Identification of Conserved and Species-Specific Functions of the *Listeria monocytogenes* PrsA2 Secretion Chaperone

Laty A. Cahoon, Nancy E. Freitag

Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, Illinois, USA

The Gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen that relies on the regulated secretion and activity of a variety of proteins that sustain life within diverse environments. PrsA2 has recently been identified as a secreted peptidyl-prolyl *cis/trans* isomerase and chaperone that is dispensable for bacterial growth in broth culture but essential for *L. monocytogenes* virulence. Following host infection, PrsA2 contributes to the proper folding and activity of secreted proteins that are required for bacterial replication within the host cytosol and for bacterial spread to adjacent cells. PrsA2 is one member of a family of Gram-positive secretion chaperones that appear to play important roles in bacterial physiology; however, it is not known how these proteins recognize their substrate proteins or the degree to which their function is conserved across diverse Gram-positive species. We therefore examined PrsA proteins encoded by a variety of Gram-positive bacteria for functional complementation of *L. monocytogenes* mutants lacking *prsA2*. PrsA homologues encoded by *Bacillus subtilis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus mutans*, *Staphylococcus aureus*, and *Lactococcus lactis* were examined for functional complementation of a variety of *L. monocytogenes* PrsA2-associated phenotypes central to *L. monocytogenes* pathogenesis and bacterial cell physiology. Our results indicate that while selected aspects of PrsA2 function are broadly conserved among diverse Gram-positive bacteria, PrsA2 exhibits unique specificity for *L. monocytogenes* target proteins required for pathogenesis. The *L. monocytogenes* PrsA2 chaperone thus appears evolutionarily optimized for virulence factor secretion within the host cell cytosol while still maintaining aspects of activity relevant to more general features of Gram-positive protein translocation.

The translocation of proteins across bacterial membranes is fundamental to bacterial movement, nutrient acquisition, complex behaviors such as biofilm formation and sporulation, and survival. While the processes underlying protein secretion and folding have been well characterized in Gram-negative bacteria (1–3), less attention has generally been focused on Gram-positive bacteria. In contrast to Gram-negative bacteria, Gram-positive organisms need only target proteins across a single membrane; however, there remain a number of challenges associated with protein folding at the Gram-positive membrane-cell wall interface. The Gram-positive cell wall consists of several peptidoglycan layers containing teichoic and lipoteichoic acids that result in a high density of negative charge as well as a capacity to bind cationic molecules (4). Proteins destined for secretion are translocated across the cell membrane in an unfolded state and must fold within the space between the membrane and the cell wall; this space is not only highly charged but freely exposed to the external environment. Proteins destined for release from the bacterium must be further translocated across the thick Gram-positive peptidoglycan cell wall.

The Gram-positive bacterium *Listeria monocytogenes* is an environmental pathogen that is capable of life as a saprophyte within the soil while also maintaining the ability to invade and replicate within mammalian cells (5). In the United States, *L. monocytogenes* is a significant health threat, as it has been associated with numerous multistate foodborne outbreaks resulting in thousands of illnesses and hundreds of deaths (6, 7). *L. monocytogenes* infections pose serious risks to immunocompromised populations, the elderly, pregnant women, and neonates, where fatality rates range from 20 to 50% (8–11). As *L. monocytogenes* transitions between life in the environment to life within the cytosol of infected mammalian host cells, the bacterium requires increased expression of a number of secreted virulence factors that facilitate intracellular

survival by promoting cell entry, bacterial escape from host vacuoles, replication within the cytosol, and spread to adjacent cells (12–17). Many of the secreted virulence factors required for bacterial survival within the host are regulated by a transcriptional activator known as PrfA (positive regulatory factor A) (18–20). PrfA becomes activated following bacterial entry into host cells, and this activation results in a significant increase in protein translocation across the bacterial membrane together with increased expression of factors that promote the folding and activity of secreted proteins, such as the posttranslocation secretion chaperone known as PrsA2 (15, 21, 22).

L. monocytogenes *prsA2* was first identified by transcriptome analysis based on its increased expression following PrfA activation (23). Subsequent proteomic analysis of *L. monocytogenes* secreted proteins indicated that levels of secreted PrsA2 were increased in strains expressing a mutationally activated form of *prfA*, known as *prfA**, that results in the constitutive expression of PrfA-regulated gene products (15). Homologues to PrsA2 appear wide-

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Address correspondence to Nancy E. Freitag, nfreitag@uic.edu.

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spread in other Gram-positive bacteria, where they are reportedly required for the folding and stability of secreted proteins (24–32). *L. monocytogenes* is now known to encode two PrsA proteins, PrsA1 and PrsA2, and these proteins have been characterized for their roles in *L. monocytogenes* protein secretion and pathogenesis (12, 15, 19, 33, 34). *L. monocytogenes* PrsA2 contributes to multiple facets of bacterial pathogenesis and is essential for virulence (33, 34), and it appears to be required for the proper folding and secretion of a number of proteins (12, 25, 35). *L. monocytogenes* mutants lacking PrsA2 exhibit decreased secreted hemolytic and phospholipase activity and are defective for cell-to-cell spread in tissue culture monolayers (33–35). Mice infected with *L. monocytogenes* Δ PrsA2 strains have up to 100,000-fold fewer CFU recovered from livers and spleens than animals infected with the wild-type strain (33, 34). In contrast, PrsA1 shares 75% amino acid similarity and 58% identity with PrsA2 but does not contribute to *L. monocytogenes* pathogenesis (33). Neither *prsA1* nor *prsA2* is required for bacterial growth in broth culture (33).

Given what appears to be a fundamental role for PrsA family members in protein folding and secretion, we were curious to determine how broadly PrsA function is conserved among Gram-positive bacteria. We had observed that the expression of *prsA1* from the *prsA2* promoter did not restore *L. monocytogenes* virulence (12), suggesting that PrsA1 and PrsA2 do not functionally substitute for each other and therefore may recognize distinct substrates. We therefore assessed the degree to which different PrsA homologues were capable of complementing PrsA2-associated activities in *L. monocytogenes*. Thus far, PrsA-like proteins have been best characterized in *L. monocytogenes* and *Bacillus subtilis* (12, 33, 36–40). *B. subtilis* contains one *prsA* gene, which is essential for viability but can be deleted in the presence of high concentrations of magnesium (41), while the single *prsA* alleles of *Streptococcus pneumoniae*, *Streptococcus mutans*, *Staphylococcus aureus*, and *Lactococcus lactis* have been shown to be nonessential (28–30, 32). Similarly to *L. monocytogenes*, *Streptococcus pyogenes* has two *prsA* alleles (31), whereas its close relative *S. pneumoniae* has only one (30). Proteomic analyses of *L. monocytogenes* and *B. subtilis* suggest that these bacteria may have a subset of overlapping PrsA substrates with roles in cell wall metabolism, swimming motility, oligopeptide transport, and membrane bioenergetics (12, 24, 41, 42). *L. monocytogenes* PrsA2 has, however, been associated with the proper folding and activity of several *L. monocytogenes* virulence factors not expressed by *B. subtilis* (12, 24, 33–36). Here we provide evidence based on the complementation of *L. monocytogenes* Δ PrsA2 strains with various *prsA* alleles that indicates both broad conservation of PrsA2 function and species-specific adaptations of the chaperone for *L. monocytogenes* pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains used in this study are listed in Table 1. *L. monocytogenes* 10403S is referred to as the wild-type strain, and *L. monocytogenes* 10403S containing an erythromycin resistance gene (*erm*) in place of *prsA2* coding sequences is the mutant strain (*L. monocytogenes* Δ PrsA2) (33) used for complementation with the designated heterologous Gram-positive *prsA* alleles. *Escherichia coli* One Shot TOP10 (Invitrogen) and S17 (a kind gift from N. Cianciotto, Northwestern University) were used as host strains for recombinant plasmids. Strains were grown in Luria broth (LB) or brain heart infusion (BHI) medium and supplemented with the appropriate antibiotic at the following concentrations: 50 μ g/ml kanamycin and 1 mg/ml streptomycin. The integration plasmid pIMK2 (43) (a kind gift from C. Hill, Cork College)

was used for providing constitutive expression of target genes for genetic complementation.

Construction of *L. monocytogenes* Δ PrsA2 strains containing heterologous Gram-positive *prsA* alleles. Genomic DNA from *L. monocytogenes* 10403S, *B. subtilis* 168, *S. pyogenes* MGAS5005 and *S. mutans* UA159 (a kind gift from M. Federle, UIC), *S. pneumoniae* R6 (a kind gift from D. Morrison, UIC), *S. aureus* USA300 (a kind gift from V. Torres, NYU), and *L. lactis* IL1403 (a kind gift from L. Tao, UIC) was used to amplify the respective *prsA* alleles using the primers specific for each sequence (see Table S1 in the supplemental material). Forward primers were designed with an NcoI restriction site, and reverse primers were designed with a ClaI restriction site, except for *L. monocytogenes* *prsA2*, for which the reverse primer had an XmaI restriction site. PCR-amplified products were cloned into the expression plasmid pIMK2, which contains a constitutive promoter for target gene expression and which integrates into a single neutral site within the *L. monocytogenes* chromosome (43). The use of the pIMK2 constitutive promoter for *prsA* gene expression provided the best current means for optimizing gene expression so as to better assess whether a specific PrsA homologue was at all capable of functional complementation. To generate constructs encoding C-terminal 6 $\times$ His-tagged Gram-positive PrsA proteins, the same forward primer as specified above was used, while a new reverse primer was designed so as to insert DNA encoding a 6 $\times$ His tag prior to the stop codon and a ClaI restriction site to allow for directional cloning into pIMK2 (43) (see Table S1). Plasmids were propagated in *E. coli* One Shot TOP10 cells, transformed into *E. coli* S17 cells, and subsequently introduced into the *L. monocytogenes* Δ PrsA2 strain by conjugation. All genes and plasmids were verified by DNA sequencing prior to introduction into *L. monocytogenes* and subsequently amplified from *L. monocytogenes* transconjugants by PCR and again verified by sequencing analysis. DNA sequencing was performed at the UIC Research Resources Center Core Genomics Facility.

Isolation of bacterial cell-associated and secreted proteins for Western blot analysis. Proteins were isolated as previously described, with minor modifications (33). Specifically, *L. monocytogenes* strains grown overnight were diluted 1:20 into BHI broth and then grown to an optical density at 600 nm (OD₆₀₀) of 0.6. Briefly, for cell-associated proteins, the bacterial pellets from normalized culture volumes were collected by centrifugation and resuspended in 2 $\times$ Laemmli sample buffer with β -mercaptoethanol (Bio-Rad). For secreted proteins, supernatants were recovered and proteins were precipitated using trichloroacetic acid (TCA) at a final concentration of 10%. The TCA-treated supernatants were incubated on ice for 30 min, and precipitated proteins were recovered by centrifugation. The supernatants were discarded, and proteins were washed with ice-cold acetone; then, protein pellets were collected by centrifugation, allowed to air dry, and resuspended in 2 $\times$ Laemmli sample buffer with β -mercaptoethanol. The samples were boiled for 10 min prior to SDS-PAGE followed by Western blot analysis using an anti-His antibody.

Swimming motility assay. Bacteria from mid-log-phase (OD₆₀₀ of ~0.8) BHI broth cultures (2 μ l) were inoculated into soft BHI agar (0.3%) plates and grown at 37°C for 24 h and then at 25°C for 24 h. The motility was measured as the diameter of the spreading colony.

Growth assays at acidic and basic pH and high ionic and nonionic osmolarity. Growth assays were performed as follows: 2 μ l of an overnight culture was inoculated into 2 ml BHI liquid broth at pH 6 or pH 9 (where HCl or NaOH was used to obtain the respective pH) or into broth containing 5% NaCl (wt/vol) or 0.7 M sucrose. These cultures were grown overnight at 37°C, and growth was measured as a function of optical density (OD₆₀₀).

Antibiotic MIC determination. The MIC to prevent bacterial growth was determined following 2 μ l inoculation from mid-log-phase (OD₆₀₀ of ~0.8) cultures into 2 ml BHI broth in 4 ml polypropylene test tubes containing various dilutions of bacitracin, vancomycin, penicillin G, or lysozyme. Cultures were grown at 37°C for 16 h, and the MIC was determined based on the complete inhibition of bacterial growth.

TABLE 1 Strains and plasmids

Strain or plasmid	Description	Designation	Source or reference
Strains			
TOP10	<i>E. coli</i> host strain used for propagation of recombinant pIMK2 plasmids		Invitrogen
S17	<i>E. coli</i> host strain used for conjugation of recombinant pIMK2 plasmids		
NF-L100	<i>L. monocytogenes</i> 10403S parent strain	<i>L. monocytogenes</i> wild type	56
NF-L1651	10403S with $\Delta prsA2::erm$	<i>L. monocytogenes</i> $\Delta prsA2$	33
NF-L3632	NF-L1651 with integrated pIMK2- <i>L. monocytogenes prsA2</i>	<i>L. monocytogenes prsA2</i>	This work
NF-L3634	NF-L1651 with integrated pIMK2- <i>L. monocytogenes prsA1</i>	<i>L. monocytogenes prsA1</i>	This work
NF-L3636	NF-L1651 with integrated pIMK2- <i>B. subtilis prsA</i>	<i>B. subtilis prsA</i>	This work
NF-L3638	NF-L1651 with integrated pIMK2- <i>S. pyogenes prsA1</i>	<i>S. pyogenes prsA1</i>	This work
NF-L3640	NF-L1651 with integrated pIMK2- <i>S. pyogenes prsA2</i>	<i>S. pyogenes prsA2</i>	This work
NF-L3642	NF-L1651 with integrated pIMK2- <i>S. pneumoniae prsA</i>	<i>S. pneumoniae prsA</i>	This work
NF-L3644	NF-L1651 with integrated pIMK2- <i>S. mutans prtM</i>	<i>S. mutans prtM</i>	This work
NF-L3646	NF-L1651 with integrated pIMK2- <i>S. aureus prsA</i>	<i>S. aureus prsA</i>	This work
NF-L3648	NF-L1651 with integrated pIMK2- <i>L. lactis pmpA</i>	<i>L. lactis pmpA</i>	This work
NF-L3650	NF-L1651 with integrated pIMK2- <i>L. monocytogenes prsA2</i> with a C-terminal 6×His tag	<i>L. monocytogenes prsA2</i> -His	This work
NF-L3652	NF-L1651 with integrated pIMK2- <i>L. monocytogenes prsA1</i> with a C-terminal 6×His tag	<i>L. monocytogenes prsA1</i> -His	This work
NF-L3654	NF-L1651 with integrated pIMK2- <i>B. subtilis prsA</i> with a C-terminal 6×His tag	<i>B. subtilis prsA</i> -His	This work
NF-L3656	NF-L1651 with integrated pIMK2- <i>S. pyogenes prsA1</i> with a C-terminal 6×His tag	<i>S. pyogenes prsA1</i> -His	This work
NF-L3658	NF-L1651 with integrated pIMK2- <i>S. aureus prsA</i> with a C-terminal 6×His tag	<i>S. aureus prsA</i> -His	This work
NF-L3660	NF-L1651 with integrated pIMK2- <i>L. lactis pmpA</i> with a C-terminal 6×His tag	<i>L. lactis pmpA</i> -His	This work
Plasmids			
pIMK2	ppL2-derived complementation integration vector		43
pNF-3662	pIMK2 with <i>L. monocytogenes prsA2</i>		This work
pNF-3664	pIMK2 with <i>L. monocytogenes prsA1</i>		This work
pNF-3666	pIMK2 with <i>B. subtilis prsA</i>		This work
pNF-3668	pIMK2 with <i>S. pyogenes prsA1</i>		This work
pNF-3670	pIMK2 with <i>S. pyogenes prsA2</i>		This work
pNF-3672	pIMK2 with <i>S. pneumoniae prsA</i>		This work
pNF-3674	pIMK2 with <i>S. mutans prtM</i>		This work
pNF-3676	pIMK2 with <i>S. aureus prsA</i>		This work
pNF-3678	pIMK2 with <i>L. lactis pmpA</i>		This work
pNF-3680	pIMK2 with <i>L. monocytogenes prsA2</i> containing a C-terminal 6×His tag		This work
pNF-3682	pIMK2 with <i>L. monocytogenes prsA1</i> containing a C-terminal 6×His tag		This work
pNF-3684	pIMK2 with <i>B. subtilis prsA</i> containing a C-terminal 6×His tag		This work
pNF-3686	pIMK2 with <i>S. pyogenes prsA1</i> containing a C-terminal 6×His tag		This work
pNF-3688	pIMK2 with <i>S. aureus prsA</i> containing a C-terminal 6×His tag		This work
pNF-3690	pIMK2 with <i>L. lactis pmpA</i> containing a C-terminal 6×His tag		This work

Hemolysin assays. Hemolytic activity was measured for strains as previously described (36). Briefly, bacterial strains were grown in 3 ml LB overnight, diluted 1:10 in fresh LB, and allowed to grow for 5 h. Supernatants were collected and normalized based on OD₆₀₀. Culture supernatant was serially diluted into PBS, pH 5, containing 1 mM dithiothreitol (PBS-DTT) and incubated at 37°C for 30 min. Then, 100 μ l of a 1:5 dilution of PBS-DTT-washed sheep red blood cells (RBCs) was added, and the mixture was incubated for an additional 30 min at 37°C. Bacterial supernatant/RBC mixtures were pelleted by centrifugation, and the supernatant dilution resulting in 50% RBC lysis was determined by visual inspection of the pellet.

Detection of phospholipase activity. Brilliance *Listeria* agar with differential supplement containing lecithin (Oxoid) was used to detect phospholipase activity. *L. monocytogenes* phospholipase hydrolyzes lecithin in the medium, which produces an opaque halo around the streak. Colonies were streaked onto the medium and incubated for 24 h at 37°C, followed by visual inspection of the zone of opacity surrounding the bacterial streaks.

L2 plaque assays. Plaque assays were conducted as previously described (44). Briefly, monolayers of L2 fibroblasts in 6-well culture dishes were infected at a multiplicity of infection (MOI) of 30:1 for 1 h. Then, infected monolayers were washed three times with PBS, pH 7, and a sub-

sequent Dulbecco's modified Eagle's medium (DMEM)-agarose overlay was added, containing 10 μ g/ml gentamicin to kill extracellular bacteria. Plaques were measured at 72 h with a micrometer.

Intravenous mouse infections. Animal procedures were approved by the UIC Animal Care Committee and were conducted in the Biological Resources Laboratory. Overnight bacterial cultures were diluted 1:20 in BHI broth and grown to an OD₆₀₀ of approximately 0.6. Then, bacteria were normalized to 6×10^8 CFU/ml, washed twice with PBS, pH 7, and then diluted and resuspended in PBS, pH 7, to a final concentration of 1×10^5 CFU/ml. Ten to fifteen 7- to 9-week-old female Swiss Webster mice (Charles River Laboratories) per treatment group were injected with 200 μ l containing 2×10^4 CFU bacteria by tail vein injection. Livers and spleens of infected animals were collected at 24, 48, and 72 h postinfection. Organs were homogenized, and 10-fold serial dilutions were plated for total CFU.

Statistical analyses. Two-tailed Student's *t* test was used for statistical analysis of data represented as bar graphs, where a *P* value of ≤ 0.05 indicates statistical significance and error bars represent the standard errors of the means. For data represented as box plots, a two-tailed Wilcoxon rank sum test was used, and a *P* value of ≤ 0.05 indicates statistical significance.

FIG 1 Evolutionary relationship and protein conservation of PrsA homologues. (A) Domain organization of *L. monocytogenes* PrsA2, where the N-terminal signal peptide (SP), N terminus, PPIase domain, and C terminus are depicted and numbers designate amino acids. (B) Phylogenetic tree of PrsA homologues from *L. monocytogenes* and other Gram-positive bacteria, created by CLUSTAL analysis (57). The percent protein similarity of each PrsA homologue with the *L. monocytogenes* PrsA2 sequence is shown in parentheses. The scale bar indicates 1 substitution for every 10 amino acid residues. PrsA homologues that have a PPIase domain are indicated by asterisks, whereas more distant homologues lack a central PPIase motif.

RESULTS

Construction of *L. monocytogenes* Δ prsA2 strains complemented with different PrsA family members. PrsA protein family members are composed of N-terminal and C-terminal regions associated with chaperone activity that flank a central peptidyl-prolyl *cis/trans* isomerase (PPIase) domain (24) (Fig. 1A); however, not all PrsA-like proteins contain the complete signature PPIase motif. *L. lactis* PmpA, *S. pneumoniae* PrsA, *S. pyogenes* PrsA1 and PrsA2, and *S. mutans* PrtM all contain residues within the central region that diverge from the signature PPIase motif (24, 29, 30, 32), while *L. monocytogenes* PrsA1 and PrsA2, *B. subtilis* PrsA, and *S. aureus* PrsA have a centrally located conserved signature PPIase motif (29, 36, 45, 46). *L. monocytogenes* PrsA1 and PrsA2, *B. subtilis* PrsA, and *S. aureus* PrsA all have been confirmed to have PPIase activity *in vitro* (36, 40, 45), whereas *S. pneumoniae* PrsA and *L. lactis* PmpA have been demonstrated to lack PPIase activity (47, 48).

L. monocytogenes PrsA2 and PrsA1 are most closely related to the *B. subtilis* PrsA and most distantly related to the PrsA of streptococcal species (Fig. 1B). PrsA homologues that contain a PPIase motif—*L. monocytogenes* PrsA2 and PrsA1, *B. subtilis* PrsA, and *S. aureus* PrsA—are more closely related than PrsA homologues that lack this motif (Fig. 1B). Based on amino acid sequence, PrsA2 is most similar to *L. monocytogenes* PrsA1 (58% identity and 75% similarity), *B. subtilis* PrsA (46% identity and 68% similarity), and the distantly related *S. pneumoniae* PrsA (33% identity and 60% similarity). *L. monocytogenes* PrsA2 is least similar to *L. lactis* PmpA (31% identity and 47% similarity), *S. mutans* PrtM (29% identity and 48% similarity), and *S. pyogenes* PrsA2 (29% identity and 48% similarity). An alignment of the PrsA homologues shows regions of conservation and variation throughout the protein sequences (Fig. 2).

L. monocytogenes mutants lacking the PrsA2 chaperone have been associated with a variety of defects in protein secretion, bacterial cell physiology, and virulence (12, 33–35); however, no phenotype has thus far been associated with the loss of PrsA1. We assessed the evolutionary and functional conservation of Gram-positive PrsA-like proteins by testing for their ability to complement an *L. monocytogenes* mutant lacking PrsA2 (*L. monocytogenes* Δ prsA2). *L. monocytogenes* prsA2 and prsA1, *B. subtilis* prsA, *S. aureus* prsA, *S. pneumoniae* prsA, *S. pyogenes* prsA1 and prsA2, *S. mutans* prtM, and *L. lactis* pmpA were placed under the control of a constitutive promoter within the integrative plasmid vector pIMK2 to optimize gene expression (43), and the plasmids were integrated into the chromosome of *L. monocytogenes* Δ prsA2 mutants (Fig. 3A). *L. monocytogenes* Δ prsA2 mutants exhibit no significant growth defects in broth culture (33), and the Gram-positive PrsA allele-complemented Δ prsA2 strains also exhibited normal patterns of growth (Fig. 3B).

Expression of the various PrsA proteins in *L. monocytogenes* was determined via the addition of C-terminal His tags to proteins encoded on the same pIMK2 expression plasmid, followed by Western blotting using antibodies directed against the His tag (33) (Fig. 3C and D). Expression of the *B. subtilis* PrsA in *L. monocytogenes* was found to be comparable to expression levels of *L. monocytogenes* PrsA2 expressed from the same constitutive promoter; however, expression of *S. aureus* PrsA, *L. lactis* PmpA, *L. monocytogenes* PrsA1, and *S. pyogenes* PrsA1 was reduced, with *S. pyogenes* PrsA1 in particular being barely detectable (Fig. 3C and D). Proteins migrating at the position of PrsA dimers were evident in many of the supernatant-derived fractions (Fig. 3C and D). The reasons underlying the reduced abundance of selected His-tagged PrsA proteins in *L. monocytogenes* are not clear, but this result was not necessarily reflective of species-dependent codon bias, as *L. monocytogenes* PrsA1 was produced at much lower levels than *L. monocytogenes* PrsA2 despite being expressed from an identical promoter (Fig. 3C). As is shown below, despite the observed differences in protein expression, sufficient protein was expressed for the assessment of complementation of a number of PrsA2-associated phenotypes, and the ability of a PrsA homologue to complement an activity did not correlate with its expression level.

PrsA proteins exhibit broad functional conservation for activities associated with swimming motility and pH tolerance but not for conditions of osmotic stress. *L. monocytogenes* strains are flagellated and motile at low temperatures but exhibit reduced motility at higher temperatures (37°C) (49). *L. monocytogenes* strains lacking prsA2 exhibit reduced swimming motility at both low and high temperatures, suggesting that PrsA2 is required for a functional flagellum and/or chemotaxis (34). In addition, flagellin has been identified as a potential PrsA2/PrsA substrate in both *L. monocytogenes* and *B. subtilis* (12, 41). Interestingly, all nine prsA alleles provided full functional complementation of swimming motility to *L. monocytogenes* Δ prsA2, including prsA alleles derived from strains that lack flagella (streptococcal species, *S. aureus*, and *L. lactis*) (Fig. 4).

L. monocytogenes is capable of withstanding broad variations in the pH of its environment (pH 4.4 to 9.6) (50), an ability which likely contributes to its survival both inside and outside an infected host. The *L. monocytogenes* Δ prsA2 mutant exhibited increased sensitivity to both acidic (pH 6) and basic (pH 9) conditions, suggesting a link between chaperone function and pH tolerance (Fig. 5A and B). Similarly to what was observed for the

FIG 2 PrsA homologue sequence conservation. Alignment of the PrsA homologues, where residues that are highly conserved are highlighted in red and less conserved in blue. The PSIPRED secondary-structure prediction of *L. monocytogenes* PrsA2 is also shown (58), where helices are indicated, strands are shown as arrows, and coils are represented as lines. The *L. monocytogenes* PrsA2 signal peptide is in orange, the N terminus is in purple, the PPIase domain is in green, and the C terminus is in brown. Homologues having a PPIase motif are shown in black, while those lacking a PPIase motif are in gray. The PPIase motif is indicated by green text.

complementation of swimming motility, all nine Gram-positive *prxA* alleles restored *L. monocytogenes* growth to wild-type levels at both pH 6 and pH 9 (Fig. 5A and B). Taken together, these results indicate a broad conservation of PrxA chaperone function as it relates to bacterial motility and pH resistance, even for homologues derived from bacterial species that lack flagella and/or exhibit only moderate levels of pH tolerance.

In addition to its resistance to extremes of pH, *L. monocytogenes* is capable of growth under conditions of high osmolarity, up to 10% (wt/vol) NaCl, where the bacterium has been shown to compensate for changes in osmolarity via the transport of com-

patible solutes (50). Growth of the *L. monocytogenes* Δ *prsA2* mutant under conditions of both ionic (5% NaCl) and nonionic (0.7 M sucrose) osmotic stress revealed significantly decreased growth in comparison to that of wild-type strains; this phenotype could be fully complemented by introduction of the *L. monocytogenes* *prsA2* allele (Fig. 5C and D). In contrast to results for swimming motility and pH resistance, the Gram-positive *prsA* alleles were not all capable of fully complementing resistance to conditions of ionic and nonionic osmotic stress (Fig. 5C and D). *B. subtilis* *prsA* and *S. pyogenes* *prsA1* fully complemented *L. monocytogenes* Δ *prsA2* for osmotic stress resistance, whereas *L. lactis* *pmpA* did

FIG 3 Growth and protein profiles of PrsA homologue-complemented strains. (A) Schematic of the *L. monocytogenes* *prsA2* mutant strain complemented by a *prsA* allele. The *prsA2* gene, nucleotides 31 to 849 of 879 in total, is replaced by an erythromycin resistance gene where the *prsA* allele is integrated elsewhere in the chromosome and expressed by a constitutive promoter. (B) Bacterial growth for the *L. monocytogenes* (*Lm*) *ΔprsA2* strains expressing the indicated Gram-positive *prsA* homologue in BHI broth, as determined by optical density (OD) measurements at 600 nm. The data shown are representative of results from three independent experiments. (C) Strains were constructed similarly to the procedure described for panel A, with the addition of a C-terminal 6×His tag. For secreted proteins, the bacterial supernatant from each strain was TCA precipitated; for cell-associated proteins, the pellet was isolated, resuspended in sample buffer with β-mercaptoethanol, and boiled. Samples were collected at equivalent bacterial densities, where 1 μl of surface-associated protein or 10 μl of secreted protein was loaded. Western analysis of protein fractions subjected to SDS-PAGE was done using an anti-His (α-His) antibody. (D) Western analysis of *L. monocytogenes* PrsA2 compared to *S. pyogenes* PrsA1 is shown. Cell-associated (CA) and secreted (S) samples were isolated as described for panel B, and relative amounts loaded are indicated.

not. Complementation by other *prsA* alleles differed for conditions of ionic versus nonionic osmotic stress (*L. monocytogenes* *prsA1*, *S. pyogenes* *prsA2*, *S. pneumoniae* *prsA*, *S. mutans* *prtM*, and *S. aureus* *prsA*), suggesting that PrsA-dependent substrates that contribute to the resistance of *L. monocytogenes* to ionic and non-ionic shock may differ between the two conditions. Overall, all strains except those expressing *L. monocytogenes* *prsA1* and *L. lactis* *pmpA* exhibited statistically significant improved growth in comparison to that of the *L. monocytogenes* *ΔprsA2* mutant, indicating some level of functional PrsA conservation.

PrsA functions associated with cell wall biosynthesis are not conserved. *L. monocytogenes* strains lacking *prsA2* have reduced levels of cell wall-modifying enzymes as well as several penicillin binding proteins (PBPs) (12) and exhibit increased sensitivity to penicillin (36) and lysozyme, an enzyme that catalyzes the hydrolysis of peptidoglycan linkages (35). Given the apparent contributions of *L. monocytogenes* PrsA2 to cell wall biosynthesis and integrity (35, 36), we assessed the sensitivity of *L. monocytogenes* *ΔprsA2* to additional antibiotics that target the cell wall, including those targeting lipid carrier recycling (bacitracin) and peptidogly-

FIG 4 Assessment of bacterial swimming motility. Motility was measured as the diameter of the swimming colony. Diameters are expressed as the averages from 4 to 9 swimming colonies from 3 independent experiments. The *L. monocytogenes* Δ prsA2 strain was complemented with the indicated prsA2 homologue under the control of the pIMK2 constitutive promoter (bracket). Error bars represent the standard errors of the means, whereas asterisks (*) and daggers (‡) indicate statistical significance of $P \leq 0.05$, by two-tailed Student's *t* test, compared to *L. monocytogenes* wild type (wt) and *L. monocytogenes* Δ prsA2, respectively.

can cross-linking (vancomycin) (Fig. 6A to D). *L. monocytogenes* Δ prsA2 mutants were more susceptible to bacitracin and vancomycin than the wild type (Fig. 6A and B), and complementation with either *L. monocytogenes* prsA2 or prsA1 restored wild-type levels of resistance to all the cell wall-targeting antibiotics tested; however, none of the other Gram-positive prsA alleles were capable of restoring resistance (Fig. 6A to D). These data suggest that *L. monocytogenes* PrsA2 and *L. monocytogenes* PrsA1 share functional substrates that contribute to cell wall physiology and that recognition of these substrates may be specific to the *L. monocytogenes* chaperones.

Partial conservation of PrsA2 function as it relates to virulence factor secretion and bacterial spread through host cell monolayers. The loss of *L. monocytogenes* prsA2 has been associated with reduced activity of proteins required for virulence (36), including listeriolysin O (LLO) and broad-range phospholipase (PC-PLC) (33, 34, 51–54). Whereas *S. pyogenes* prsA1, *S. aureus* prsA, and *L. lactis* pmpA were unable to complement the loss of *L. monocytogenes* prsA2 with respect to secreted LLO hemolytic activity (Fig. 7A), *L. monocytogenes* prsA1, *B. subtilis* prsA, *S. pyogenes* prsA2, *S. pneumoniae* prsA, and *S. mutans* prtM were all capable of an intermediate level of complementation of the *L. monocytogenes* Δ prsA2 mutant (Fig. 7A). Notably, the ability of a heterologous prsA allele to partially complement for LLO secretion was not restricted to those bacteria that produce a related cholesterol-dependent pore-forming toxin (such as streptolysin O [SLO] produced by *S. pyogenes*), as species (such as *B. subtilis*) that lack toxin production nevertheless expressed a prsA allele that restored LLO secretion and activity. All nine Gram-positive prsA alleles restored secreted PC-PLC activity, based on the assessment of lecithinase activity via growth of the bacteria on solid medium containing lecithin (Fig. 7B).

The ability of selected alleles of prsA to complement virulence factor secretion suggested that complementation may extend to

activities necessary for intracellular growth and cell-to-cell spread. The *L. monocytogenes* Δ prsA2 mutant is defective for intracellular growth and cell-to-cell spread in monolayers of infected mouse L2 fibroblasts, forming zones of cell clearing (plaques) that are much smaller in size than those formed by wild-type *L. monocytogenes* (33–35) (Fig. 8). Despite intermediate complementation of secreted LLO by some prsA alleles and functional complementation of PC-PLC activity by all prsA alleles, only *L. monocytogenes* prsA2 was able to fully complement *L. monocytogenes* Δ prsA2 based on plaque formation in L2 monolayers (Fig. 8). However, most of the prsA alleles were observed to provide a partial level of complementation, with the exception of the *S. mutans* prtM allele (Fig. 8). These results indicate functional conservation of a subset of PrsA2-associated activities that contribute to *L. monocytogenes* intracellular growth and/or cell-to-cell spread but suggest that species-specific activities are required for full complementation.

Functional complementation of *L. monocytogenes* prsA2 does not extend to *in vivo* infection. *L. monocytogenes* mutants lacking prsA2 are severely attenuated for virulence in mouse infection models, with at least 100,000-fold fewer CFU observed in mouse livers and spleens at 72 h postinfection than in mice infected with wild-type *L. monocytogenes* (33, 34). Given that several of the Gram-positive prsA alleles restored virulence factor secretion and provided for some restoration of intracellular growth and/or cell-to-cell spread (Fig. 9), we assessed whether any degree of functional complementation could be observed when strains were tested for virulence in mice (Fig. 9). For these experiments, we focused on those prsA alleles that supported appreciable levels of intracellular growth and/or cell-to-cell spread as indicated by plaque formation in L2 monolayers, i.e., *B. subtilis* prsA, *S. pyogenes* prsA1, and *S. aureus* prsA, and for contrast we included one allele with negligible complementation, *L. lactis* pmpA (Fig. 9). Complemented strains were examined for their ability to colonize, persist, and/or multiply in target organs of intravenously infected

FIG 5 Bacterial resistance to acidic pH, basic pH, and osmotic shock. (A) Acidic-pH sensitivity. The growth is shown as optical density (OD₆₀₀) of strains inoculated from a saturated culture into liquid broth at pH 6. (B) Basic-pH sensitivity. Shown is the growth (OD₆₀₀) of strains inoculated from a saturated culture into liquid broth at pH 9. (C) Ionic shock. Shown is the growth (OD₆₀₀) of strains inoculated from a saturated culture into liquid broth containing 5% NaCl. (D) Nonionic shock. Growth (OD₆₀₀) of strains in liquid broth containing 0.7 M sucrose inoculated from a saturated culture is shown. Error bars represent the standard errors of the means from 3 to 6 cultures, whereas asterisks (*) and daggers (‡) indicate statistical significance of $P \leq 0.05$, by two-tailed Student's *t* test, compared to *L. monocytogenes* wt and *L. monocytogenes* ΔprsA2, respectively.

mice. At 24 h postinfection, all bacterial strains could be detected in livers and spleens of infected animals; however, only animals infected with the ΔprsA2 mutant complemented with *L. monocytogenes* prsA2 had bacterial burdens that were equivalent to those infected with wild-type *L. monocytogenes* (Fig. 9A). Animals infected with ΔprsA2 mutants containing the *B. subtilis* prsA and *S. pyogenes* prsA1 alleles exhibited bacterial burdens in both spleen and liver that were significantly increased in comparison to burdens after infection with the *L. monocytogenes* ΔprsA2 mutant without complementation (Fig. 9A). However, by 48 h postinfection, bacterial burdens continued to increase in liver and spleen for mice infected with the wild-type and *L. monocytogenes* prsA2-complemented strains, while the burdens associated with the remaining strains remained similar to those observed at 24 h postinfection (data not shown). By 72 h postinfection, bacterial burdens for mice infected with the wild-type and *L. monocytogenes* prsA2-complemented strain continued to increase, while the burdens in the remaining animals either remained similar to those observed

at 24 h postinfection or decreased dramatically (Fig. 9A and B). These data demonstrate that only *L. monocytogenes* prsA2 is able to fully functionally complement the *L. monocytogenes* ΔprsA2 mutant, indicating that while some aspects of PrsA2 function may be broadly conserved, the full spectrum of activities required for virulence within infected animals appears to have been adapted specifically to *L. monocytogenes* PrsA2.

DISCUSSION

Gram-positive bacteria face challenges distinct from those faced by Gram-negative bacteria when it comes to the translocation of bacterial proteins across the cytoplasmic membrane. Membrane- and cell wall-associated proteins, as well as those secreted away from the cell, must fold in an environment that is both highly charged and freely accessible to external solutes and chemicals (4). Here we have described the functional characterization of a critical secretion chaperone, *L. monocytogenes* PrsA2, and its related homologues based on the ability of different prsA alleles to func-

FIG 6 Bacterial resistance to cell wall-active antibiotics. The MIC to prevent growth of *L. monocytogenes* strains was determined by bacterial inoculation into broth containing dilutions of the indicated cell wall-active antibiotics. (A) MIC of bacitracin, which inhibits lipid carrier recycling. (B) MIC of vancomycin, which inhibits peptidoglycan transglycosylation and transpeptidation. (C) MIC of penicillin, which inhibits peptidoglycan transpeptidation. (D) MIC of lysozyme, which catalyzes the hydrolysis of peptidoglycan. Error bars represent the standard errors of the means from 4 to 8 cultures, whereas asterisks (*) and daggers (‡) indicate statistical significance of $P \leq 0.05$, by two-tailed Student's *t* test, compared to *L. monocytogenes* wt and *L. monocytogenes ΔprsA2*, respectively.

tionally complement *L. monocytogenes* strains lacking *prsA2*. Our data indicate a surprisingly broad degree of functional complementation for complex activities such as flagellum-mediated swimming motility and pH resistance, while other aspects of bacterial physiology, such as cell wall synthesis and bacterial replication within an infected animal, require functions specific to *L. monocytogenes* PrsA2. PrsA2 is thus a member of a family of chap-

erones that appear to maintain both broadly conserved and species-specific functions (Fig. 10).

Chaperones often work in a stoichiometric rather than a catalytic manner, inhibiting protein aggregation while promoting folding (55); thus, the overall levels of PrsA2 protein produced are likely important for its functional activity. Each heterologous *prsA* allele was placed under the control of the

FIG 7 Assessment of *L. monocytogenes* secreted hemolysin and phospholipase. (A) Hemolytic activity. Dilutions of bacterial culture supernatants were assessed for their ability to lyse sheep red blood cells (RBCs) *in vitro*. The reciprocal of the supernatant dilution that resulted in 50% RBC lysis (hemolytic units) was determined for 4 independent experiments. Error bars represent the standard errors of the means, whereas asterisks (*) and daggers (‡) indicate statistical significance of $P \leq 0.05$, by two-tailed Student's *t* test, compared to *L. monocytogenes* wt and *L. monocytogenes* Δ prsA2, respectively. (B) Phospholipase activity was determined by the incubation of each strain on *Listeria* selective agar plates containing lecithin that is hydrolyzed, producing a zone of opacity surrounding the bacterial streak.

same constitutive promoter, and yet protein levels clearly varied between *L. monocytogenes* strains expressing the different constructs (Fig. 3C and D). The reason underlying the variation in PrsA protein levels is not clear, as differences were observed even between *L. monocytogenes* *prsA1* and *prsA2*, where, for example, potential codon bias is not anticipated to be present. Although differences in the levels of PrsA protein expressed were clearly evident, there was in general no direct correlation observed between the levels of the various PrsA proteins and the ability of each protein to functionally complement an *L. monocytogenes* Δ prsA2 strain. For example, *L. monocytogenes* PrsA1 complemented most PrsA2-associated activities, and yet its expression

was severalfold lower than that of PrsA2 (Fig. 3C). *B. subtilis* PrsA was expressed at levels nearly equivalent to *L. monocytogenes* PrsA2, and it complemented a number of activities, but so did the poorly expressed *S. pyogenes* PrsA1, which was barely detectable. We interpret these results to indicate that, in most cases, sufficient levels of PrsA protein were present for functional complementation if complementation was possible. It should be noted, however, that increased expression of *prsA1* from the pIMK2 promoter did enable complementation of secreted LLO and phospholipase activity in Δ prsA2 strains, a complementation activity that was not observed when *prsA1* was expressed either from its own promoter or from the *prsA2* promoter (12).

FIG 8 Bacterial intracellular growth and cell-to-cell spread as measured by plaque assays. Monolayers of mouse L2 fibroblasts were infected with the indicated *L. monocytogenes* strains, and plaque formation in the presence of gentamicin was determined 72 h postinfection. At least 25 plaques were measured in 3 to 5 independent experiments for all strains. Measurements represent plaque size with respect to *L. monocytogenes* wt (set at 100%). Error bars represent the standard errors of the means, whereas asterisks (*) and daggers (‡) indicate statistical significance of $P \leq 0.05$, by two-tailed Student's *t* test, compared to *L. monocytogenes* wt and *L. monocytogenes ΔprsA2*, respectively.

The failure of even the abundantly expressed *B. subtilis* PrsA to complement *L. monocytogenes ΔprsA2* intracellular growth and virulence in mice suggests that this version of PrsA does not recognize the full spectrum of substrates or interacting partners recognized by *L. monocytogenes* PrsA2.

All tested PrsA homologues were capable of restoring flagellum-mediated swimming motility and resistance to extremes of pH in *L. monocytogenes ΔprsA2* strains (Fig. 4 and 5A and B). Restoration of both activities occurred regardless of whether the bacterium from which the *prsA* allele was derived was capable of swimming motility or of exhibiting resistance to extremes of pH. With respect to swimming motility, *L. monocytogenes ΔprsA2* mutants produce visible flagella (data not shown), so it is not a complete absence of flagellar assembly that leads to the motility defect but may instead be the assembly of a nonfunctional flagellum and/or a defect in chemotactic ability. We suspect that the broad complementation of bacterial motility and pH resistance by the various *prsA* alleles reflects a conserved contribution of PrsA to some fundamental structural property of the bacterial surface that in turn leads to the correct assembly of membrane proteins that contribute to these two activities. Consistent with this hypothesis, there are several membrane-localized proteins that contribute to *L. monocytogenes* acid resistance, including the GAD and ADI transport proteins and the multicomponent F_0F_1 ATPase that extrudes protons, while swimming motility clearly requires the assembly of a large and complex flagellar motor.

In contrast to the broad complementation of swimming motility and pH resistance, only the *L. monocytogenes* PrsA homologues were capable of complementing for antibiotic resistance related to *L. monocytogenes* cell wall biosynthesis. Complementation with *L. monocytogenes* PrsA1 or PrsA2 restored resistance to antibiotics that targeted lipid carrier recycling, transglycosylation,

transpeptidation, and peptidoglycan biosynthesis, while none of the other *prsA* alleles was capable of restoring any significant level of resistance (Fig. 6). This suggests that PrsA contributions to cell wall physiology are species specific, perhaps reflecting chaperone specificity for distinct cell wall enzymes. *L. monocytogenes ΔprsA2* mutants have been shown to have reduced levels of a number of cell wall-modifying enzymes as well as several penicillin binding proteins (PBPs) (12). Similarly, PrsA has been associated with cell wall integrity and biosynthesis in several other Gram-positive organisms, including *B. subtilis*, *S. mutans*, and *S. aureus* (28, 32, 41).

With respect to the complementation of *L. monocytogenes ΔprsA2* mutants for activities directly associated with bacterial pathogenesis, many of the heterologous *prsA* alleles restored secreted LLO and PC-PLC activity (Fig. 7) (33, 36). Interestingly, PrsA homologues derived from bacterial species, such as *B. subtilis*, that do not express a cholesterol-dependent cytolysin (CDC) restored secreted LLO activity, whereas PrsA1 derived from *S. pyogenes*, which expresses the CDC streptolysin O (SLO) (31), did not (Fig. 7A). *S. pyogenes* PrsA2 did complement for LLO secretion, and it is possible that SLO is a substrate for *S. pyogenes* PrsA2 but not for its PrsA1. Overall, these results indicate that PrsA substrate specificity cannot easily be predicted based on one PrsA being required for secretion of a family member of a particular type of secreted protein, such as a CDC. This observation indicates diversity between PrsA family members in substrate recognition, even between multiple alleles expressed by a single species.

With the exception of *L. monocytogenes prsA2*, none of the PrsA homologues was capable of restoring virulence to *L. monocytogenes ΔprsA2* strains (Fig. 9). It thus appears that neither the complementation of cell wall integrity (*L. monocytogenes* PrsA1) nor the restoration of virulence factor secretion (*B. subtilis* and streptococcal homologues) suffices for restoration of *L. monocytogenes*

FIG 9 *L. monocytogenes* PrsA2 is required for full virulence in mice. Mice were intravenously infected with 2×10^4 CFU of the indicated strain. At 24 h (A) and 72 h (B) postinfection, the spleens and livers were harvested, homogenized, and plated for bacterial CFU. Box plots are shown, where each point represents one mouse. A dotted line indicates the limit of detection of 500 CFU. Data were obtained from two independent experiments. Asterisks (*) and daggers (§) indicate statistical significance of $P \leq 0.05$, by two-tailed Wilcoxon rank sum test, compared to *L. monocytogenes* wt and *L. monocytogenes* Δ prsA2, respectively.

Δ prsA2 virulence. *L. monocytogenes* PrsA1, *B. subtilis* PrsA, and *S. pyogenes* PrsA1 were all capable of partial restoration of intracellular growth and cell-to-cell spread in cell monolayers based on plaque formation; however, *L. monocytogenes* Δ prsA2 strains expressing these alleles remained as severely attenuated as *L. monocytogenes* Δ prsA2 mutants alone based on bacterial burdens recovered from the livers and spleens of infected animals (Fig. 9). These

results indicate the degree to which PrsA2 has become specifically adapted for aspects of *L. monocytogenes* physiology necessary for animal infection.

Overall, the data presented here indicate that PrsA homologues have aspects of functional activity that are both broadly conserved (such as those activities required for swimming motility) and highly specialized (*L. monocytogenes* virulence in mice) (Fig. 10).

FIG 10 Model indicating conserved and species-specific aspects of *L. monocytogenes* PrsA2 activity. Shown is the *L. monocytogenes* bacterial membrane, extracellular space, and cell wall. The PrsA homologue is shown in the extracellular space; arrows indicate conserved functions (green) and functions that are not conserved (orange).

It is interesting that some bacterial species maintain and express multiple PrsA homologues with at least some overlapping function (*L. monocytogenes* *prsA1* and *prsA2*, *S. pyogenes* *prsA1* and *prsA2*) (12, 31, 33, 36), whereas other bacteria have a single allele that may or may not be essential (*B. subtilis*, *L. lactis*) (29, 41). For *L. monocytogenes*, we have yet to identify a phenotype associated with *prsA1* deletion strains. *L. monocytogenes* *prsA1* is clearly expressed and functional; however, it is not clear yet as to whether it recognizes substrates that may be distinct from those recognized by PrsA2. It is possible that *L. monocytogenes* PrsA1 contributes to aspects of *L. monocytogenes* fitness that are relevant to bacterial survival in the outside environment rather than inside a mammalian host. For future studies, *L. monocytogenes* may prove to be an ideal organism for the molecular dissection of PrsA substrate specificity and activity, given that neither PrsA1 nor PrsA2 is required for growth of *L. monocytogenes* in broth culture and based on the ease with which mutations can be generated and assessed for functional consequences with respect to both bacterial physiology and virulence.

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