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Listeria monocytogenes PrsA2 Is Required for Virulence Factor Secretion and Bacterial Viability within the Host Cell Cytosol[▽]

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In the course of establishing its replication niche within the cytosol of infected host cells, the facultative intracellular bacterial pathogen *Listeria monocytogenes* must efficiently regulate the secretion and activity of multiple virulence factors. *L. monocytogenes* encodes two predicted posttranslocation secretion chaperones, PrsA1 and PrsA2, and evidence suggests that PrsA2 has been specifically adapted for bacterial pathogenesis. PrsA-like chaperones have been identified in a number of Gram-positive bacteria, where they are reported to function at the bacterial membrane-cell wall interface to assist in the folding of proteins translocated across the membrane; in some cases, these proteins have been found to be essential for bacterial viability. In this study, the contributions of PrsA2 and PrsA1 to *L. monocytogenes* growth and protein secretion were investigated *in vitro* and *in vivo*. Neither PrsA2 nor PrsA1 was found to be essential for *L. monocytogenes* growth in broth culture; however, optimal bacterial viability was found to be dependent upon PrsA2 for *L. monocytogenes* located within the cytosol of host cells. Proteomic analyses of *prsA2* mutant strains in the presence of a mutationally activated allele of the virulence regulator PrfA revealed a critical requirement for PrsA2 activity under conditions of PrfA activation, an event which normally takes place within the host cell cytosol. Despite a high degree of amino acid similarity, no detectable degree of functional overlap was observed between PrsA2 and PrsA1. Our results indicate a critical requirement for PrsA2 under conditions relevant to host cell infection.

During the course of infection, bacterial pathogens are dependent upon the secretion of multiple protein products that modulate host cell physiology and facilitate bacterial growth. A number of protein secretion systems have been identified and functionally characterized for Gram-negative bacteria for which the existence of both an inner and outer membrane presents a significant barrier to protein translocation (12, 17, 20, 35, 62, 79, 120). In Gram-positive bacteria, secreted proteins are translocated across the single bacterial cell membrane in an unfolded state and delivered to the compartment existing between the membrane and the cell wall (80). The cell walls of Gram-positive bacteria consist of a thick matrix of peptidoglycan layers and glycopolymers, including teichoic acids and lipoteichoic acids (111), and these abundant anionic polymers have a high capacity to bind divalent metal ions and cationic molecules (5, 70, 108). Proteins that are translocated across the bacterial membrane therefore enter a challenging environment for protein folding based on the high density of negative charge, high concentrations of cations, and low pH (80, 108). Within this environment, secreted proteins may additionally require further posttranslational modification, proteolytic activation, or sequestration prior to release for interaction with host cell targets. It should be noted that not all secreted proteins are found in the extracellular milieu, as many are specifically localized at the membrane or within the cell wall. Proteins present in bacterial culture supernatants thus constitute a group of exoproteins to which numerous pathogenic traits can be attributed (17).

For the facultative intracellular pathogen *Listeria monocytogenes*, protein secretion has been reported to occur primarily via the Sec-mediated secretion pathway (16). Proteins secreted via Sec-dependent secretion include well-characterized virulence factors, such as the internalins InlA and InlB, which mediate host cell invasion (6, 32, 50–52, 60, 72, 87), listeriolysin-O (LLO) and the broad-range phosphatidyl-choline phospholipase (PC-PLC), which mediate vacuole membrane lysis (21, 36, 37, 41, 47, 65, 82–84, 101, 118), and the surface protein ActA, which mediates actin polymerization and cell-to-cell spread within the host (3, 9, 18, 54, 90, 91, 93, 115). These proteins are critical for the establishment of the *L. monocytogenes* replication niche within the cytosol of infected host cells (28, 40, 86, 102).

L. monocytogenes PrsA1 and PrsA2 are secreted proteins that are predicted to function as parvulin-type peptidyl-prolyl isomerase (PPIase) chaperones at the bacterial membrane-cell wall interface to assist in the folding and stability of secreted proteins (1). PrsA2 appears to be primarily adapted for *L. monocytogenes* pathogenesis, based on the regulation of *prsA2* expression by the central virulence transcriptional activator PrfA and on the essential requirement for PrsA2 for bacterial virulence in mice (1, 74, 121). The loss of PrsA2 dramatically reduces bacterial cell-to-cell spread in monolayers of mouse fibroblast cells and also reduces LLO stability and impedes the processing of PC-PLC to its enzymatically active form (1, 13, 121). Zemansky et al. have additionally demonstrated that *prsA2* deletion mutants are defective for bacterial flagellum-mediated swimming motility, an observation that suggests multiple roles for PrsA2 both inside and outside infected host cells

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Description	Designation	Reference
TOP10	*E. coli* host strain used for recombinant pPL2 plasmids		
SM10	*E. coli* host strain for conjugation of pPL2 plasmids		
NF-L100	*L. monocytogenes* 10403S parent strain		
NF-L1167	10403S *actA-gus-neo prfA*(*L140F*)	L140F strain	116
NF-L1651	10403S with Δ*prsA2*::*erm*	Δ*prsA2* mutant	1
NF-L1656	NF-L1651 (Δ*prsA2*::*erm*) with integrated pPL2-*prsA2* (pNF1255)	Δ*prsA2* + pPL2-*prsA2* mutant	1
NF-L1483	HEL 402-10403S with a *prsA1* in-frame deletion	Δ*prsA1* mutant	1
NF-L1637	NF-L1167 [*prfA*(L140F)] with Δ*prsA2*::*erm*	*prfA*(*L140F*) Δ*prsA2* mutant	1
NF-L1631	NF-L1438 (Δ*prsA1*) transduced with Δ*prsA2*::*erm*	Δ*prsA1* Δ*prsA2* mutant	This work
NF-L1605	10403S with a Δ*htrA* in-frame deletion	Δ*htrA* mutant	113
NF-L1633	NF-L1605 (Δ*htrA*) transduced with Δ*prsA2*::*erm*	Δ*htrA* Δ*prsA2* mutant	This work
NF-L1665	NF-L1633 (Δ*htrA* Δ*prsA2*) with integrated pPL2-*prsA2* (pNF1255)	Δ*htrA* Δ*prsA2* + pPL2-*prsA2* mutant	This work
NF-L1670	NF-L1651 (Δ*prsA2*::*erm*) with integrated pPL2-P_{prsA2}-*prsA1* (pNF1611)	Δ*prsA2* + pPL2-P_{prsA2}-*prsA1* mutant	This work
pNF1255	pPL2 containing the *prsA2* open reading frame for complementation	pPL2-*prsA2* mutant	1
pNF1611	pPL2 containing P_{prsA2}-*prsA1* SOE product	pPL2-P_{prsA2}-*prsA1* mutant	This work

(121). In contrast to its homologue in *Bacillus subtilis*, PrsA2 is not required for *L. monocytogenes* viability, and Δ*prsA2* mutants replicate very similarly to wild-type strains in broth culture and on agar medium (1, 80).

Unlike *L. monocytogenes* Δ*prsA2* mutants, strains lacking *prsA1* are fully virulent in mouse models of infection (1). *prsA1* is not required for bacterial growth in broth culture, and its potential contributions to other aspects of *L. monocytogenes* physiology are as yet undefined. PrsA2 and PrsA1 are highly similar at the amino acid sequence level; thus, it is possible that PrsA2 and PrsA1 share some degree of functional overlap (1). In *B. subtilis*, the depletion of PrsA leads to the induction of the CssR/S two-component system and increased expression of the HtrA chaperone/protease in response to the accumulation of misfolded proteins at the bacterial membrane-cell wall interface (48, 80). The loss or depletion of both PrsA2 and PrsA1 in *L. monocytogenes* could potentially elicit a similar membrane stress response if one or both are required for the folding of a large number of secreted proteins.

In this study, we investigated the potential functional overlap of PrsA2 and PrsA1 through the construction of an *L. monocytogenes* Δ*prsA1* Δ*prsA2* double mutant. In addition, exoproteomic analyses were used to identify proteins whose secretion or localization was altered due to the lack of either PrsA2 or PrsA1. Our findings indicate a critical PrsA1-independent role for PrsA2 in the maintenance of full bacterial viability in the host cell cytosol, further strengthening the link between PrsA2 function and *L. monocytogenes* virulence.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. All bacterial strains used in this study are listed in Table 1. *L. monocytogenes* 10403S (NF-L100) and the 10403S *prfA*(*L140F*) strain (NF-L1167) were used as the parent strains for the construction of gene deletion mutants (67, 74, 116). All strains were grown overnight at 37°C with agitation in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) or Luria broth (LB) (Invitrogen, Carlsbad, CA) unless otherwise specified. Antibiotics were used at the following concentrations: streptomycin (200 μg/ml), chloramphenicol (7.5 and 5 μg/ml), erythromycin (1 μg/ml), and neomycin (10 μg/ml).

Construction of *L. monocytogenes* Δ*prsA1* Δ*prsA2* and Δ*htrA* Δ*prsA2* mutant strains. Strains containing multiple in-frame deletion mutations were generated by phage-mediated transduction of *L. monocytogenes* Δ*prsA1* (NF-L1483) and Δ*htrA* (NF-L1605) mutant strains (1, 113). U153 bacteriophage-mediated transduction was performed as previously described (1, 116). Briefly, phage lysates were prepared from the *L. monocytogenes* Δ*prsA2*::*erm* mutant (NF-L1651) (1). Lysates were mixed at a 1:1 ratio with bacteria (10^8 phage to 10^8 CFU of each strain) and incubated at room temperature for 40 min in the presence of $CaCl_2$ and $MgCl_2$ (final concentration, 10 mM). Mixtures of bacteria and bacteriophage lysates were then spread onto BHI plates containing 1 μg/ml erythromycin to select for transductants. For each transductant, the replacement of the wild-type *prsA2* allele with the *prsA2*::*erm* mutation was confirmed by PCR amplification of the appropriate chromosomal region. Confirmed mutant strains were designated as follows: Δ*prsA1* Δ*prsA2* (NF-L1631) and Δ*htrA* Δ*prsA2* (NF-L1633) double mutants.

Construction of an *L. monocytogenes* Δ*prsA2* strain expressing *prsA1* under the regulation of the *prsA2* promoter. A P_{prsA2}-*prsA1* (*prsA2* promoter to *prsA1* open reading frame [ORF]) fusion was generated as follows: two DNA fragments were generated by PCR using primer pairs P2A (5′-GGC-GAGCTC-CCTAAAATC AATCAAC-3′)/P2B (5′-CATCACTTTTTTTAATTTTGTCAATAAATAAAA CACACTCCTTAG-3′) and P1A (5′-CTAAGGAGTGTGTTTTATTTATTGA CAAAATTAAAAAAAGTGATG-3′)/P1B (5′-GGC-GGTACC-TTAGTTAG ATGTAGTCGTTGA-3′). The fragments were purified and used in a splicing-by-overlap extension (SOE) PCR along with primer pair P2A and P1B to generate a 1,520-bp fragment. The fragment was digested with KpnI and SacI and subcloned into the plasmid vector pPL2 to generate pNF1611. pNF1611 was introduced into the *L. monocytogenes* Δ*prsA2* mutant strain (NF-L1651) via conjugation as previously described (29, 57). The resultant strain was designated NF-L1670.

Growth curves and bacterial cell viability assays. Bacterial growth was measured in BHI broth beginning with a 1:20 dilution of overnight culture into fresh BHI. Growth was measured each hour by determining the absorbance at an optical density at 600 nm (OD_{600}) in a spectrophotometer. For measurement of growth in terms of the number of CFU, 1-ml culture aliquots were removed at each hour and serially diluted into phosphate-buffered saline (PBS) (0.144 g/ml KH_2PO_4, 9 g/ml NaCl, 0.795 g/ml Na_2HPO_4 [anhydrous]) and CFU were enumerated after overnight incubation on BHI plates at 37°C. Cell viability was measured using the Live/Dead *Bac*Light bacterial viability kit (Molecular Probes, Invitrogen, Carlsbad, CA). Briefly, bacterial strains were diluted 1:20 from an overnight culture into 19 ml of BHI. Cultures were grown for 3 h to an approximate OD_{600} of ~0.6, at which point 10 ml of each culture was removed and centrifuged at 8,500 rpm for 15 min. The bacterial pellets were washed with 1 ml of PBS and resuspended in 1 ml of PBS prior to staining. A 2× propidium iodide-Cyto9 solution was prepared according to the manufacturer's instructions in 5 ml of water and mixed 1:1 with the bacterial cell suspension. After incubation for 15 min at room temperature, 8 μl of cell suspension was placed onto a glass microscope slide and immediately examined using a DeltaVision fluorescent microscope (Applied Precision, Issaquah, WA). Images were acquired using Softworx Image Acquisition software (Applied Precision, Issaquah, WA). A minimum of 10 fields were viewed for each strain and total bacteria per field (live and dead) from at least three independent experiments were enumerated.

Bacterial intracellular growth in tissue culture cells. J774 macrophage-like cells were maintained as previously described (10, 68, 96). Macrophages (2×10^6) were seeded onto glass coverslips in tissue culture dishes the night prior to infection. Overnight cultures of *L. monocytogenes* were used to infect cells at a multiplicity of infection of 0.1 bacterium to 1 macrophage. The infection was

allowed to proceed for 30 min, followed by three washes with PBS and the addition of fresh medium containing gentamicin (30 μg/ml) to kill extracellular bacteria. At the indicated time points, coverslips were removed and lysed in 5 ml H_2O with vigorous vortexing. Lysates were spread onto LB agar plates and incubated at 37°C overnight. Bacterial CFU were enumerated the following day. The data shown (see Fig. 2) are representative of results from three independent experiments.

Plaque assays. Plaque assays were conducted as previously described (95). Briefly, L2 fibroblasts were infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 30 to 1. After 1 h, gentamicin was added in a Dulbecco's modified Eagle's medium (DMEM) agarose (0.7%) overlay. Plaque formation was monitored at 72 h after staining with Neutral Red solution (Sigma, St. Louis, MO) in at least three independent experiments.

Hemolysin assays. Hemolytic activity assays were conducted as previously described with some modifications (1, 11, 53). Overnight cultures of *L. monocytogenes* in LB broth were diluted 1:10 in fresh LB and grown for 5 h at 37°C with shaking. OD_{600} readings were taken, and 1.2 ml of culture was centrifuged at maximum speed for 5 min in a tabletop centrifuge. Bacterial supernatants were normalized based on the OD_{600} of the original cultures to account for any differences in culture density, such that supernatants from cultures with greater optical densities were diluted into a suitable volume of LB to match the culture with the lowest OD_{600}. Serial dilutions of normalized supernatants were made in PBS (pH 5.5) containing 1 mM dithiothreitol (DTT) and 5% washed sheep's red blood cells (RBCs) (Cocalico Biologicals, Reamstown, PA), and samples were incubated for 30 min at 37°C. Hemolytic units are described as the reciprocal of the dilution required for 50% lysis of RBCs and are derived from the results of at least five independent experiments.

Detection of PC-PLC activity. Egg yolk agar was used to measure phospholipase activity (68). Chicken egg yolks were separated and mixed 1:1 (vol/vol) with PBS by vortexing, and 5 ml of the egg yolk suspension was then mixed with molten LB agar at 42°C containing 0.2% activated charcoal (Fisher Scientific, Pittsburgh, PA) and 25 mM glucose-6-phosphate (Sigma, St. Louis, MO) to enhance *plcB* expression (119). The degree of phospholipase activity was detected as a zone of opacity surrounding the bacterial streak after overnight growth at 37°C.

Isolation of bacterial exoproteins for two-dimensional gel electrophoresis. Bacterial exoproteins were prepared from culture supernatants as previously described (1, 74). Bacterial strains [10403S and the Δ*prsA2*, Δ*prsA1*, Δ*prsA1* Δ*prsA2*, *prfA*(*L140F*), and Δ*prsA2 prfA*(*L140F*) mutants] were grown in 20 ml BHI overnight at 37°C with shaking. Bacteria were diluted 1:20 into 200 ml fresh BHI, and growth was monitored for 5 h to an approximate OD_{600} of 1.2. Bacterial cells were centrifuged at 9,000 rpm for 20 min to recover the supernatant to which trichloroacetic acid was added to a final volume of 10%. The exoproteins were precipitated on ice for 30 min, and the protein pellets were recovered following centrifugation at 9,000 rpm for 15 min followed by a wash in 12 ml of ice-cold acetone and an additional centrifugation at 9,000 rpm for 15 min. The pellets were air dried and resuspended in 400 μl 1× SDS boiling buffer without β-mercaptoethanol (5% SDS, 10% glycerol, 60 mM Tris, pH 6.8). Total protein was determined by a bicinchoninic acid (BCA) assay (Fisher Scientific, Pittsburgh, PA) and was used to normalize loading onto two-dimensional (2-D) polyacrylamide gels. Prior to 2-D SDS-PAGE, all samples were run on 1-D SDS polyacrylamide gels to verify the consistency and quality of the sample preparation. All samples were prepared on three independent occasions and used for 2-D SDS-PAGE, resulting in a minimum of two biological replicates for all gels. Samples from biological replicates were each analyzed a minimum of three times, resulting in three technical replicates to validate consistency between samples.

Two-dimensional polyacrylamide gel electrophoresis. Two-dimensional electrophoresis was performed according to the carrier Ampholine method of isoelectric focusing (IEF) (69) by Kendrick Labs, Inc. (Madison, WI), as follows: isoelectric focusing was carried out in a glass tube with an inner diameter of 2.0 mm by using 2% Ampholine mix (pH 4 to 8) (GE Healthcare, Piscataway, NJ, and Serva, Heidelberg, Germany) for 9,600 V · h. One microgram of an IEF internal standard, tropomyosin, was added to the sample. This protein migrated as a doublet with a lower polypeptide spot that had a molecular weight (MW) of 33,000 and a pI of 5.2. A tube gel pH gradient plot was determined with a surface pH electrode. After isoelectric focusing and equilibration for 10 min in buffer O (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 h at 15 mA/gel. The following proteins were used as molecular-weight standards: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000) (Sigma-Aldrich, St. Louis, MO). These standards appear along the basic edge of the Coomassie blue-stained 10% acrylamide slab gel. The Coomassie blue-stained gels were dried between sheets of cellophane with the acid edge to the left.

Protein identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS). All proteomics data were acquired from two independent biological replicates and two or more technical replicates per strain. Gel spots were considered unique if present in one gel but completely absent in another as determined by visual inspection of gels on a light box. Modest differences in protein abundances were thus excluded for these profiles. All unique protein spots were cut out of gels using a fresh scalpel blade and placed into Eppendorf tubes containing 200 μl of sterile water. The remainder of the protein extraction and digestion was performed by the proteomics core facility at the University of Illinois at Chicago Research Resources Center. Briefly, identified spots were cut into 1-mm cubes with a scalpel followed by being washed with 100 mM ammonium bicarbonate, reduction with dithiothreitol in ammonium bicarbonate, and alkylation with iodoacetamide in the dark. Samples were digested overnight with Promega modified sequencing-grade trypsin in ammonium bicarbonate (Promega, Madison, WI). Peptides were liberated using three consecutive extractions with ammonium bicarbonate at 37°C, followed by sample concentration using a SpeedVac.

LC-MS/MS was carried out using a Thermo Scientific LTQ FT instrument equipped with a Dionex UltiMate 3000 two-dimensional microcapillary high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA). Peptides were separated on a C_{18} column after being eluted with a gradient. Generated peak lists were extracted from the resulting chromatograms as mascot generic format (MGF) files by using ReAdW (Institute for Systems Biology, Seattle, WA) and in-house software and then searched using a Mascot 2.2 search engine (Matrix Science, Boston, MA) against the List_monocyt *Listeria monocytogenes* NCBI database (53,458 sequences; 14,892,194 residues) by using a peptide tolerance of 10 ppm and carbamidomethylation of cysteine and oxidation of methionine as variable modifications. Scaffold 2.4 (Proteome Software, Portland, OR) software was used to merge and display only the results with a 95% confidence and 2 or more unique peptide matches. The average false discovery rate was between 3% and 5%, as estimated by Mascot and automated decoy database searching.

Mouse intravenous infections. Animal procedures were IACUC approved and performed in the Biological Resources Laboratory at the University of Illinois at Chicago. Bacterial cells were prepared as previously described (1). Female Swiss Webster mice (6 to 8 weeks old) were inoculated via the tail vein with 200 μl PBS containing 2×10^4 CFU of each bacterial strain tested. After 72 h, mice were sacrificed and both the liver and spleen were isolated. Each organ was homogenized in 5 ml of H_2O using a Tissue Master 125 homogenizer (Omni, Marietta, GA), and dilutions were spread onto BHI agar plates to quantify the bacterial burden in each organ.

Statistics. Statisical analysis was performed using Prism Software (GraphPad version 2.0) and is described in the figure legends of experiments for which statistics were necessary. Where appropriate, a Student *t* test or one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to identify statistically significant differences. In all cases, a P value of <0.05 was considered significant.

RESULTS

***In vitro* characterization of an *L. monocytogenes* Δ*prsA1* Δ*prsA2* double mutant.** *L. monocytogenes* Δ*prsA1* and Δ*prsA2* mutants exhibit apparently normal growth characteristics in broth culture (1). Given that PrsA homologues fulfill essential functions in some Gram-positive bacteria (such as *B. subtilis*), we sought to determine if *L. monocytogenes* PrsA1 and PrsA2 shared any functional redundancy that contributed to bacterial growth in broth culture. In contrast to PrsA's essential function in *B. subtilis*, an *L. monocytogenes* mutant lacking both *prsA1* and *prsA2* was fully viable (Fig. 1A). In tissue culture cells, the growth of the Δ*prsA1* Δ*prsA2* mutant resembled that of the single Δ*prsA2* mutant based on the slightly lower levels of bacterial uptake into J774 macrophages and a leveling off of bacterial growth at late time points (Fig. 1B). The successful construction of the Δ*prsA1* Δ*prsA2* double mutant demonstrated that neither gene product serves an essential function for *L. monocytogenes* growth *in vitro*.

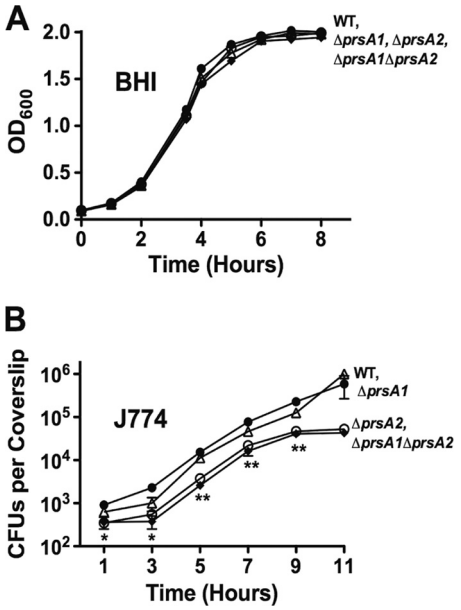

FIG. 1. *L. monocytogenes* Δ*prsA1* Δ*prsA2* mutants exhibit normal growth patterns in broth culture and resemble single Δ*prsA2* mutants in infected tissue culture cells. (A) Bacterial growth in BHI broth as determined by optical density measurements at 600 nm. The data shown are representative of results from three independent experiments, each performed in duplicate. (B) J774 macrophage-like cells were infected at an MOI of 0.1 bacterium to 1 macrophage, and intracellular bacterial replication was measured at the indicated time points following the addition of gentamicin (30 μg/ml) at 1 h postinfection. Data shown are the averages of results from three independent experiments. ●, wild type; ○, Δ*prsA2* mutant; △, Δ*prsA1* mutant; ◆, Δ*prsA1* Δ*prsA2* mutant. Statistical significance was determined using one-way analysis of variance with Tukey's multiple comparison test (*, $P < 0.01$; **, $P < 0.001$).

Loss of *prsA1* does not exacerbate the virulence-associated defects of a Δ*prsA2* mutant. The loss of *prsA2* reduces LLO-associated hemolytic activity as well as PC-PLC-associated phospholipase activity and severely diminishes the capacity of *L. monocytogenes* to spread to adjacent cells (1, 121). Δ*prsA2* mutants are highly attenuated in a mouse infection model, with a greater than 100,000-fold reduction in bacterial burdens recovered from the livers and spleens of infected animals (1, 121). In contrast, a *prsA1* deletion mutant exhibits none of the *prsA2*-associated defects either *in vitro* or *in vivo* (1). To determine whether PrsA1 shared any detectable degree of functional overlap with respect to PrsA2-associated activities in *L. monocytogenes*, the Δ*prsA1* Δ*prsA2* double mutant was assessed for hemolytic activity, phospholipase activity, cell-to-cell spread in tissue culture cells, and bacterial virulence in infected mice. In all cases, the phenotypes observed for the double-deletion mutant resembled those of the single *prsA2* deletion mutant, with a 2-fold reduction in hemolytic activity and a similar reduction in phospholipase activity on egg yolk agar plates (Fig. 2A and B). The Δ*prsA1* Δ*prsA2* mutant also resembled the Δ*prsA2* mutant with respect to its ability to spread cell-to-cell in monolayers of L2 fibroblast cells (plaque formation) (Fig. 2C) and in the magnitude of its virulence defect in infected mice (Fig. 3). Additionally, expression of *prsA1* under the control of the *prsA2* promoter in a Δ*prsA2* background was unable to complement any *in vitro* defect

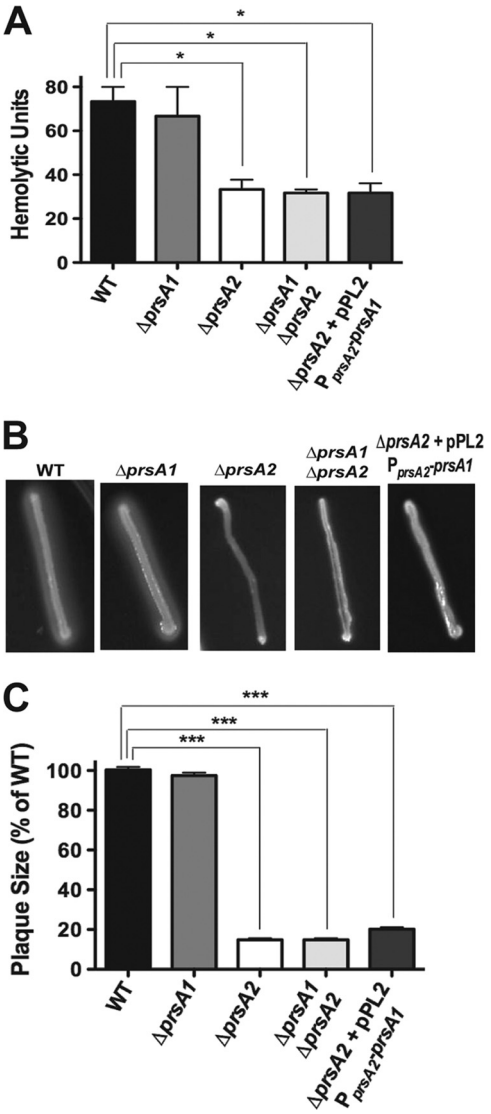

FIG. 2. PrsA1 shares no apparent functional overlap with PrsA2 in *in vitro* assays. All assays were conducted using the wild-type strain or Δ*prsA2*, Δ*prsA1*, Δ*prsA1* Δ*prsA2*, or Δ*prsA2* plus pPL2-P_{prsA2}-*prsA1* mutant strains. (A) Measurement of LLO-associated bacterial hemolytic activity. Dilutions of bacterial culture supernatants were assessed for their ability to lyse sheep's red blood cells (RBCs) *in vitro*. The reciprocal of the supernatant dilution that resulted in 50% lysis of RBCs (hemolytic units) was determined in a minimum of five independent experiments, each conducted in duplicate, and the average of results is shown. (B) Phospholipase activity as determined by the incubation of bacterial strains on egg yolk agar plates followed by the observation of a zone of opacity surrounding the bacterial streak. A representative image from one of five plates is shown. (C) Plaque formation in L2 fibroblast monolayers in the presence of gentamicin. At 72 h postinfection, plaques were measured using a micrometer and the plaque size for the wild type was set to 100%. Plaque assays were repeated at least three times, with results from a minimum of 20 plaques averaged per experiment. Statistical significance was determined using one-way analysis of variance with Tukey's multiple comparison test (*, $P < 0.01$; ***, $P < 0.0001$).

associated with a *prsA2* mutant (Fig. 2). Thus, although PrsA2 and PrsA1 share a high degree of amino acid similarity, PrsA1 does not compensate to any measurable degree for any PrsA2-associated phenotype.

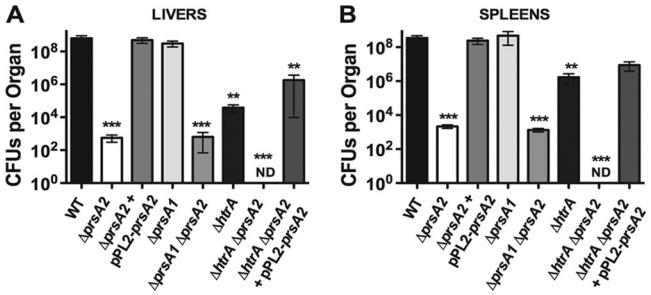

FIG. 3. Loss of *htrA* significantly impairs the survival of Δ*prsA2* mutants in mice. Mice were injected via the tail vein with 2×10^4 CFU of either the wild-type strain or Δ*prsA2*, Δ*prsA2* plus pPL2-*prsA2*, Δ*prsA1*, Δ*prsA1* Δ*prsA2*, Δ*htrA*, Δ*htrA* Δ*prsA2*, or Δ*htrA* Δ*prsA2* plus pPL2-*prsA2* mutant strains. The liver and spleen of infected mice were recovered at 72 h postinoculation, and the bacterial burden in each organ was determined. A minimum of 5 mice were inoculated per strain tested, and the means and standard deviations are shown. Statistical significance was determined using one-way analysis of variance with Tukey's multiple comparison test (**, $P < 0.001$; ***, $P < 0.0001$).

Proteomic analyses reveal altered exoprotein patterns for strains lacking *prsA2* but not those lacking *prsA1*. It has been previously reported that both LLO stability and activity as well as the processing of the phospholipase PC-PLC to its mature form are reduced in *L. monocytogenes* Δ*prsA2* strains (1, 121). Altered SDS-PAGE polypeptide profiles have also been observed for supernatant proteins derived from Δ*prsA2* strains versus those derived from the wild type (121). We sought to better define how the loss of *prsA2* or *prsA1* affects the *L. monocytogenes* exoproteome by identifying changes in the supernatant protein profiles for the Δ*prsA1*, Δ*prsA2*, and Δ*prsA1* Δ*prsA2* mutant strains in comparison to the wild type by using two-dimensional polyacrylamide gel electrophoresis.

An examination of the exoprotein profiles for supernatants derived from the wild-type and Δ*prsA2*, Δ*prsA1*, and Δ*prsA1* Δ*prsA2* mutant strains resulted in a number of interesting observations (Fig. 4). First, consistent with the absence of a detectable phenotype for a Δ*prsA1* mutant in comparison to the wild-type strain (Fig. 1 to 3), no notable differences in secreted polypeptides were observed for this mutant compared to those of the wild type (Fig. 4A and 4C). In contrast, the supernatant protein profiles derived from a Δ*prsA2* mutant exhibited striking differences compared to the wild-type and Δ*prsA1* mutant profiles, while the Δ*prsA1* Δ*prsA2* double mutant profile resembled that of the Δ*prsA2* mutant (Fig. 4). Table 2 lists the identities of the proteins isolated based on differences in exoprotein profiles. Twenty-three proteins were identified based on their presence in the supernatants derived from wild-type strains (and the Δ*prsA1* mutant) in comparison to strains lacking *prsA2*. The majority of the identified proteins (16/23) could be classified into four functional categories: virulence factors,

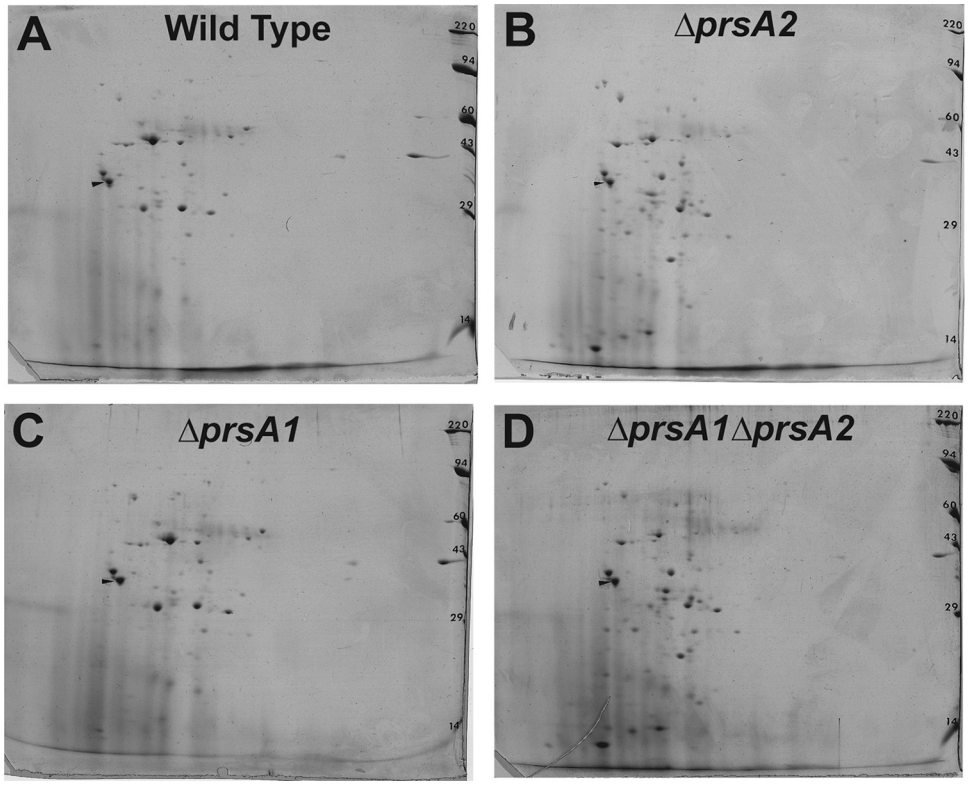

FIG. 4. Two-dimensional electrophoresis of bacterial exoproteins reveals significantly altered protein profiles for Δ*prsA2* mutants. Supernatants from the wild-type strain or Δ*prsA1*, Δ*prsA2*, or Δ*prsA1* Δ*prsA2* mutant strains were trichloroacetic acid (TCA) precipitated and subjected to two-dimensional SDS-PAGE, revealing a significantly altered protein profile for Δ*prsA2* mutant supernatants (B) in comparison to wild-type supernatants (A). A Δ*prsA1* mutant supernatant profile (C) closely resembled that of the wild type, while a Δ*prsA1* Δ*prsA2* mutant profile (D) was remarkably similar to the Δ*prsA2* single mutant.

TABLE 2. Proteins present in wild-type gels but absent from Δ*prsA2* mutant gels

Function or functional class	Gene no.	Protein name	Protein description	No. of peptide matches	% coverage	Protein score	Reference(s)
Virulence factors	lmo0202	LLO	Listeriolysin-O	17	47	586	83 and 86
Cell surface and cell wall metabolism	lmo2467		Chitin binding protein/carbohydrate binding protein	10	35	979	59
	lmo1521		*N*-Acetylmuramoyl-L-alanine amidase	8	30	1298	
	lmo1883		Chitinase	4	16	477	59
	lmo2522		LysM domain penicillin binding protein	2	12	443	74
	lmo2039	PbpB	Penicillin binding protein	5	8.9	414	43
	lmo1892	PbpA	Penicillin binding protein 2A	6	11	257	43
	lmo0540		Penicillin binding protein, putative	5	20	118	43
	lmo2591		GW repeat surface protein	3	24	210	
	lmo2504		Peptidase M48 family	5	21	309	
Transport/binding proteins and lipoproteins	lmo2196	OppA	Oligopeptide ABC transporter, oligopeptide binding protein	9	26	489	8
	lmo0135	CtaP	Cysteine transport-associated protein (lipoprotein)	14	38	1668	74 and 117
Detoxification	lmo1439	Sod	Superoxide dismutase	3	16	670	2 and 100
	lmo0927	IspB	Sulfatase family protein	4	9.8	456	110
	lmo2079		Putative lipoprotein	6	30	1099	
Mobility and chemotaxis	lmo0690	FlaA	Flagellin	2	11	81	109
Hypotheticals	lmo1752		Hypothetical protein	4	22	249	
Metabolism	lmo2459	Gap	Glyceraldehyde 3-phosphate dehydrogenase	6	20	1323	81
	lmo1620		Similar to dipeptidase PepV	7	18	420	
	lmo2455	Eno	Enolase	2	20	74	81
Specific pathways	lmo0429		Glycosyl hydrolase	2	2	20	
Membrane bioenergetics	lmo0013	QoxA	AA3-600 quinol oxidase subunit II	5	17	464	
Amino-acyl tRNA synthetases	lmo1755	GatA	Glutamyl-tRNA (Gln) amidotransferase subunit A	2	6	110	

cell surface/cell wall metabolism, transport/binding proteins, and proteins involved in stress responses/detoxification/adaptation to atypical conditions. Included among the virulence proteins was LLO, whose stability has already been linked to PrsA2 activity (1, 121). Additional proteins were identified with functions associated with motility, metabolism, and membrane bioenergetics, as well as a hypothetical protein and amino-acyl tRNA synthetase.

Table 3 lists exoproteins identified in Δ*prsA2* mutant supernatants but absent in supernatants derived from the wild-type and Δ*prsA1* strains. In contrast to the majority of the proteins identified in wild-type supernatants, none of the 24 differing proteins identified within Δ*prsA2* mutant supernatants were predicted to be bona fide secreted proteins based on the presence of a recognizable signal peptide sequence. All were predicted to be located within the bacterial cytosol with roles in protein folding, protein synthesis, amino acid synthesis, sugar and lipid metabolism, and glycolysis, as well as other physiological pathways. Despite the lack of a secretion signal sequence, nine of the proteins predicted to reside within the bacterial cytosol have been previously identified in *L. monocytogenes* exoproteome analyses (23, 97). It thus appears that a number of additional bacterial proteins with cytosolic functions have altered localization in the absence of functional PrsA2.

The presence of 15 proteins of predicted cytosolic functions within Δ*prsA2* mutant supernatants could potentially reflect compromised bacterial membrane permeability and/or bacterial lysis. While differences in bacterial growth rates and cell densities were not apparent based on optical density measurements (Fig. 1A), limited bacterial cell lysis could have occurred in late-log- or stationary-phase cultures, resulting in the release of cytosolic proteins into the supernatant. Bacterial viability was therefore directly measured for Δ*prsA2* mutants by the assessment of the number of bacterial CFU during growth in broth culture. Wild-type and Δ*prsA2* mutant strains exhibited no detectable differences either in growth rate or in the numbers of viable cells, as the total number of CFU of the Δ*prsA2* mutant remained nearly identical to that of the wild type (Fig. 5A). Similarly, an assessment of membrane integrity and cell viability via Live/Dead staining revealed no differences between the wild-type and Δ*prsA2* strains (Fig. 5B). Thus, the presence of bacterial cytosolic proteins in Δ*prsA2* mutant supernatants does not appear to be due to increased levels of cell lysis or to gross changes in cell membrane permeability.

PrfA activation dramatically alters the exoproteome of a Δ*prsA2* mutant. PrfA is the major transcriptional regulator of *L. monocytogenes*-secreted virulence factors (28, 30). PrfA exists in a low-activity state during bacterial growth in broth culture but becomes activated upon contact with host cells and

TABLE 3. Proteins present in Δ*prsA2* mutant gels but absent from wild-type gels

Function or functional class	Gene no.	Protein name	Protein description	No. of peptide matches	% coverage	Protein score	Reference(s)
Detoxification/adaptation to atypical conditions	lmo2785	Kat	Catalase	13	41	791	26 and 58
	lmo1439	Sod	Superoxide dismutase	8	51	536	2 and 100
	lmo2468	ClpP	ATP-dependent Clp-protease	3	27	146	33 and 34
	lmo0943	Fri	Nonheme iron-containing ferritin	5	50	970	25
Hypotheticals	lmo0995		Hypothetical, similar to *B. subtilis* YkrP protein	2	13	100	
	lmo1597		Hypothetical protein	2	9	126	
Protein folding	lmo1267	Tig	Trigger factor (prolyl isomerase)	7	27	395	7
Protein synthesis	lmo0239	CysS	Cysteinyl tRNA synthetase	5	17	263	61
	lmo1657	Tsf	Translation elongation factor Ts	11	51	1091	
	lmo1314	Frr	Ribosome recycling factor	2	14	102	
	lmo0250	RplJ	50S ribosomal protein L10	5	36	362	27
Termination	lmo2543	Prf1	Peptide chain release factor 1	2	8	63	
Amino acid synthesis	lmo0223	CysK	Cysteine synthase A	11	59	977	22
Metabolism	lmo2459	Gap	Glyceraldehyde-3-phosphate dehydrogenase	5	19	228	81
Metabolism of lipids	lmo1372		2-Oxoisovalerate dehydrogenase E1 component	3	17	187	27
Main glycolytic pathways	lmo2456	Pgm	Phosphoglycerate mutase	12	36	723	
	lmo2457	Tpi	Triose-phosphate isomerase	2	16	169	
Specific pathways	lmo2556	FbaA	Fructose 1,6-bisphosphate aldolase	4	28	149	
	lmo2103	Pta	Phosphotransacetylase	5	29	251	42
	lmo1571	Pfk	6-Phosphofructokinase	6	29	651	112
			1-Phosphofructokinase	3	17	131	
	lmo0210	Ldh	L-Lactate dehydrogenase	2	4	106	
	lmo0191		Phospho-beta-glucosidase	2	16	85	
	lmo1376		6-Phosphogluconate dehydrogenase	13	34	749	

bacterial entry into the cytosol, resulting in the expression and secretion of a number of gene products required for host cell invasion, bacterial intracellular growth, and cell-to-cell spread (86). The signal that leads to PrfA activation is not known, but mutations have been identified within *prfA* that result in constitutive PrfA activation in broth culture (*prfA** mutations) (66, 67, 77, 88, 116). Given that *prsA2* expression is PrfA regulated (74) and that *L. monocytogenes prfA** strains constitutively express significant amounts of secreted virulence gene products (68, 74, 88), we performed additional exoproteome analyses in Δ*prsA2* strains in the presence of *prfA** (Fig. 6A and B). As previously observed, the presence of the *prfA** allele *prfA*(*L140F*) dramatically increased the number and abundance of *L. monocytogenes* exoproteins (74) (compare Fig. 4A with Fig. 6A). In the absence of *prsA2*, the *prfA*(*L140F*) exoprotein profile was dramatically altered (Fig. 6B). Numerous polypeptides that were abundant in *prfA*(*L140F*) strain supernatants were completely absent in Δ*prsA2 prfA*(*L140F*) strains, and an increased number of lower-molecular-weight spots were observed, suggestive of increased levels of protein degradation.

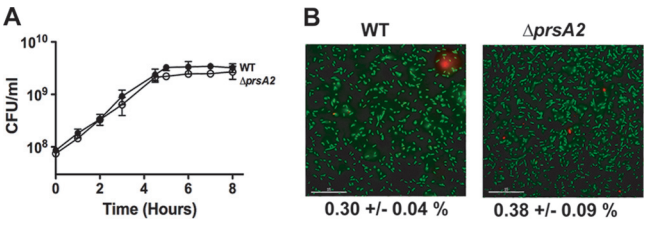

FIG. 5. Δ*prsA2* mutants are fully viable in broth culture despite an increased abundance of bacterial cytosol-associated proteins in culture supernatants. (A) Broth culture growth curves of the wild type and the Δ*prsA2* mutant as measured by plating dilutions of bacterial culture onto solid medium at the indicated time points. Data represent results from one of three experiments performed in duplicate. ●, wild type; ○, Δ*prsA2* mutant. (B) Bacterial Live/Dead staining as performed on log-phase cultures of the wild type or a Δ*prsA2* mutant. Live bacterial cells are stained with Cyto9 (green), while dead or membrane-compromised bacterial cells are stained with propidium iodide (red). The percentage of cells positive for propidium iodide (red) was determined by counting the number of total cells in a minimum of 10 randomly chosen fields from two independent experiments. Statistical significance was determined using a Student *t* test ($P > 0.05$).

Ten proteins were identified in the supernatants derived from *prfA*(*L140F*) strains that were not present in Δ*prsA2 prfA*(*L140F*) mutant supernatants (Table 4). Of note were four well-characterized virulence gene products (ActA, LLO, PC-PLC, and Mpl), two of which (LLO and PC-PLC) have been functionally associated with PrsA2 activity (1, 121). Additional proteins involved in stress responses/detoxification/adaptation to atypical conditions, cell surface/wall metabolism, and transport/binding were identified. In contrast to the 10 proteins identified as present in *prfA*(*L140F*) strain supernatants, a

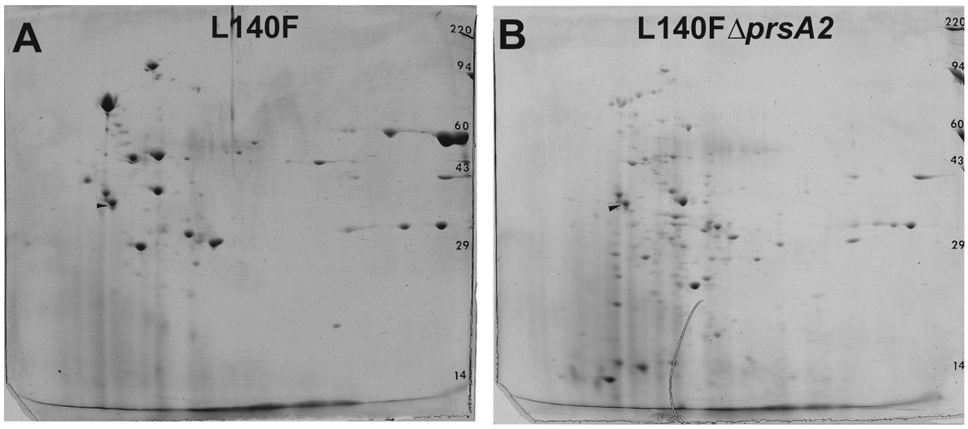

FIG. 6. Two-dimensional electrophoresis of bacterial exoproteins in the presence of PrfA activation reveals significantly altered protein profiles for Δ*prsA2* mutants. Supernatant fractions derived from *prfA*(*L140F*) or Δ*prsA2 prfA*(*L140F*) mutant strains were TCA precipitated and subjected to two-dimensional SDS-PAGE, thereby revealing a significantly altered protein profile for the Δ*prsA2 prfA*(*L140F*) strain (B) in comparison to the *prfA*(*L140F*) strain with the wild-type *prsA2* strain (A). As previously noted, the *prfA*(*L140F*) strain supernatant protein profile (A) is distinct from that of wild-type supernatants in which PrfA is not mutationally activated (Fig. 3A).

large number of polypeptides were identified as present in Δ*prsA2 prfA*(*L140F*) mutant supernatants but absent in *prfA*(*L140F*) strain supernatants (Table 5). Fifteen of the 44 identified gene products were predicted to be secreted proteins and may potentially represent PrsA2 substrates whose abundance and/or localization is altered in the absence of *prsA2*. Virulence factors, cell surface/cell wall proteins, and transport/binding proteins were included within this group. The virulence factors identified were identical to those found in *prfA*(*L140F*) strain supernatants (Table 5); however, they were excised from portions of the gel that reflected smaller-than-expected molecular masses and thus likely represent degradation products or proteins with altered mobility. Similar to a number of the proteins associated with Δ*prsA2* mutant supernatants, 29 proteins were predicted to be cytosolic and to serve as intermediates in metabolic pathways (Table 5). The increased abundance of bacterial cytosolic proteins present in the supernatant fraction of Δ*prsA2 prfA*(*L140F*) strains suggests that PrfA activation enhances and/or exacerbates the aberrant transit of these proteins across the bacterial membrane.

Mutants lacking *prsA2* exhibit decreased viability and altered membrane integrity in the presence of mutationally activated *prfA.** As the supernatants derived from Δ*prsA2 prfA*(*L140F*) strains contained increased numbers of cytosolic proteins in comparison to Δ*prsA2* strains containing the wild-type *prfA* allele (29 versus 15 bacterial cytosolic proteins, respectively), we examined whether *prfA* activation exacerbated the phenotype of the Δ*prsA2* mutant and resulted in compromised membrane integrity or reduced bacterial viability. In the presence of *prfA**, the Δ*prsA2* mutant was dramatically impaired for growth in broth culture, as indicated both by optical density measurements at 600 nm and by enumeration of bacterial CFU (Fig. 7A and B). The assessment of the membrane integrity of the Δ*prsA2 prfA*(*L140F*) mutant by using Live/Dead staining reagents indicated a substantial number of cells that stained positive for propidium iodide uptake [19.16% ± 1.06% (mean ± standard deviation) for the Δ*prsA2*

TABLE 4. Proteins present in L140F strain gels but absent from Δ*prsA2*(*L140F*) mutant gels

Function or functional class	Gene no.	Protein name	Protein description	No. of peptide matches	% coverage	Protein score	Reference(s)
Virulence factors	lmo0204	ActA	Actin assembly-inducing protein	14	28	1239	18 and 54
	lmo0202	LLO	Listeriolysin-O	22	58	3398	83 and 86
	lmo0205	PlcB	Phospholipase-C	13	32	745	92 and 101
	lmo0203	Mpl	Zinc metalloproteinase precursor	1	2.7	62	63, 64, 75, and 76
Detoxification	lmo0927	IspB	Sulfatase family protein	13	32	874	110
	lmo0644		Sulfatase family protein	9	21	526	110
Cell surface and cell wall metabolism	lmo1540		*N*-Acetyl muramoyl L-alanine amidase	11	25	522	
	lmo2505	P45	Peptidoglycan lytic protein	3	11	280	85
	lmo2504		Peptidase M48 family	2	4.4	273	
Transport/binding proteins and lipoproteins	lmo2416		Conserved hypothetical lipoprotein	4	20	174	

TABLE 5. Proteins present in Δ*prsA2 prfA*(*L140F*) mutant gels but absent from *prfA*(*L140F*) gels

Function or functional class	Gene no.	Protein name	Protein description	No. of peptide matches	% coverage	Protein score	Reference(s)
Virulence factors	lmo1786	InlC	Internalin C	8	29	568	19 and 24
	lmo0204	ActA	Actin assembly-inducing protein	7	33	1819	18 and 54
	lmo0202	LLO	Listeriolysin-O	12	44	1444	83 and 86
	lmo0205	PlcB	Phospholipase-C	3	17	36	92 and 101
	lmo0203	Mpl	Zinc metalloproteinase precursor	2	9	179	63, 64, 75, and 76
Cell surface and cell wall metabolism	lmo2505	P45	Peptidoglycan lytic protein	9	23	4374	85
	lmo2522		LysM domain-containing protein	5	34	736	74
	lmo1547	MreC	Rod shape-determining protein	4	11	468	98
Transport/binding proteins and lipoproteins	lmo2416		ABC transporter, substrate binding protein	2	11	204	
	lmo0369		Conserved hypothetical protein	2	14	109	
	lmo1388	TcsA	$CD4^+$ T cell-stimulating antigen (lipoprotein)	2	6.9	227	74
	lmo1757		Putative lipoprotein	2	8.4	395	
	lmo2637		Hypothetical pheromone-like protein	3	13	687	74
	lmo1847		ABC transporter, manganese binding protein	4	15	642	
	lmo1002	PtsH	PTS phosphocarrier protein (Hpr)	4	41	782	14
Protein folding	lmo1474	GrpE	Heat shock protein/cochaperone	4	36	246	
	lmo1267	Tig	Trigger factor (prolyl isomerase)	8	27	1379	7
Metabolism							
Carbohydrates	lmo2455	Eno	Enolase	16	50	3741	81
	lmo2367	Pgi	Glucose-6-phosphate isomerase	2	6.2	225	
	lmo2456	Pgm	Phosphoglycerate mutase, 2,3-biphosphoglycerate independent	10	31	1668	
	lmo2459	Gap	Glyceraldehyde 3-phosphate dehydrogenase	2	8.6	416	81
Lipids	lmo1808	FabD	Malonyl coenzyme A acyl carrier protein transacylase	3	13	296	
Coenzymes	lmo2101		Pyridoxine biosynthesis protein	4	22	744	
Amino acids	lmo2414	SufD	Hypothetical FeS assembly protein	3	11	246	
Nucleic acids	lmo2611	Adk	Adenylate kinase	5	26	875	
Detoxification/adaptation to atypical conditions	lmo0927	IspB	Sulfatase family protein	6	13	820	110
	lmo2785	Kat	Catalase	2	5.1	102	26 and 58
	lmo1439	Sod	Superoxide dismutase	6	37	2726	2 and 100
	lmo2468	ClpP	ATP-dependent Clp protease proteolytic subunit	3	20	602	33 and 34
	lmo0943	Fri	Nonheme iron-binding ferritin	4	36	181	25
Regulation and transcription	lmo0443		Transcriptional regulator (putative)	8	28	1212	
	lmo1280	CodY	Transcriptional repressor	7	37	1050	4
	lmo1496	GreA	Transcription elongation factor	5	44	606	
Protein synthesis	lmo0239	CysS	Cysteinyl-tRNA synthetase	2	7.6	284	61
	lmo1657	Tsf	Translation elongation factor Ts	7	26	853	
	lmo2654	Fus	Elongation factor G	2	4.6	479	
	lmo1314	Frr	Ribosome recycling factor	5	31	676	
	lmo0250	RplJ	50S ribosomal protein L10	4	24	617	27
	lmo0251	RplL	50S ribosomal protein L7/L12	2	20	305	
Amino acid synthesis	lmo0223	CysK	Cysteine synthase A	8	43	631	22
Specific pathways	lmo2556	FbaA	Fructose bisphosphate aldolase	5	18	1045	
	lmo1339		Glucokinase	2	8.4	363	
	lmo0429		Glycosyl hydrolase	2	8	205	
	lmo0811		Carbonic anhydrase	2	11	399	

prfA(*L140F*) mutant compared to 0.38% ± 0.09% for the Δ*prsA2* mutant and 0.13% ± 0.03% for the *prfA*(*L140F*) mutant]. These results strongly suggest that PrsA2 plays a critical role in maintaining bacterial cell viability and membrane integrity under the conditions of increased protein secretion resulting from constitutive PrfA activation.

The loss of the *htrA*-encoded chaperone/protease further exacerbates the viability defects associated with Δ*prsA2* in cytosolic bacteria. In *B. subtilis*, PrsA and the secreted chaperone/protease HtrA appear to be functionally linked (48, 80). Hyyryläinen et al. have demonstrated that depletion of *B. subtilis* PrsA results in an increase in HtrA expression, and it has

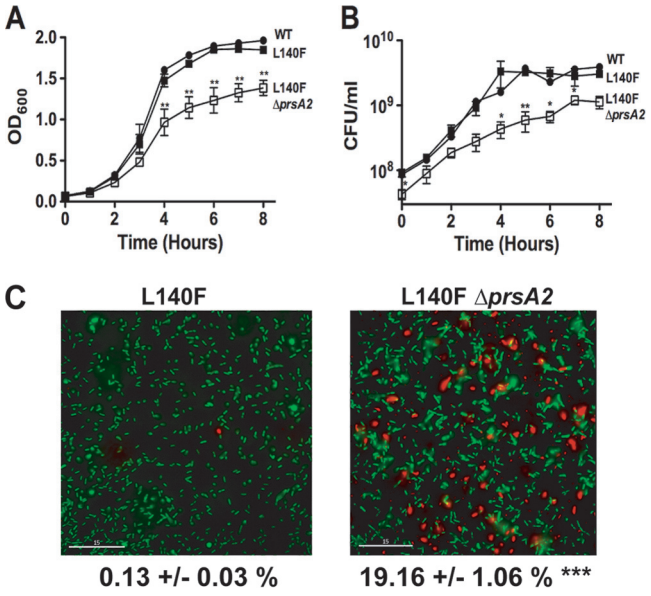

FIG. 7. PrfA activation reduces the viability of Δ*prsA2* mutants. (A and B) Broth culture growth of the wild-type strain and *prfA*(*L140F*) and Δ*prsA2 prfA*(*L140F*) mutants as measured by optical density at 600 nm or by plating dilutions of bacterial culture onto solid medium at the indicated time points. Data represent results from one of three experiments performed in duplicate. ●, wild type; ■, *prfA*(*L140F*) strain; □, Δ*prsA2 prfA*(*L140F*) mutant. (C) Bacterial Live/Dead staining as performed on log-phase cultures of the *prfA*(*L140F*) or Δ*prsA2 prfA*(*L140F*) mutant. Live bacterial cells are stained with Cyto9 (green), while dead cells or cells with a compromised membrane are stained with propidium iodide (red). The percentage of cells positive for propidium iodide (red) was determined by counting the number of total cells in a minimum of 10 randomly chosen fields from two independent experiments. Statistical significance for panels A and B was determined using a one-way analysis of variance with Tukey's multiple comparison test (*, $P < 0.01$; **, $P < 0.001$) and for panel C using a Student *t* test (***, $P < 0.0001$).

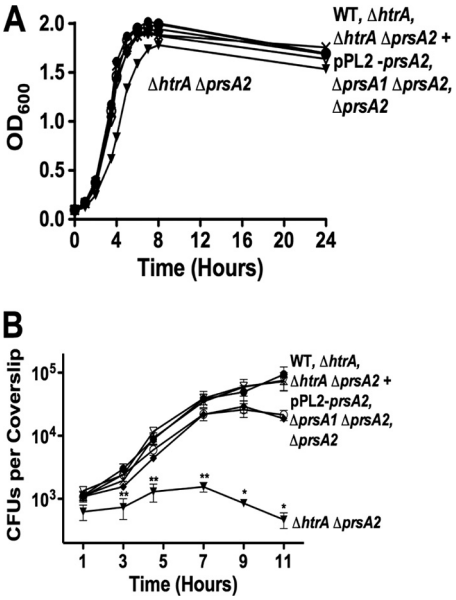

FIG. 8. The loss of *htrA* exacerbates Δ*prsA2*-associated bacterial viability defects within infected host cells. (A) Bacterial growth in BHI broth was determined via optical density measurements at 600 nm. The data shown represent results from one of three independent experiments, each performed in duplicate. (B) J774 macrophage-like cells were infected at an MOI of 0.1 bacterium to 1 macrophage, and bacterial intracellular growth was measured in the presence of gentamicin at the indicated time points. Data shown are the averages of results from three independent experiments. ●, wild type; ○, Δ*prsA2* mutant; X, Δ*htrA* mutant; ◆, Δ*prsA1* Δ*prsA2* mutant; ▼, Δ*htrA* Δ*prsA2* mutant; ▽, Δ*htrA* Δ*prsA2* plus pPL2-*prsA2* mutant. Statistical significance was determined using one-way analysis of variance with Tukey's multiple comparison test (*, $P < 0.01$; **, $P < 0.001$).

been hypothesized that HtrA functions to reduce the accumulation of misfolded proteins at the bacterial membrane-cell wall interface that occurs in the absence of PrsA (48). Given that the viability of a Δ*prsA2* mutant in *L. monocytogenes* is severely compromised in the presence of constitutively activated PrfA (Fig. 7) and that the loss of PrsA2 is likely to result in an increase of misfolded proteins at the bacterial membrane, we examined whether the loss of *L. monocytogenes* HtrA might further exacerbate the Δ*prsA2* defects associated with protein secretion under more natural conditions of PrfA activation. A Δ*prsA2* Δ*htrA* double-deletion mutant exhibited a modest defect in bacterial growth, as measured in rich broth culture and as evidenced by its slightly increased doubling time (67.4 ± 0.58 min for the Δ*prsA2* Δ*htrA* mutant compared to an average of approximately 57.5 ± 1.4 min for the wild type and single-deletion mutants) (Fig. 8A). In contrast, the growth of the Δ*prsA2* Δ*htrA* mutant within the cytosol of J774 macrophages was dramatically reduced in comparison to the wild-type and single-mutant strains (Fig. 8B). The number of Δ*prsA2* Δ*htrA* mutant CFU was observed to increase modestly during the first 5 h postinfection; however, after this time point, bacterial numbers declined, indicative of a loss of bacterial viability. Consistent with the dramatic reduction in intracellular bacterial viability, no bacteria were detected in the livers or spleens of mice infected with Δ*prsA2* Δ*htrA* mutants at 72 h postinfection (Fig. 3A and B). These results indicate that the combined loss of *prsA2* and *htrA* dramatically impacts *L. monocytogenes* viability under conditions of PrfA activation.

DISCUSSION

As a facultative intracellular bacterial pathogen, *L. monocytogenes* relies on the secretion and activity of a number of factors that enable the bacterium to establish its replicative niche within host cells (28, 40, 86, 102). Proteins essential for bacterial virulence must be rapidly translocated across the bacterial membrane and properly folded to carry out target functions. PrsA1 and PrsA2 were recently identified as posttranslocation chaperones with predicted roles in protein folding and stability at the *L. monocytogenes* membrane-cell wall interface (1). Experimental evidence indicated that PrsA2 was distinct from PrsA1 in its requirement for *L. monocytogenes* virulence; however, it remained possible that PrsA1 and PrsA2 shared a limited degree of functional overlap. Here, we demonstrate that PrsA2 alone has been functionally adapted for a role in *L. monocytogenes* virulence and for maintaining bacterial viability within infected host cells.

The successful construction of the Δ*prsA1* Δ*prsA2* double mutant indicates that neither gene product is required for bacterial viability in broth culture, a result which contrasts with

the essential requirement for the single *prsA* gene in *B. subtilis* (107). The Δ*prsA1* Δ*prsA2* double mutant was indistinguishable from a Δ*prsA2* single mutant for all *in vitro* and *in vivo* phenotypes examined, further substantiating the absence of functional redundancy between the two proteins. It remains possible that PrsA1 contributes to *L. monocytogenes* protein secretion under as-yet-undefined environmental conditions that may be encountered outside infected hosts. PrsA1 is present in *L. monocytogenes* broth culture supernatants (F. Alonzo and N. Freitag, unpublished data), but little information exists thus far regarding its expression patterns or functional activity. It is interesting that despite the high degree of amino acid sequence similarity shared between PrsA1 and PrsA2 (58% identify and 75% similarity), PrsA1 was not capable of compensating for the loss of PrsA2 even when the gene was expressed from the *prsA2* promoter (Fig. 2). This result suggests that unique residues and/or regions of PrsA2 are critical for its functional differentiation from PrsA1.

PrsA2 plays an important role in *L. monocytogenes* pathogenesis, as indicated by the greater than 100,000-fold reduction in bacterial burdens recovered from the livers and spleens of mice infected with the Δ*prsA2* mutant in comparison to mice infected with wild-type *L. monocytogenes* (1, 121). Based on the mutant's severely attenuated phenotype, it was somewhat surprising that Δ*prsA2* mutants exhibited only relatively modest reductions in secreted LLO and PC-PLC activity. Previous work has indicated that *L. monocytogenes* mutants that produce as little as one-tenth of the LLO normally secreted in broth culture are still capable of efficient escape from host cell vacuoles (31). Consistent with this observation, the Δ*prsA2* mutant gained access to the host cytosol with kinetics that resembled those observed for cells infected with the wild-type strain (1). PC-PLC has been demonstrated to facilitate bacterial escape from the secondary vacuoles formed during cell-to-cell spread; however, a complete-loss-of-function Δ*plcB* mutant is attenuated for virulence in mice by only approximately 10-fold (92, 101). While modest reductions in LLO and PC-PLC activity seem unlikely to account for the full magnitude of the virulence defect observed for Δ*prsA2* mutants, it remains possible that the reduction in LLO and PC-PLC activity combined with a loss in activity of other PrsA2-dependent substrate proteins might be sufficient to account for the severe attenuation. Proteomic analyses of bacterial supernatants have identified a number of proteins whose localization and/or stability was altered in the absence of PrsA2 (Fig. 4). While these proteins may not all be bona fide PrsA2 substrates, it seems feasible that a subset of these proteins represent PrsA2-interacting partners with roles in bacterial virulence. At least 3 of the proteins identified have been associated with PrsA2 activity and/or PrsA2-dependent phenotypes (LLO, PC-PLC, and FlaA) (1, 121). Additionally, similar proteomics-based approaches have been used successfully to identify substrates for PrsA of *B. subtilis* and for the SurA PPIase chaperone of *Escherichia coli* (103, 106).

Proteins identified based on differential expression patterns in the presence or absence of PrsA2 included recognized virulence factors as well as a substantial number of proteins with reported roles in cell surface and cell wall metabolism. Several gene products were identified as potential penicillin binding proteins (PBPs), including Lmo2522, Lmo2039, Lmo1892, and Lmo0540 (43, 74). PBPs are associated with the synthesis and structural integrity of the bacterial cell wall via their transglycosylase and transpeptidase activities and have been most studied in *L. monocytogenes* as targets of β-lactam antibiotics (38, 39, 44–46, 55, 56, 71, 73, 99, 104, 105). It is possible that in the absence of PrsA2, altered PBP activity negatively impacts the integrity of the cell wall and results in the aberrant secretion of a number of proteins. PrsA-dependent maintenance of PBP activity/stability was recently demonstrated to be critical for the maintenance of cell wall integrity in *B. subtilis* (49). In addition to PBPs, a number of transport/binding proteins were identified, including OppA and CtaP. OppA and CtaP are oligopeptide- and cysteine-associated transport proteins (respectively) that contribute to bacterial survival within the host (8, 74, 117). CtaP secretion is increased following PrfA activation, and the loss of CtaP has been associated with alterations in bacterial membrane integrity; thus, CtaP could potentially contribute to the integrity defect observed for Δ*prsA2 prfA*(*L140F*) mutants (74, 117). Lastly, proteins involved in the protection of the bacterium from reactive oxygen species (catalase and superoxide dismutase) were found to be altered in localization in *prsA2* mutant strains. However, neither Δ*prsA2* nor Δ*prsA2 prfA* (*L140F*) mutants demonstrated any increase in sensitivity to reactive oxygen intermediates in *in vitro* assays (F. Alonzo and N. Freitag, unpublished).

One informative finding resulting from the proteomic analyses of both Δ*prsA2* and Δ*prsA2 prfA*(*L140F*) mutant-derived supernatants was the identification of multiple proteins associated with functional roles in the bacterial cytosol. For Δ*prsA2* mutants, at least 9 of these cytosol-associated proteins have previously been detected in *L. monocytogenes* supernatants, and no obvious alteration in membrane permeability or evidence of increased bacterial cell lysis/death was observed for Δ*prsA2* mutants in comparison to wild-type strains (Fig. 5) (23, 97). An increase in the number of proteins associated with the bacterial cytosol was especially evident in the supernatants derived from Δ*prsA2 prfA*(*L140F*) strains, and these mutants were confirmed to exhibit significant alterations in membrane integrity as well as increased cell lysis. Based on these observations, we speculate that PrsA2 plays a critical role not only in facilitating normal protein secretion but also in maintaining bacterial viability following PrfA activation and the associated increased secretion of PrfA-dependent virulence gene products.

For the examination of the effects of PrfA activation on PrsA2-dependent functions, the mutationally activated *prfA** allele was a useful tool for rapidly comparing differences in exoprotein profiles in culture (74). PrsA2-associated defects in bacterial viability were not obvious for strains containing wild-type *prfA* in broth culture (conditions under which PrfA is not activated) but were detectable for bacteria located within the cytosol after several hours of growth, as shown by a plateau in bacterial numbers beginning at approximately 9 h postinfection (Fig. 1 and 8). The bacterial growth defect observed for intracellular Δ*prsA2* mutants was exacerbated by the deletion of a second posttranslocation chaperone with protease activity, HtrA (94, 113, 114). Interestingly, work with *E. coli* has demonstrated that the loss of the PPIase chaperone SurA in combination with HtrA is lethal, inducing cell death in response to the accumulation of misfolded proteins at the membrane (15,

78, 89). For *L. monocytogenes*, the most significant decrease in bacterial viability was evident only under conditions of PrfA activation (within the host cell cytosol or within infected mice) (Fig. 3 and 8), indicating that neither PrsA2 nor HtrA is required for normal *L. monocytogenes* physiology in broth culture.

PrsA2 thus appears to have been adapted to facilitate *L. monocytogenes* survival under conditions of increased protein secretion, such as occurs during PrfA activation within host cells. We speculate that the dramatic attenuation observed for Δ*prsA2* mutants in animal infection models reflects not only the diminished activity of PrsA2 substrates (such as LLO) but also the decrease in bacterial viability that results from the accumulation of misfolded proteins at the membrane-cell wall interface. It will be interesting to determine if the combined loss of PrsA2 and HtrA under conditions of PrfA activation serves to trigger the induction of a membrane stress response that ultimately leads to bacterial cell death, similar to the loss of SurA and HtrA in *E. coli* (15, 78, 89).

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