPL2-*prsA2* fully restored he-

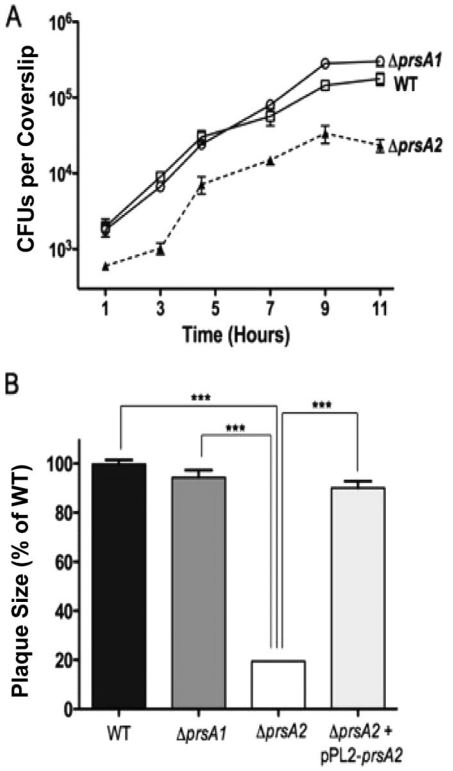

FIG. 3. The Δ*prsA2*::*erm* mutant is capable of intracellular growth but is defective for cell-to-cell spread in tissue culture cells. (A) J774 macrophage-like cells were infected with the WT or the Δ*prsA1* or Δ*prsA2*::*erm* mutant at an MOI of 0.1. After 30 min, the monolayers were washed and gentamicin (50 μg/ml) was added at 1 h postinfection. The data shown are representative of at least three independent experiments done in duplicate. □, wild type; ○, *prsA1*; ▲, Δ*prsA2*::*erm*. (B) Plaque formation in mouse L2 fibroblast cells. Monolayers of L2 cells were infected with the WT or Δ*prsA1*, Δ*prsA2*::*erm*, or Δ*prsA2*::*erm* + pPL2-*prsA2* mutant for 1 h and washed with PBS, and 20 μg/ml gentamicin was added. At least 15 plaques were measured in three independent experiments for all strains; measurements represent plaque size comparisons with respect to the WT (set at 100%). ***, statistically significant value ($P < 0.0001$) as calculated using a one-way way analysis of variance with Tukey's multiple comparison test (GraphPad v.6.0A).

molytic activity (55 ± 6.5 U). The modest reduction in secreted hemolytic activity was likewise evident in the presence of the mutationally activated *prfA*(*L140F*) allele, which confers constitutive high-level expression of PrfA-dependent gene products, including LLO and PC-PLC (45, 47, 72, 78). When the Δ*prsA2*::*erm* mutation was introduced into the *prfA*(*L140F*) strain by phage transduction, hemolytic activity in *prfA*(*L140F*) Δ*prsA2*::*erm*-derived supernatants was decreased approximately threefold in comparison to that of the *prfA*(*L140F*) strain containing WT *prsA2* or the *prsA1* deletion [111 ± 8.9 U for *prfA*(*L140F*) Δ*prsA2*::*erm* versus 373 ± 53.3 U for *prfA*(*L140F*) Δ*prsA1* and 396 ± 29.5 U for *prfA*(*L140F*)] and again was fully restored upon complementation with pPL2-*prsA2* (360 ± 40.0 U) (Fig. 4A). The presence of *prfA*(*L140F*) increased LLO-dependent hemolytic activity four- to fivefold for all strains in comparison to strains carrying the WT *prfA* allele.

PC-PLC activity was assessed following growth of the bac-

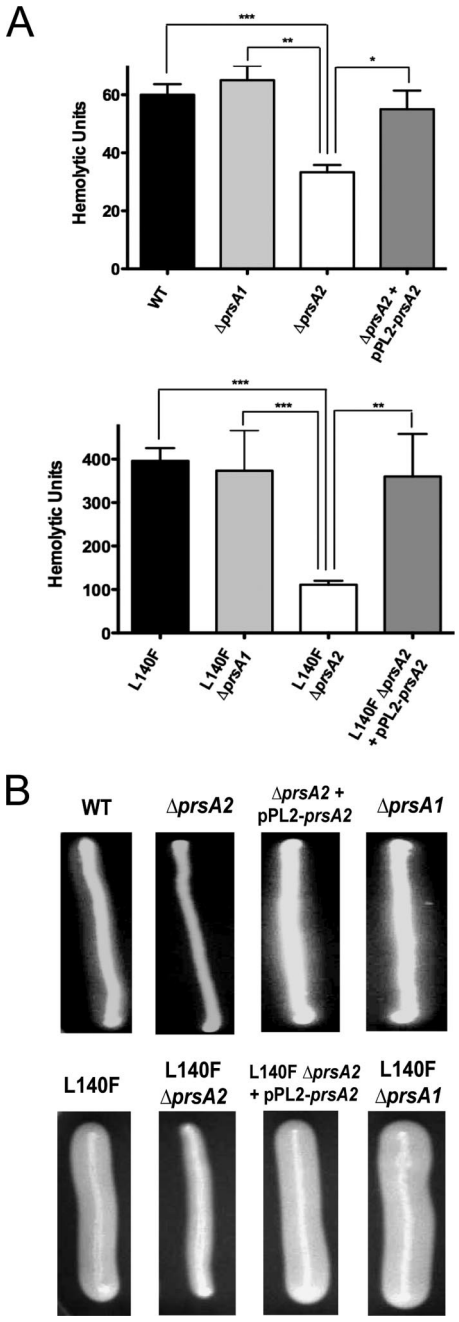

FIG. 4. Δ*prsA2*::*erm* strains have decreased LLO and PC-PLC activity. (A) The hemolytic activity of culture supernatants derived from WT and *prfA*(*L140F*) strains in the presence and absence of *prsA1* or *prsA2* and its complement was determined based on 50% lysis of sheep RBCs. The average values of four independent experiments are shown. Statistically significant differences are indicated as determined by one-way analysis of variance with Tukey's multiple comparison test (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$) (GraphPad V.5.0). (B) Detection of PC-PLC-related phospholipase activity of the Δ*prsA1* or Δ*prsA2*::*erm* mutant and its complement in the presence of the WT (top panel) and mutationally activated *prfA* [*prfA*(*L140F*)] (bottom panel). *prfA*(*L140F*), *prfA*(*L140F*) Δ*prsA2*::*erm*, *prfA*(*L140F*) Δ*prsA2*::*erm* + pPL2-*prsA2*, and *prfA*(*L140F*) Δ*prsA1* strains incubated on egg yolk agar plates exhibited zones of opacity indicative of PC-PLC activity. A representative image from 1 of 10 plates is shown.

teria on solid medium containing egg yolk and was based on the ability of bacterially secreted PC-PLC-associated lecithinase activity to yield a white precipitate. PC-PLC activity is difficult to detect for strains containing WT *prfA* as the expression of *plcB* (which encodes PC-PLC) is low in bacteria grown on standard agar media; however, the addition of charcoal and phosphorylated glucose has been shown to enhance PC-PLC expression (81), as does the introduction of the *prfA*(*L140F*) allele (72, 78). PC-PLC activity was detected for all strains as visible zones of precipitation (Fig. 4B). Strains lacking *prsA2* exhibited significantly reduced levels of PC-PLC activity; these levels could be fully restored following the introduction of the pPL2-*prsA2* plasmid (Fig. 4B). *S*trains lacking *prsA1* exhibited levels of secreted PC-PLC activity equivalent to those of strains containing WT *prsA1* in the presence of either WT *prfA* or the *prfA*(*L140F*) allele (Fig. 4B). PrsA2 is therefore unique from PrsA1 in its requirement for optimal activity of both secreted LLO and PC-PLC, most notably under conditions where PrfA is activated.

PrsA2 contributes to LLO stability and processing of pro-PC-PLC to its mature form. Based on the apparent reduction in activity of LLO and PC-PLC observed for strains containing *prsA2* mutations, we next examined the amount of LLO and PC-PLC protein secreted by WT and Δ*prsA2*::*erm* strains as well as protein associated with the bacterial cell surface. In strains expressing the WT *prfA* allele, Western blot analysis of secreted LLO revealed approximately 2.5-fold-lower levels of LLO secreted by the Δ*prsA2*::*erm* mutant in comparison to WT strains (Fig. 5). LLO secretion was restored to the mutant by the introduction of the pPL2-*prsA2* plasmid. No reduction in LLO was observed for protein associated with the bacterial cell surface of *prsA2* mutants. Levels of detectable PC-PLC in secreted fractions were similar for all strains, with no visible protein retained at the cell surface (Fig. 5). Under these conditions, PC-PLC was only detected as the unprocessed pro-form of the enzyme based on its molecular weight. Western blot analysis of secreted levels of LLO and PC-PLC for Δ*prsA1* strains indicated no difference from WT bacteria (data not shown).

In the presence of the activated *prfA*(*L140F*) allele, levels of both secreted and cell-associated LLO and PC-PLC were increased in strains with WT *prsA2* as well as the Δ*prsA2*::*erm* mutant (Fig. 6). However, a number of lower-molecular-weight bands that reacted with the anti-LLO antibody were evident in the secreted protein fractions, suggesting that degradation of LLO was occurring along with the increase in LLO synthesis. Interestingly, degradation products were more abundant and exhibited a different peptide banding pattern for secreted LLO isolated from the Δ*prsA2*::*erm* mutant from the parent strain (Fig. 6). LLO degradation products were also evident for cell-associated protein fractions. Comparison of relative amounts of full-length LLO between WT and Δ*prsA2*::*erm* strains with the *prfA*(*L140F*) allele indicated an approximately 2.5-fold decrease in LLO protein secreted by the Δ*prsA2*::*erm* strain, with no significant difference observed for cell-associated LLO (Fig. 6). Overall, these results indicate that (i) bacteria with WT *prsA2* secrete more full-length LLO than strains lacking *prsA2*, (ii) induction of LLO synthesis results in increasingly greater amounts of LLO degradation for strains with and without *prsA2*, and (iii) induction of LLO synthesis in strains lacking

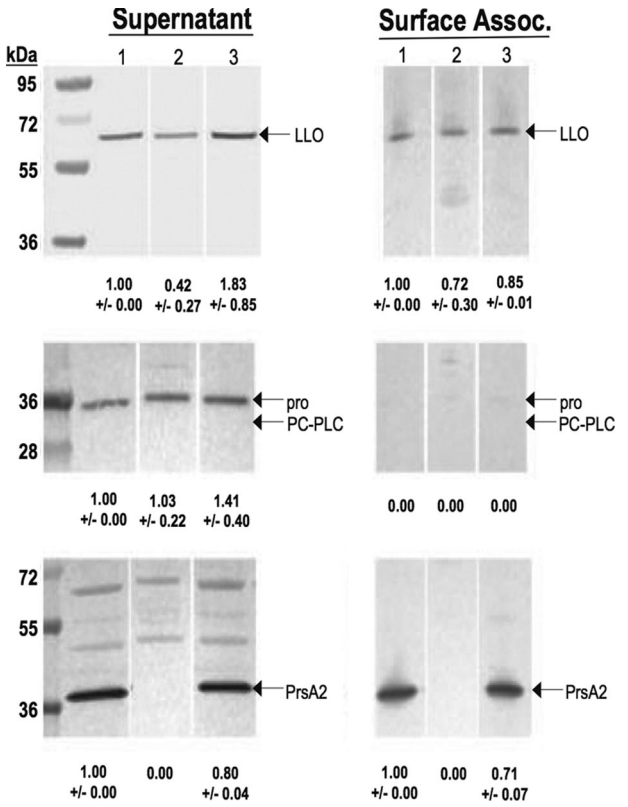

FIG. 5. Strains lacking PrsA2 secrete reduced amounts of LLO and PC-PLC. Bacterial supernatant and surface-associated proteins from the WT or Δ*prsA2*::*erm* or Δ*prsA2*::*erm* + pPL2-*prsA2* mutant were isolated by TCA precipitation (secreted proteins) or boiling in 2% SDS (surface-associated proteins). Sample volumes were adjusted to reflect equivalent bacterial densities (OD_{600} of ~1.4). Western analysis of protein fractions subjected to SDS-PAGE was done using rabbit polyclonal anti-LLO, rabbit polyclonal anti-PrsA2, and rabbit polyclonal anti-PC-PLC antibody to detect LLO, PrsA2, and PC-PLC, respectively. Arrows indicate bands of interest (either LLO, PrsA2, or PC-PLC) in each panel. The amount of protein detected for each sample in comparison to the WT lane (set at 1.0) as determined by densitometry is indicated at the bottom of each panel. Lane 1, WT; lane 2, Δ*prsA2*::*erm*; lane 3, Δ*prsA2*::*erm* + pPL2-*prsA2*.

prsA2 results in increased amounts of LLO degradation and different degradation peptide patterns in comparison to bacteria with WT *prsA2*. The altered banding patterns of the LLO degradation products in *prsA2* mutant strains may be indicative of an altered LLO structure that is more sensitive to proteolytic cleavage, a result that would be consistent with a role for PrsA2 in proper LLO folding.

Secretion of PC-PLC was increased in the presence of *prfA*(*L140F*) for both WT and *prsA2* mutant strains, with the predominant form of the enzyme remaining the unprocessed pro-form (Fig. 6). Strains lacking *prsA2* secreted slightly reduced amounts of pro-PC-PLC in comparison to strains with WT *prsA2* and approximately three- to fourfold-less mature PC-PLC. Examination of cell-associated PC-PLC in the presence of *prfA*(*L140F*) revealed slightly reduced amounts of pro-PC-PLC associated with the cell surface of Δ*prsA2*::*erm* strains; however, strains with WT *prsA2* had significantly increased levels of processed PC-PLC associated with the cell surface in

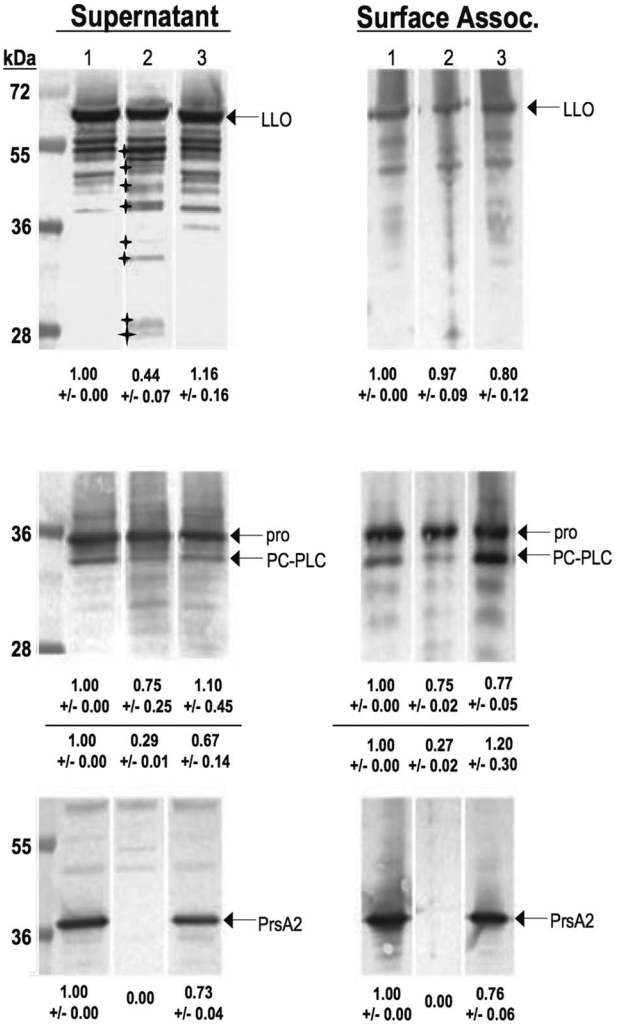

FIG. 6. Strains lacking PrsA2 exhibit increased LLO degradation and reduced processing of pro-PC-PLC in the presence of activated PrfA. Bacterial supernatant and surface-associated proteins from the mutationally activated *prfA*(*L140F*), *prfA*(*L140F*) Δ*prsA2*::*erm*, or *prfA*(*L140F*) Δ*prsA2*::*erm* + pPL2-*prsA2* mutant were isolated by TCA precipitation (secreted proteins) or boiling in 2% SDS (surface-associated proteins). Sample volumes were adjusted to reflect equivalent bacterial densities (OD_{600} of ~1.4). Western analysis of protein fractions subjected to SDS-PAGE was done using rabbit polyclonal anti-LLO antibody, rabbit polyclonal anti-PrsA2, and rabbit polyclonal anti-PC-PLC antibody to detect LLO, PrsA2, and PC-PLC respectively. Arrows indicate bands of interest (either LLO, PrsA2, pro-PC-PLC, or PC-PLC) in each panel. Small stars indicate the altered LLO degradation pattern found in *prfA*(*L140F*) Δ*prsA2*::*erm* strains compared to that of *prfA*(*L140F*) and complement strains. The amount of protein detected for each sample in comparison to the WT lane (set at 1.0) as determined by densitometry is indicated at the bottom of each panel. The top and bottom rows of numbers located at the bottom of the PC-PLC Western blot represent the total amounts of pro-PC-PLC and PC-PLC, respectively. Lane 1, *prfA*(*L140F*); lane 2, *prfA*(*L140F*) Δ*prsA2*::*erm*; lane 3, *prfA*(*L140F*) Δ*prsA2*::*erm* + pPL2-*prsA2*.

comparison to Δ*prsA2*::*erm* strains (approximately three- to fourfold-more mature PC-PLC enzyme) (Fig. 6). *L. monocytogenes* is known to synthesize and sequester PC-PLC until the bacterium encounters the appropriate host environment for the release of active enzyme; thus, the cell-associated processed PC-PLC observed in Fig. 6 may reflect sequestered enzyme (41). These results implicate a role for PrsA2 in promoting the processing of pro-PC-PLC to its mature form.

Reduced PC-PLC activity in *prsA2* strains is functionally detectable following *L. monocytogenes* infection of human epithelial cells. Infection of J774 macrophages with the Δ*prsA2*::*erm* strain indicated that the mutant was fully capable of mediating bacterial escape from the primary phagosome (Fig. 3A). Although reduced numbers of bacteria were internalized, bacterial replication rates for the first 9 h of infection were similar (Fig. 3A), and examination of bacteria associated with host cell actin (a marker for cytosolic entry) indicated no difference in the rate of escape of the Δ*prsA2*::*erm* mutant from primary phagosomes in comparison to that of WT *L. monocytogenes* (data not shown). Consistent with these observations, efficient phagosome lysis has been previously demonstrated with *L. monocytogenes* mutants expressing low levels of LLO in broth culture (approximately 10-fold below WT levels) (15), and the Δ*prsA2*::*erm* mutant exhibits only an approximately two- to threefold reduction in secreted LLO activity in comparison to WT *L. monocytogenes* (Fig. 4A). However, the decline in bacterial numbers in J774 cells beginning approximately 9 h postinfection and the small-plaque phenotype of the mutant in fibroblast cells (Fig. 3B) are strongly suggestive of a defect in bacterial cell-to-cell spread. PC-PLC activity has been shown to be an important contributor to lysis of secondary vacuoles formed during bacterial spread to adjacent cells (40, 41, 63, 80). To more fully investigate the potential consequences on cellular infection of reduced PC-PLC activity in Δ*prsA2*::*erm* mutants, we examined intracellular growth of Δ*prsA2*::*erm* mutants in Henle human epithelial tissue culture cells in the presence and absence of *hly*. Unlike most tissue culture cell lines, LLO (encoded by *hly*) is not required for vacuole lysis in human epithelial cells; this function instead can be fully provided by active PC-PLC, thereby enabling intracellular assessment of PC-PLC activity based on intracellular bacterial growth and cell-to-cell spread (39, 46, 48). Henle human epithelial cell monolayers were infected with WT and Δ*prsA2*::*erm* mutant strains in the presence and absence of *hly*. Bacterial growth was monitored in the presence of 50 μg/ml gentamicin added at 1 h postinfection. As previously reported, Δ*hly* strains lacking LLO activity efficiently escaped the vacuoles of Henle cells and replicated within the cytosol (Fig. 7A). Strains lacking *prsA2* grew similarly to WT *L. monocytogenes*, with a slight reduction in the number of bacteria initially entering host cells observable at 1 h postinfection and a consistent leveling off of bacterial numbers by 11 h postinfection. In contrast, the Δ*hly* Δ*prsA2*::*erm* double mutant exhibited a pronounced defect in intracellular growth, with bacterial numbers increasing modestly only after 7 h postinfection and continuing to lag behind the WT up to 11 h postinfection (Fig. 7A). The Δ*hly* Δ*prsA2*::*erm* double mutant was, however, capable of limited intracellular bacterial replication based on its increased numbers in comparison to those of strains completely lacking LLO and PC-PLC activity (the Δ*hly* Δ*plcB* double mutant), which remain trapped within host cell vacuoles (39) (Fig. 7A). Visual inspection of infected monolayers by microscopy at 10.5 h postinfection indicated cells infected with the Δ*hly* Δ*prsA2*::*erm* double mutant primarily contained single bacteria, suggesting that the double mutants were significantly im-

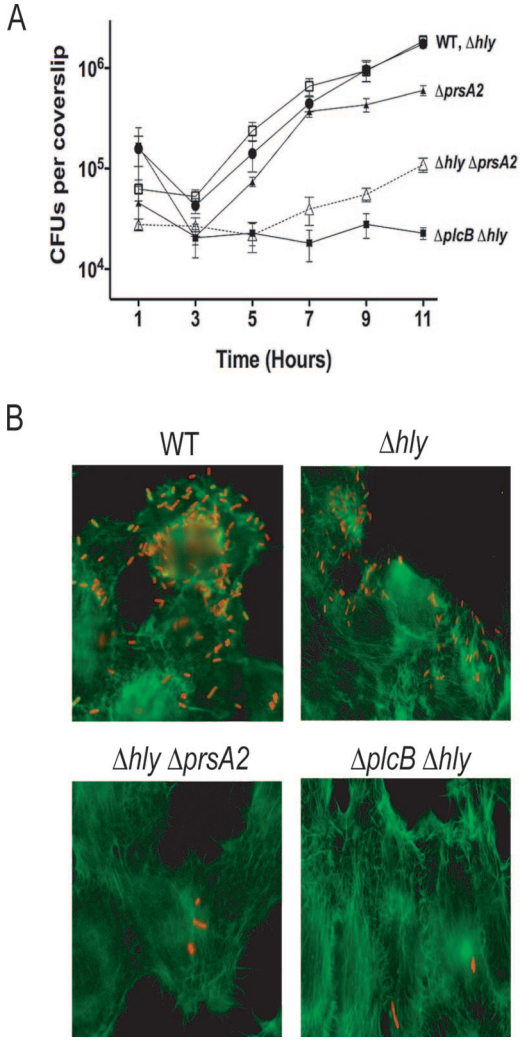

FIG. 7. Reduced PC-PLC activity in Δ*prsA2*::*erm* strains impairs bacterial vacuolar escape, resulting in reduced intracellular replication. (A) Henle human epithelial cells were infected with the WT or Δ*prsA2*::*erm*, Δ*hly*, Δ*prsA2*::*erm* Δ*hly*, or Δ*plcB* Δ*hly* mutant as described in Materials and Methods. After 30 min, infected cells were washed with PBS followed by the addition of gentamicin (50 μg/ml) at 1 h postinfection. The data shown are representative of three independent experiments done in duplicate. □, WT; ▲, Δ*prsA2*::*erm*; ●, Δ*hly*; △, Δ*prsA2*::*erm* Δ*hly* double mutant; ■, Δ*plcB* Δ*hly* double mutant. (B) Coverslips were also processed for microscopy at 10.5 h postinfection. Cell monolayers were fixed, permeabilized, and stained for *L. monocytogenes* using an anti-*Listeria* antibody and tetramethylrhodamine-conjugated goat anti-rabbit secondary antibody to detect bacteria (red) and NBD (7-nitrobanz-2-oxa-1,3-diazole)-phallacidin to detect F-actin (green) at a ×1,000 magnification.

paired for vacuolar escape and hence intracellular replication (Fig. 7B). These results indicate that the secretion of active PC-PLC is significantly reduced in Δ*prsA2*::*erm* strains during cellular infection.

DISCUSSION

L. monocytogenes secretes numerous proteins that enable the organism to invade mammalian cells, gain access to the cytosol, and exploit this protected environment for bacterial replication and systemic spread (55, 71). The expression and activity of secreted virulence factors have been shown to be regulated at multiple steps and include transcriptional, posttranscriptional, and posttranslational regulation (25, 40, 41, 50–53, 58, 77). In this report, we describe the identification of PrsA2, a predicted postsecretion chaperone that is essential for *L. monocytogenes* pathogenesis. Although *L. monocytogenes* encodes two highly similar PrsA-like proteins, only PrsA2 was found to influence LLO activity and stability and to promote the processing of pro-PC-PLC to its activated form. Based on our results and on functional analysis of PrsA homologues in other gram-positive bacteria (13, 23, 24, 31, 32, 38, 73, 75), we propose that PrsA2 contributes to *L. monocytogenes* pathogenesis by promoting the folding, stability, and hence the activity of targeted secreted virulence factors that are essential for bacterial intracellular replication and cell-to-cell spread.

The substantial 5- to 6-log virulence defect observed for mutants lacking *prsA2* based upon bacterial recovery from the livers and spleens of infected mice serves to emphasize the essential contributions of PrsA2 to *L. monocytogenes* virulence. The magnitude of the *prsA2* virulence defect is comparable to that observed for strains completely lacking *hly* or *prfA*, genes encoding two well-established essential virulence factors (51, 54). This was somewhat surprising given that Δ*prsA2*::*erm* strains are not completely lacking in secreted LLO and PC-PLC activity; however, it is probable that PrsA2 contributes to the folding and/or activity of virulence factors in addition to LLO and PC-PLC. Preliminary analysis of secreted and surface-associated proteins from WT and *prsA2* mutant strains indicates that a number of proteins appear to be dependent upon PrsA2 activity for secretion and surface association (data not shown). The reduction in bacterial uptake by macrophages (Fig. 3A), also observed for some additional cell lines (data not shown), may indicate a role for PrsA2 in proper folding of proteins required for bacterial adhesion and/or invasion. PrsA2 function may therefore be required for proper function of additional *L. monocytogenes*-secreted proteins, and it remains to be determined how many of these have functions related to host infection.

Western blot analysis of secreted and cell surface-associated proteins strongly suggests that PrsA2 contributes to LLO stability. PrsA2's role in LLO stability was especially evident in the presence of the mutationally activated *prfA*(*L140F*) allele, which increases the expression of *L. monocytogenes* virulence genes to levels comparable to those observed for cytosolic bacteria (45, 47, 72, 78). Degradation of LLO was evident for both secreted and cell-associated forms of the protein for strains containing WT *prsA2* as well as the Δ*prsA2*::*erm* mutation; however, strains lacking *prsA2* exhibited more extensive degradation (Fig. 6). Changes in protease susceptibility of a given protein are often used to detect changes in protein structure or conformation (22, 45, 66); thus, the appearance of novel degradation products observed in the absence of functional PrsA2 may indicate altered LLO structure or reflect improper folding of the protein. Alternatively, PrsA2 may be required for proper folding of a factor that inhibits LLO degradation. Similarly, PrsA2 may assist in the folding of pro-PC-PLC such that it is more efficiently recognized by the Mpl protease for processing (3, 81), or PrsA2 may be required for full activity of Mpl so that it is capable of pro-PC-PLC pro-

cessing. Definition of the precise role of PrsA2 with respect to LLO and PC-PLC activity awaits detailed biochemical analyses.

It is worth noting that the virulence defect observed for strains lacking *prsA2* was substantially greater than that observed for the *prsA2* plasmid insertion mutant (a 5- to 6-log difference versus a 2-log difference in the number of bacterial CFU recovered from the livers of infected mice, respectively) (47). This difference likely reflects the stability of the Δ*prsA2*::*erm* mutant versus the temperature-sensitive plasmid insertion. The construction of a stable *prsA2* mutation also allowed the detection of the fitness defect conferred by the loss of *prsA2* in comparison to WT strains—a defect that was evident in mixed cultures but not in monocultures (see Fig. S1 in the supplemental material). The fitness defect observed for the *prsA2* mutants in mixed cultures may reflect a physiological change in the mutant that contributes to the reduction in bacterial virulence observed in infected mice (Fig. 2). Confirmation of this hypothesis awaits a more detailed understanding of PrsA2's role in *L. monocytogenes* physiology and protein secretion.

It is striking that while *L. monocytogenes* encodes two PrsA-like proteins, only one is clearly required for bacterial virulence. PrsA1 and PrsA2 share significant amounts of amino acid homology (58% identity and 75% similarity) (Fig. 1A). It is not yet known whether PrsA2 has evolved functional capacities distinct from PrsA1 or whether the failure of *prsA1* to compensate for loss of *prsA2* is due to a lack of *prsA1* expression in the appropriate environment. *prsA1*, unlike *prsA2*, does not appear to contain a PrfA binding site within its 5′ upstream region, and little is currently known regarding overall expression patterns for either gene. The construction of *L. monocytogenes* mutant strains that contain *prsA1* coding sequences under the control of the *prsA2* promoter should help to clarify the individual roles of PrsA1 and PrsA2. Unlike *prsA* of *B. subtilis*, neither *prsA1* nor *prsA2* appears essential for bacterial viability; however, it is possible that while PrsA1 cannot compensate for the PrsA2 virulence defect, it may share redundant function with respect to an essential role in *L. monocytogenes*. The PrsA-like protein of *L. lactis* is not essential for bacterial viability, and Drouault et al. (13) have hypothesized that PrsA-like proteins may be more important for bacteria that are highly dependent on extracellular enzymes for nutrient acquisition, as is the case for soil-dwelling *B. subtilis* but not for *L. lactis* grown in milk (13). If the essential nature of PrsA activity is indeed linked to extracellular nutrient acquisition, it will be interesting to determine if *L. monocytogenes* uses one PrsA-like protein for life in the soil (PrsA1) while having adapted a second PrsA-like protein for life within the host (PrsA2).

PrsA-like proteins have been described in other gram-positive bacteria, including *L. lactis* (13), *Streptococcus pyogenes* (38), and *Bacillus anthracis* (76), and additional homologues to PrsA have been identified in the genomes of both pathogenic and nonpathogenic bacteria (13). Most recognized *prsA* genes encode a leader peptide followed by a cysteine, indicative of gene products that are lipoproteins. *B. subtilis* PrsA has been reported to retain activity as a foldase without lipid modification; however, strains with non-lipid-modified PrsA were reduced for the secretion of PrsA-dependent proteins, presumably due to insufficient concentrations of PrsA at the cell surface (36). We first identified PrsA2 as a secreted protein (47); however, preliminary results indicate that a substantial portion of the protein is localized to the cell membrane and cell wall fractions (data not shown). Baumgartner el al (1) have recently confirmed that PrsA2 is indeed lipid modified, although two processed forms of the protein were detected for reasons that were not clear. Some lipoproteins are detectable in secreted protein fractions (1), and it remains to be determined what form of PrsA2 is important for activity and whether PrsA2 activity is restricted to the cell surface, or if secreted PrsA2 fulfills a specific function, perhaps distinct from cell-associated PrsA2. The role of the potential peptidyl-prolyl isomerase activity of PrsA2 also needs to be explored; this activity appears dispensable for the foldase activity of at least some PrsA-like proteins (13, 73).

Recently, Zemansky et al. (82) reported a role for PrsA2 in *L. monocytogenes* virulence. Their work did not distinguish PrsA1 from PrsA2 but did confirm the attenuation of *prsA2* mutants in animal models and the role of PrsA2 in LLO and PC-PLC activity and stability (82). Clearly, PrsA2 is important to the pathogenesis of *L. monocytogenes*. Its apparently unique role (from PrsA1) in the stability of LLO and in pro-PC-PLC processing is intriguing, and it will be interesting to determine what other secreted factors require PrsA2 for full activity. Clarification of the role of PrsA2 in *L. monocytogenes* pathogenesis should provide additional insight into the mechanisms by which gram-positive bacterial pathogens use postsecretion chaperones to regulate secretion and promote virulence within the infected host.

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