Posttranslocation Chaperone PrsA2 Regulates the Maturation and Secretion of *Listeria monocytogenes* Proprotein Virulence Factors[▽]

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PrsA2 is a conserved posttranslocation chaperone and a peptidyl prolyl *cis-trans* isomerase (PPIase) that contributes to the virulence of the Gram-positive intracellular pathogen *Listeria monocytogenes*. One of the phenotypes associated with a *prsA2* mutant is decreased activity of the broad-range phospholipase C (PC-PLC). PC-PLC is made as a proenzyme whose maturation is mediated by a metalloprotease (Mpl). The proforms of PC-PLC and Mpl accumulate at the membrane-cell wall interface until a decrease in pH triggers their maturation and rapid secretion into the host cell. In this study, we examined the mechanism by which PrsA2 regulates the activity of PC-PLC. We observed that in the absence of PrsA2, the proenzymes are secreted at physiological pH and do not mature upon a decrease in pH. The sensitivity of the *prsA2* mutant to cell wall hydrolases was modified. However, no apparent changes in cell wall porosity were detected. Interestingly, synthesis of PC-PLC in the absence of its propeptide lead to the secretion of a fully active enzyme in the cytosol of host cells independent of PrsA2, indicating that neither the propeptide of PC-PLC nor PrsA2 is required for native folding of the catalytic domain, although both influence secretion of the enzyme. Taken together, these results suggest that PrsA2 regulates compartmentalization of Mpl and PC-PLC, possibly by influencing cell wall properties and interacting with the PC-PLC propeptide. Moreover, the ability of these proproteins to respond to a decrease in pH during intracellular growth depends on their localization at the membrane-cell wall interface.

Protein secretion in Gram-positive bacteria is comprised of two steps. Proteins are first translocated across the bacterial cytoplasmic membrane to the membrane-cell wall interface. These proteins are then secreted across the bacterial cell wall. Following translocation, proteins rely on various folding factors to reach their native state. These folding factors, located on the *trans* side of the cell membrane, include disulfide bond oxidoreductases, peptidyl prolyl *cis-trans* isomerases (PPIases), and chaperones (10, 34). In general, chaperones assist proteins folding into their native conformations by inhibiting improper protein-protein interactions and altering the activation energy necessary for proper folding (11). PPIases serve as chaperones in addition to their isomerase activity and contribute to cell envelope integrity (10). PrsA, a parvulin-type PPIase of the Gram-positive bacterium *Bacillus subtilis*, contributes to the folding of penicillin binding proteins (PBPs) (18). PBPs catalyze the cross-linking of glycan chains with short peptides forming peptidoglycan and transglycosylase reactions during cell wall biosynthesis (15). Peptidoglycan of the *B. subtilis prsA* mutant shows a cross-linking defect, causing changes in cell morphology and a decrease in viability (44). PrsA2, a PrsA ortholog from the Gram-positive pathogen *Listeria monocytogenes*, is not essential for viability during extracellular growth, but the mutant leaks cytoplasmic proteins, suggest-

ing that it is important for cell envelope homeostasis (1, 3, 47).

Listeria monocytogenes is a facultative intracellular pathogen and the causative agent of the food-borne disease listeriosis (43). Following entry into a host cell, membrane-bound bacteria rapidly escape vacuoles to access the host cytosol where they multiply (42). *L. monocytogenes* uses an actin-based mechanism of motility to spread from cell to cell without exiting to the extracellular milieu. During cell-to-cell spread, the bacteria become entrapped in double-membrane vacuoles from which they must escape to perpetuate their intracellular growth cycle. Escape from vacuoles is mediated by a pore-forming hemolysin called listeriolysin O (LLO) (14) and two phospholipases of type C (9, 39). PrsA2 contributes to LLO folding and pore-forming ability and to the activity of the broad-range phospholipase C (PC-PLC) (2, 47). Accordingly, the intracellular growth and cell-to-cell spread of *L. monocytogenes* are affected in the absence of PrsA2. In addition, *L. monocytogenes* is highly attenuated *in vivo* in the absence of PrsA2. Interestingly, the PPIase activity of PrsA2 is not required for PC-PLC activity, but it is needed for optimal LLO pore-forming ability and *L. monocytogenes* virulence (3).

PC-PLC and the metalloprotease of *L. monocytogenes* (Mpl) are synthesized as inactive proenzymes containing N-terminal propeptides. During intracytosolic growth, these proteins translocate across the bacterial cytoplasmic membrane and accumulate at the membrane-cell wall interface (28, 31, 40). Upon cell-to-cell spread, bacteria in vacuoles experience a decrease in pH, which initiates Mpl autocatalysis, leading to the proteolytic maturation of PC-PLC by mature Mpl (6, 12, 27). Concomitant to maturation, both proteins are rapidly se-

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TABLE 1. *L. monocytogenes* strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant feature(s)	Reference
Strains		
10403S	Wild-type (serotype 1/2a)	5
NF-L943	*prfA* with G155S mutation in 10403S background (overexpresses PrfA-dependent genes)	37
DP-L1075	10403S *prfA*::Tn*917*-LTV3	13
DP-L1935	Internal in-frame deletion of *plcB* in 10403S background	39
DP-L2296	Internal in-frame deletion of *mpl* in 10403S background	26
DP-L2343	Deletion of *mpl* structural gene in 10403S background	36
DP-L5596	10403S *prsA2::Himar1*	47
DP-L5601	Internal in-frame deletion of *prsA2* from positions 29 to 291 in 10403S background (Δ*prsA2*)	47
DP-L5603	DP-L5601 carrying pJZ065 integrated at the tRNA^{Arg} site	47
DP-L5749	10403S carrying pBHE573	35
DP-L5750	10403S expressing PSA bacteriophage holin/lysin and carrying pBHE573	35
DP-L5751	DP-L5601 carrying pBHE573	This study
DP-L5755	10403S carrying pJZ095 integrated at the tRNA^{Arg} site	This study
EJ-L12	10403S Δ*inlA*	4
HEL-335	10403S *plcB*Δpro	46
HEL-981	NF-L943 Mpl-Flag$_{N-cat}$[a]	12
HEL-1216	DP-L2296 *prsA2::Himar1*	This study
HEL-1230	NF-L943 Mpl-Flag$_{N-cat}$ *prsA2::Himar1*	This study
HEL-1232	NF-L943 Mpl-Flag$_{N-cat}$ *prsA2::Himar1* carrying pJZ065 integrated at the tRNA^{Arg} site	This study
HEL-1405	HEL-335 *prsA2::Himar1*	This study
NF-L1166	10403S with *actA-gus-neo-plcB* and *prfA* with the L140F mutation	30
Plasmids		
pBHE573	Firefly luciferase with the cytomegalovirus promoter	35
pJZ065	*prsA2* promoter, without *prfA* box, and open reading frame cloned into pPL2	47
pJZ095	*prsA2* promoter-*gus* cloned into pPL2 (P$_{PrsA2}$-*gus*)	This study
pPL2	Site-specific shuttle integration vector	22

[a] Mpl-Flag$_{N-cat}$ is an Mpl construct that contains a Flag tag at the N terminus of its catalytic domain.

creted across the bacterial cell wall. The secretion of PC-PLC in response to pH is dependent on Mpl, but the secretion of Mpl is not dependent on PC-PLC (12, 46).

In this study, we investigated the mechanism by which PrsA2 affects the activity of PC-PLC. Our results indicated that PrsA2 is required to maintain the Mpl zymogen and the proform of PC-PLC associated with bacteria during infection, possibly as a result of its influence on cell wall composition. Interestingly, the secreted proenzymes did not undergo maturation upon a decrease in intracellular pH, indicating that Mpl autocatalysis is spatially controlled in addition to being controlled by pH. Also, phospholipase activity was independent of PrsA2 when PC-PLC was produced in the absence of its propeptide. Therefore, it appears that the PC-PLC maturation defect observed in the absence of PrsA2 results from premature secretion of the Mpl zymogen, possibly caused by changes in the biochemical properties of the cell wall, and from the inability of the zymogen to undergo autocatalysis postsecretion.

MATERIALS AND METHODS

Bacterial strains. All *Listeria monocytogenes* strains used in this study are listed in Table 1. *L. monocytogenes* strains were grown in brain heart infusion (BHI) medium or Luria-Bertani (LB) broth supplemented with 50 mM morpholinepropanesulfonic acid (MOPS) adjusted to pH 7.3, 0.2% activated charcoal, and 25 mM glucose-1-phosphate (LB-MOPS-G1P). *Escherichia coli* bacteria with pKSV7-derived plasmids were cultured in LB broth supplemented with ampicillin (100 μg/ml). *E. coli* bacteria containing pPL2-derived plasmids were cultured in LB broth supplemented with chloramphenicol (25 μg/ml). *L. monocytogenes* strains harboring pPL2-derived or pKSV7-derived plasmids were cultured in BHI medium supplemented with chloramphenicol (10 μg/ml).

Transduction of *prsA2::Himar1*. Transductions were performed as described previously (17, 47). Briefly, U153 phage grown on donor strain *L. monocytogenes*

10403S *prsA2::Himar1* (DP-L5596) was used to infect recipient *Listeria* strains (Table 1). Transductants were selected at 37°C on semisolid medium supplemented with lincomycin (25 μg/ml) and erythromycin (2 μg/ml).

Construction of *prsA2* promoter-*gus* fusion plasmid. Splicing by overlap extension (SOEing) PCR was used to fuse the promoter of *prsA2* to *gus* (gene encoding β-glucuronidase [Gus]). The promoter of *prsA2* and the *gus* structural gene were amplified by PCR using primer pair JZ-241 (47) and BK-011 (5′-GTTCTACAGGACGGACCATTAAATAAAACACACTCCTTAGTTGTTGGAA-3′) and primer pair BK-012 (5′-TTCCAACAACTAAGGAGTGTGTTTTATTTAATGGTCCGTCCTGTAGAAAC-3′) and BK-013 (5′-TTTGTCGACTCATTGTTTGCCTCCCTGCTG-3′), respectively, and *L. monocytogenes* NF-L1166 (kindly provided by Nancy Freitag) genomic DNA as the template (Table 1). The respective 307-bp and 1,851-bp PCR products were used in a SOEing PCR with primers JZ-241 and BK-013. The 2,108-bp product was digested with EagI and SalI and ligated into the shuttle integration vector pPL2, thus generating pJZ095 (Table 1).

Conjugation experiments. All pPL2-derived plasmids (Table 1) were introduced into *Listeria* by conjugation as described previously (32). Chloramphenicol-resistant conjugants were screened for plasmid integration at the *attB* site of the tRNA^{Arg} gene by PCR using primers NC16 and PL95 (22).

Metabolic labeling and immunoprecipitation assays. The specific detection of metabolically labeled broad-range phospholipase C (PC-PLC) or metalloprotease (Mpl) synthesized by intracellular bacteria was performed as described previously (31, 38, 40). Secreted PC-PLC and Mpl were immunoprecipitated from cleared host cell lysates, whereas bacterium-associated PC-PLC was immunoprecipitated from the lysate of intracellular bacteria. For pulse-chase experiments, radiolabeled infected cells were chased in tissue culture medium containing unlabeled methionine (5 mM), cysteine (1 mM), and chloramphenicol (20 μg/ml) for the times indicated below in Results and the figure legends. To detect PC-PLC or Mpl as a function of pH, radiolabeled infected cells were chased in a potassium-based buffer adjusted to either pH 7.3 or pH 6.5 and supplemented with nigericin (10 μM). Immunoprecipitates were resolved by SDS-PAGE and detected by autoradiography or phosphorimaging.

To determine the half-life of PC-PLC, the intensities of protein bands detected by phosphorimaging from pulse-chase experiments were quantified using ImageJ (rsbweb.nih.gov/ij/). A regression curve was generated and used to calculate the half-life of PC-PLC.

Immunofluorescence staining of Mpl. Bacterium-associated Mpl-Flag$_{N-cat}$ (an Mpl construct that contains a Flag tag at the N terminus of its catalytic domain) was detected by immunofluorescence as described previously (12). Briefly, HeLa cells infected with *L. monocytogenes* were fixed and then treated with purified *L. monocytogenes*-specific phage endolysin Ply118. To detect bacterium-associated Flag-tagged Mpl (Mpl-Flag), mouse monoclonal anti-Flag M2 (Sigma) was used followed by a donkey anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC). Bis-benzimide (Hoechst 33258) was used to detect *Listeria*.

Expression of *prsA2*. Levels of *prsA2* expression were measured using a quantitative Gus assay as described previously (37). *L. monocytogenes* strains containing pJZ095 were grown in BHI medium for 8 h. At each hour, optical density measurements (optical density at 600 nm [OD$_{600}$]) were taken, and bacterial pellets were resuspended in 100 mM potassium phosphate (pH 7.0), 100 mM NaCl, and 0.1% Triton X-100. Gus substrate (4-methylumbelliferyl-β-D-glucuronide [4-MUGlcU]) was added to lysates at a concentration of 0.4 mg/ml for 1 h at room temperature. Fluorescence measurements were taken after adding 200 mM sodium carbonate to each sample. Fluorescence measurements ($\lambda_{excitation}$ = 360 nm; $\lambda_{emission}$ = 460 nm) were made using a Horiba-Fluoro-Max-3 fluorometer and normalized to OD$_{600}$ readings. Normalized fluorescence measurements were converted to concentrations of Gus using a standard curve of fluorescence intensity to concentrations of 4-methylumbelliferone.

Preparation of cell wall fractions. Bacterial cell wall fractions were isolated as described previously (40). Briefly, bacteria were grown in LB-MOPS-G1P to an OD$_{600}$ of ~1.2. Bacterial cells were suspended in protoplast buffer supplemented with phage endolysin Ply118 to a calculated OD$_{600}$ of 12. Protoplast formation was monitored by microscopy. The protoplasts were pelleted by centrifugation at 15,000 × *g* for 5 min at 4°C, and the cell wall proteins were recovered in the supernatant. The proteins were precipitated using 5% trichloroacetic acid, resolved by SDS-PAGE, and detected with Coomassie brilliant blue R-250. The purity of the cell wall fraction was verified by performing Western immunoblotting for detection of InlA, a cell wall protein, and PrfA, a cytoplasmic protein as described previously (40).

Cell wall hydrolysis assays. Bacteria were grown in BHI medium at 37°C and 200 rpm to an OD$_{600}$ of ~0.5, pelleted, and suspended in 20 mM Tris-HCl (pH 8.0) to a calculated OD$_{600}$ of 1.0. Chicken egg white lysozyme (Sigma) or phage endolysin Ply118 was added to a final concentration of 50 μg/ml. OD$_{600}$ measurements were taken every minute for 30 min at 37°C using a BioTek Power-Wave XS microplate spectrophotometer. Three experimental replicate assays were performed.

Bacteriolysis luciferase assay. The luciferase assay to test for bacterial lysis in infected cells was performed as described previously (35). Briefly, bone marrow-derived macrophages from IFNAR$^{-/-}$ mice (IFNAR stands for IFN-α/β receptor) were infected with *L. monocytogenes* strains expressing firefly luciferase under the control of a cytomegalovirus promoter (pBHE573; Table 1). Six hours postinfection, luciferase reagent (Sigma) was added to infected host cell lysates, and luciferase activity was measured.

Detection of phospholipase activity. PC-PLC activity was detected on LB egg yolk agar plates as described previously (46). Bacteria were spot inoculated onto plates and incubated for 48 h at 37°C.

Plaque assay. The efficiency of the cell-to-cell spread of intracellular bacteria was measured using the plaque assay as described previously (41). The infected monolayers of mouse L2 fibroblast cells were stained with neutral red, and plaque areas were calculated. Plaque area is reported as a percentage of the wild-type plaque size. Three independent experiments were performed.

Statistical analysis. Cell wall hydrolysis assay results were analyzed using a two-tailed paired *t* test to determine whether the *prsA2* mutant was more sensitive to cell wall hydrolases than the wild-type strain. Plaque assay results were analyzed using a one-way analysis of variance (ANOVA) with a Tukey-Kramer multiple-comparison test to determine whether the plaque size of each of the strains tested was significantly different. A significance level (α) of 0.05 was set for both tests.

RESULTS

PC-PLC and Mpl are no longer found associated with bacteria in the absence of PrsA2. Previous studies have reported that broad-range phospholipase C (PC-PLC) secretion and activity are reduced in the absence of PrsA2 (2, 47). PC-PLC and metalloprotease of *L. monocytogenes* (Mpl) are translocated across the bacterial membrane as inactive proenzymes and accumulate at the membrane-cell wall interface (40).

Upon a decrease in pH, which is normally experienced when bacteria are in vacuoles, Mpl undergoes autocatalysis and mediates the maturation of PC-PLC (6, 12). This maturation process is followed by the rapid secretion of these two enzymes across the bacterial cell wall (12, 28, 40). We questioned the role of PrsA2 in regulating the compartmentalization of PC-PLC and Mpl. Infected cells were pulse-labeled, and bacterium-associated PC-PLC was immunoprecipitated from lysates of intracellular bacteria. The proform of PC-PLC (proPC-PLC) was detected from the wild-type but not from the *prsA2* bacterium lysate (Fig. 1A). We were unsuccessful at detecting Mpl from immunoprecipitates using either anti-Flag or anti-Mpl antibodies, possibly as a result of nonspecific interactions between Mpl and other proteins from the bacterial lysates or a result of proteolytic degradation. Therefore, we used an immunofluorescence assay to detect bacterium-associated Mpl. We observed Mpl associated with bacteria in cells infected with wild-type bacteria, but not in cells infected with the *prsA2* mutant (Fig. 1B). Complementation of the mutant restored the wild-type phenotype for PC-PLC and Mpl. This result indicated that PrsA2 is necessary for the accumulation of PC-PLC and Mpl within bacteria, suggesting that these proteins are degraded rapidly posttranslocation or secreted prematurely in the absence of PrsA2.

PC-PLC and Mpl are prematurely secreted from bacteria in the absence of PrsA2. The rate of protein secretion across the bacterial cell wall could be influenced by the biophysical and biochemical properties of the cell wall, protein structure, protein-protein interactions, and ionic interactions. Considering that PrsA2 is likely to act as a chaperone and therefore assist in the folding of proteins posttranslocation, we hypothesized that the absence of PrsA2 could influence the rate of PC-PLC and Mpl secretion. Therefore, we assessed whether PC-PLC and Mpl were secreted prematurely in the absence of PrsA2. Infected cells were pulse-labeled and then chased for 1, 2, or 5 min postinfection. PC-PLC was immunoprecipitated from the secreted bacterial fraction in lysates of infected cells and from lysates of intracellular bacteria. The results showed that PC-PLC was found exclusively in the secreted fraction of the *prsA2* mutant immediately after the pulse but remained associated with bacteria for the entire time of the chase with the wild-type strain (Fig. 2A). Mpl was also immunoprecipitated from the secreted bacterial fraction in lysates of infected cells. Similar to PC-PLC, Mpl was secreted rapidly from the *prsA2* mutant, but not from wild-type bacteria (Fig. 2B). Although we were unable to isolate Mpl from intracellular bacterial lysates, these results indicated that wild-type bacteria maintain a pool of Mpl, whereas the *prsA2* mutant does not (Fig. 1B).

Mpl controls the secretion of PC-PLC across the cell wall. In the absence of Mpl, PC-PLC remains associated with bacteria upon a decrease in pH. The ability of Mpl to facilitate the secretion of PC-PLC is independent of its role in mediating the maturation of PC-PLC, suggesting that perhaps Mpl serves to transport PC-PLC across the cell wall (46). Therefore, we asked whether the secretion of PC-PLC from the *prsA2* mutant was dependent on Mpl. Infected cells were pulse-labeled, and PC-PLC was immunoprecipitated from bacteria that lacked either Mpl and/or PrsA2. As previously reported, PC-PLC remained associated with bacteria at physiological pH in the presence or absence of Mpl (Fig. 3, lanes 1 and 5). However,
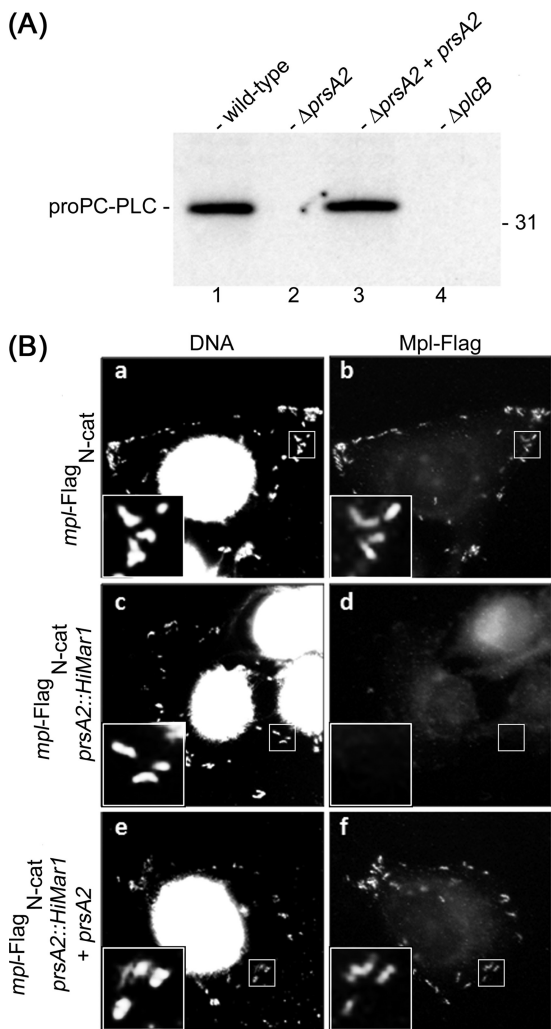
(A)

(B)

FIG. 1. Broad-range phospholipase C (PC-PLC) and metalloprotease of *L. monocytogenes* (Mpl) are not associated with bacteria in the absence of PrsA2. (A) Bacterium-associated PC-PLC was immunoprecipitated from intracellular bacterial cell lysates. *L. monocytogenes* strains 10403S (wild type), DP-L5601 (Δ*prsA2* mutant) (lane 2), DP-L5603 (Δ*prsA2* mutant carrying pJZ065, which contains the *prsA2* gene), and DP-L1935 (Δ*plcB* mutant) were tested. The position of the 31-kDa protein standard is indicated to the right of the gel, and the position of the proform of PC-PLC (proPC-PLC) is indicated to the left of the gel. (B) Infected HeLa cells were fixed and stained for fluorescence microscopy. Bis-benzamide was used to detect host cell nuclei and bacterial chromosome, and anti-Flag M2 antibody was used to detect Flag-tagged Mpl (Mpl-Flag). *L. monocytogenes* strains HEL-981 (a and b), HEL-1230 (c and d), and HEL-1232 (e and f) were studied. Mpl-Flag$_{N-cat}$ is an Mpl construct that contains a Flag tag at the N terminus of its catalytic domain.

in the absence of PrsA2, PC-PLC was secreted independently of Mpl (Fig. 3, lanes 4 and 8). Together, these results indicated that, at physiological pH, PrsA2 is required (i) to prevent the premature secretion of PC-PLC and Mpl across the bacterial cell wall and (ii) to enable Mpl to control PC-PLC secretion.

Maturation of PC-PLC and Mpl does not occur in the absence of PrsA2 during intracellular infection. We previously hypothesized that the maturation of PC-PLC by Mpl in re-

sponse to a decrease in pH occurs prior to secretion because secretion of PC-PLC synthesized in the absence of its propeptide is very rapid as opposed to secretion of the proform (46). To test this hypothesis, we assessed whether a decrease in pH would lead to the maturation of PC-PLC in the cytosol of infected cells, as the *prsA2* mutant does not retain the proform of PC-PLC and Mpl associated with bacteria. Infected cells were pulse-labeled with [^{35}S]methionine/cysteine and chased under conditions that maintain the host cell cytosol at physiological pH or mimic the acidified vacuolar pH to induce PC-PLC maturation. Secreted PC-PLC was immunoprecipitated from host cell lysates and detected by autoradiography. As previously reported, mature PC-PLC was detected in the host cell lysate following a decrease in intracellular pH in cells infected with wild-type bacteria (Fig. 4). However, pH-mediated maturation of PC-PLC did not occur in cells infected with the *prsA2* mutant.

The absence of PC-PLC maturation in response to a decrease in pH could be due to a failure by Mpl either to undergo autocatalysis or to process PC-PLC postsecretion. To answer this question, we performed the same experiment as described above, except that secreted Mpl, not PC-PLC, was immunoprecipitated. Mature Mpl was detected in the host cell lysate following a decrease in intracellular pH in cells infected with wild-type bacteria (Fig. 4). However, Mpl maturation did not occur in response to a decrease in pH in cells infected with the *prsA2* mutant. Together, these results suggested that the effect of pH on Mpl autocatalysis and Mpl-mediated maturation of PC-PLC is spatially controlled and that PrsA2 is required to maintain the proproteins at the membrane-cell wall interface where maturation occurs.

Cell wall permeability does not appear to be increased in the absence of PrsA2. Since the *B. subtilis* PrsA is important for peptidoglycan cross-linking (18) and the *L. monocytogenes* PrsA2 is associated with cell wall integrity (3), we suspected that perhaps the cell wall of the *prsA2* mutant was more porous than the cell wall of wild-type bacteria. To assess differences in cell wall porosity, we opted to examine the profile of proteins that are present within the cell wall or are retained at the membrane-cell wall interface of *L. monocytogenes*. Before performing this experiment, we determined the temporal regulation of *prsA2* expression in broth culture to assess when a defect in *prsA2* expression was most likely to affect cell wall integrity. A *prsA2* promoter and *gus* structural gene transcriptional fusion was constructed, and levels of β-glucuronidase (Gus) were measured (Fig. 5). Gus was detected early during exponential growth phase, peaked in late log phase, and dropped during stationary phase, suggesting that PrsA2 was most important during the exponential phase of bacterial growth.

Cell wall porosity was assessed using bacteria in the exponential phase of growth. Protoplasts were generated using a purified cell wall hydrolase. To verify the purity of the cell wall fraction, we assessed the presence of a cell wall protein, InlA, and of a cytoplasmic protein, PrfA, by Western immunoblotting (40). The results confirmed the presence of InlA and the absence of PrfA in the cell wall fraction of each sample, indicating that it was devoid of cytoplasmic proteins (Fig. 6A).

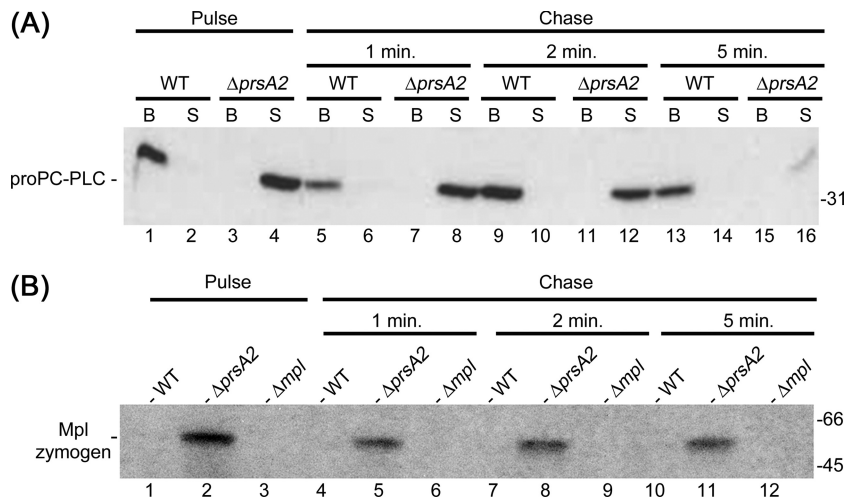This cell wall fraction is expected to contain cell wall-an-

FIG. 2. PC-PLC and Mpl are prematurely secreted into the host cytosol in the absence of PrsA2. Infected mouse J774 macrophage-like cells were pulse-labeled and then chased with cold Met/Cys and chloramphenicol for either 1, 2, or 5 min as indicated in the figure. (A) Bacterium-associated (B) and secreted (S) PC-PLC were immunoprecipitated from bacterial and host cell lysates, respectively. *L. monocytogenes* strains 10403S (wild type [WT]) and DP-L5601 (Δ*prsA2* mutant) were tested. (B) Secreted Mpl was immunoprecipitated from the host cell lysates. *L. monocytogenes* strains 10403S (WT), DP-L5601 (Δ*prsA2* mutant), and DP-L2343 (Δ*mpl* mutant) were studied. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the gel.

chored proteins as well as translocated proteins that have not yet been secreted across the cell wall. If the *prsA2* mutant cell wall were more porous, we would expect to detect either fewer or fainter protein bands in this fraction. Although we observed minor differences, overall the protein patterns for the wild-type strain, the *prsA2* mutant, and the complemented mutant strain were very similar (Fig. 6B). These results suggested that there is no gross difference in the cell wall porosity of the wild-type *L. monocytogenes* and the *prsA2* mutant.

Susceptibility of *L. monocytogenes* to cell wall hydrolases is modified in the absence of PrsA2. To further investigate potential differences in the biochemical properties of the cell wall from wild-type and *prsA2* mutant bacteria, we assessed the susceptibility of the cell wall to lysozyme and to an *L. monocytogenes*-specific cell wall hydrolase. Lysozyme cleaves the β(1,4) linkage between the *N*-acetylglucosamine and *N*-acetyl-muramic acid residues of peptidoglycan. Independent of its enzymatic activity, lysozyme has also been shown to induce cell lysis through the activation of autolysins (21). To determine

whether the *prsA2* mutant was more susceptible to lysozyme-mediated lysis, the optical density of exponential-phase wild-type and *prsA2* bacteria incubated with or without lysozyme was measured as a function of time (Fig. 7A). In the presence of lysozyme, the *prsA2* mutant exhibited a slight but significant increase in the rate of lysis compared to wild-type bacteria, beginning at 10 min ($P < 0.05$). The same assay was performed with purified Ply118, an *L. monocytogenes*-specific phage endolysin that cleaves the peptidoglycan peptide backbone between the L-alanine and D-glutamate residues (24). The *prsA2* mutant was slightly more sensitive to Ply118 during the first 10 min of incubation (Fig. 7B). However, as incubation progressed, the mutant showed more resistance to Ply118 than wild-type bacteria. This difference was significant beginning at
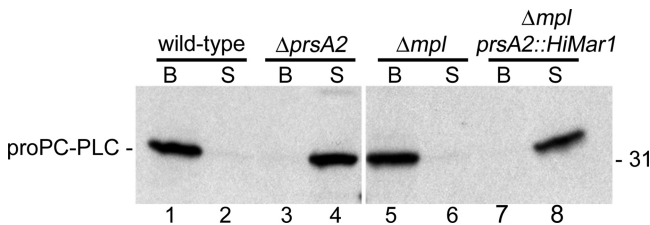

FIG. 3. PC-PLC is secreted into the host cytosol independent of Mpl in the absence of PrsA2. Infected mouse J774 macrophage-like cells were pulse-labeled. Bacterium-associated (B) and secreted (S) PC-PLC were immunoprecipitated from bacterial and host cell lysates, respectively. *L. monocytogenes* strains 10403S (wild type), DP-L5601 (Δ*prsA2* mutant), DP-L2343 (Δ*mpl* mutant), and HEL-1216 (Δ*mpl prsA2::Himar1* mutant) were tested. The position of the 31-kDa protein standard is indicated to the right of the gel.
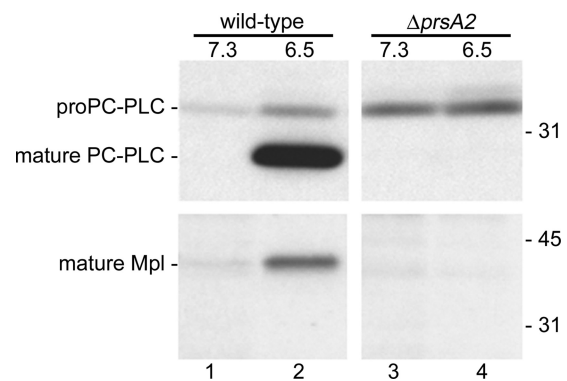

FIG. 4. pH-dependent PC-PLC maturation and Mpl autocatalysis do not occur in the absence of PrsA2. Infected mouse J774 macrophage-like cells were pulse-labeled and then chased in a nigericin-containing potassium buffer equilibrated to pH 7.3 or pH 6.5. Secreted PC-PLC and Mpl were immunoprecipitated from host cell lysates. *L. monocytogenes* strains 10403S (wild type) and DP-L5601 (Δ*prsA2* mutant) were tested. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the gel.
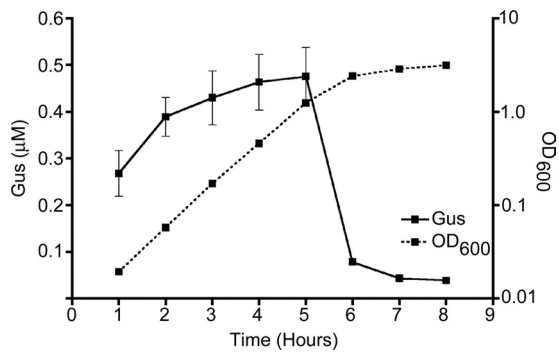
FIG. 5. *prsA2* is expressed during exponential growth in broth culture. *L. monocytogenes* strain 10403S carrying pJZ095 (strain DP-L5755) was grown for 8 h in BHI medium. Each hour, an OD_{600} measurement was taken, and the β-glucuronidase (Gus) level from 1 ml of culture was measured. The levels of Gus were normalized to the OD_{600} measurements of the culture at each time point. The error bars represent the standard errors of the means. Data were collected from duplicate samples from three independent experiments.

25 min ($P < 0.05$) (Fig. 7B). Taken together, these results indicated that there are biochemical differences, albeit minor, in the peptidoglycan cell wall between the wild type and the *prsA2* mutant.

The absence of PrsA2 does not increase the susceptibility of *L. monocytogenes* to intracellular lysis. Next, we investigated whether differential susceptibility of wild-type and *prsA2* mutant bacteria to cell wall hydrolases would predispose the *prsA2* mutant to lysis during intracellular growth. Host cells were infected with bacteria carrying the gene coding for luciferase on a plasmid (35). The luciferase-coding gene is under the control of a cytomegalovirus promoter whose expression is dependent on host cell transcription factors, thus on bacterial lysis. No significant differences were observed between host cells infected with the wild-type and *prsA2* mutant bacteria (Fig. 8). For a positive control for this assay, we infected cells with bacteria expressing PSA bacteriophage holin and lysin. This bacterial strain underwent autolysis, and the infected host cells generated high levels of luciferase activity. This result indicated that intracellular bacteria lacking PrsA2 are not predisposed to intracellular lysis to a larger extent than wild-type bacteria.

PC-PLC is less stable when produced in the absence of PrsA2. The rapid secretion of PC-PLC and Mpl across the bacterial cell wall in the absence of PrsA2 could result from modifications in protein structure. To assess whether PrsA2 assists in the folding of PC-PLC and Mpl, the stability of the proteins secreted in the cytosol of infected cells was assessed. Pulse-labeled bacterial proteins from infected cells were chased for incremental periods of time. Secreted PC-PLC and Mpl were immunoprecipitated from the host cell lysate, fractionated by SDS-PAGE, and detected by phosphorimaging. Because wild-type bacteria secrete smaller amounts of proproteins than the *prsA2* mutant, the wild-type sample gels were exposed two to three times longer than the *prsA2* mutant sample gels were. The intensities of protein bands were quantified using ImageJ (Fig. 9), and protein half-lives were calculated. The half-lives of PC-PLC made by the *prsA2* mutant and wild-type strain were 5 min and greater than 10 min, respec-
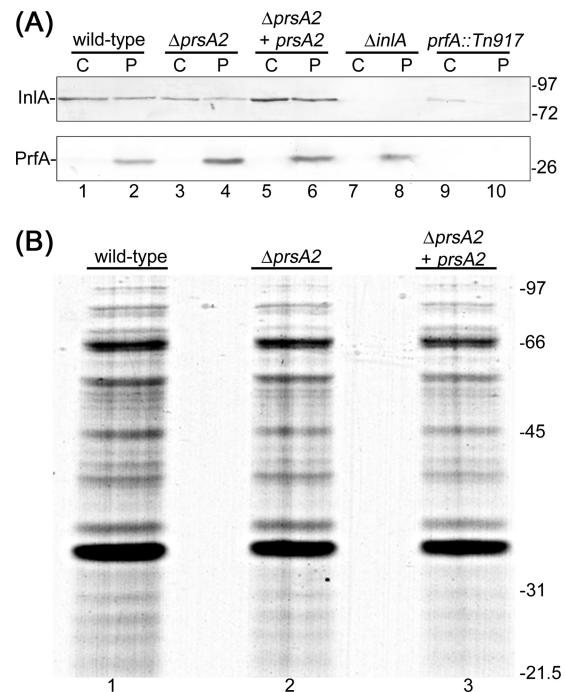

FIG. 6. (A) Detection of InlA and PrfA from cell wall (C) and protoplast (P) fractions by Western immunoblotting. *L. monocytogenes* strains 10403S (wild type), DP-L5601 (Δ*prsA2* mutant), DP-L5603 (Δ*prsA2* mutant carrying pJZ065, which contains the *prsA2* gene), EJL-12 (Δ*inlA* mutant), and DP-L1075 (*prfA::*Tn*917*) were tested. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the gel. (B) Protein profiles of *L. monocytogenes* wild-type and *prsA2* mutant cell wall fractions. Proteins that localize in the cell wall and at the membrane-cell wall interface were isolated as described in Materials and Methods. Equivalent amounts of culture OD units were loaded per lane. Proteins were resolved by SDS-PAGE and detected by Coomassie brilliant blue staining. The heavy band above the 31-kDa marker corresponds to phage endolysin Ply118. *L. monocytogenes* strains 10403S (wild type), DP-L5601 (Δ*prsA2* mutant), and DP-L5603 (Δ*prsA2* mutant carrying pJZ065, which contains the *prsA2* gene) were tested. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the gel.

tively (Fig. 9 and 2A, respectively). In contrast, Mpl was very stable in the absence or presence of PrsA2 (Fig. 2B and data not shown). These results suggested that the secreted proform of PC-PLC, but not the Mpl zymogen, is folded differently in the absence of PrsA2, and thus more sensible to proteolysis.

Deletion of the propeptide rescues the PC-PLC activity defect in the absence of PrsA2. PC-PLC synthesized in the absence of its propeptide is rapidly secreted across the bacterial cell wall as an active enzyme, and this phenomenon is independent of pH and of Mpl (46). We questioned whether PrsA2 contributes to the folding of the catalytic domain of PC-PLC by examining phospholipase activity generated by a propeptide deletion mutant (Δpro*plcB*). Wild-type and mutant bacteria were spotted on egg yolk agar, where a zone of opacity around the colony is indicative of PC-PLC activity. As previously reported, the *prsA2* mutant generated less activity than the wild-type strain, and the propeptide deletion mutant generated a higher level of phospholipase activity than the wild-type strain (46, 47) (Fig. 10A, panels 1, 2, and 4). Interestingly, the level of
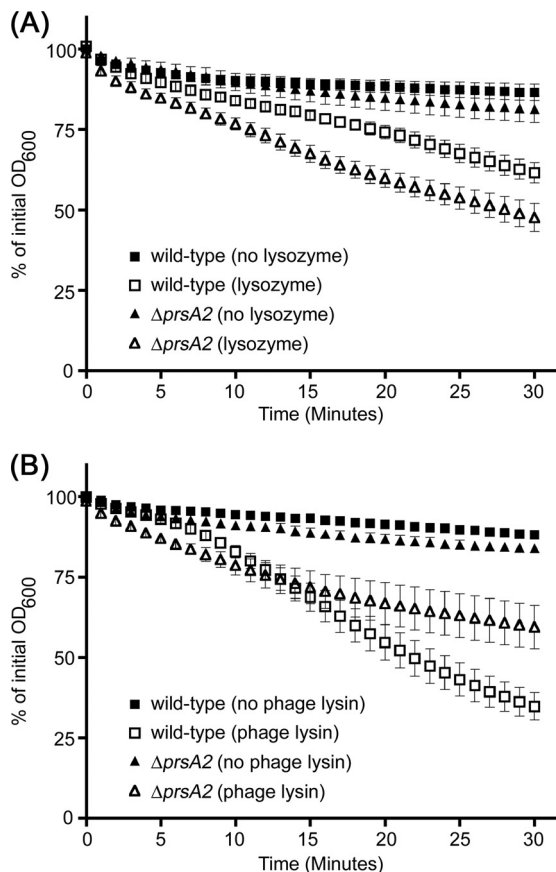
FIG. 7. Deletion of *prsA2* affects the cell wall of *L. monocytogenes*. The sensitivity of mid-log phase bacteria to lysis in the presence (open symbols) or absence (filled symbols) of lysozyme (A) or phage endolysin Ply118 (B) was monitored by spectrometry. OD$_{600}$ measurements were normalized to the initial optical density prior to incubation. The error bars represent the standard errors of the means from three independent experiments. The *L. monocytogenes* strains tested include 10403S (wild type) (squares) and DP-L5601 (Δ*prsA2* mutant) (triangles).

phospholipase activity generated by the propeptide deletion mutant was independent of PrsA2 (Fig. 10A, panel 5).

We next wished to determine whether deleting the propeptide of PC-PLC would partially restore the cell-to-cell spread defect of the *prsA2* mutant in infected cells. To address this question, we used a plaque assay in which the size of the plaques is proportional to the efficiency of cell-to-cell spread of the bacteria (Fig. 10B). As previously reported, the *prsA2* mutant makes plaques that are 25.5% the size of wild-type plaques (2, 47), whereas the *plcB* mutant generates plaques that are 40.8% the size of wild-type plaques, indicating that the plaque defect observed in the *prsA2* mutant is not due to its effect on PC-PLC alone. The Δpro*plcB* mutant makes plaques that are close in size to wild-type plaques (87.3% of wild-type) (45). The *prsA2* Δpro*plcB* double mutant makes plaques that are 44.5% the size of wild-type plaques, which is an intermediate size between the *prsA2* and Δpro*plcB* single mutants. Together, these results indicated that deleting the propeptide of PC-PLC rescues the PC-PLC activity defect associated with the absence of PrsA2 but only partially restores the cell-to-cell spread de-
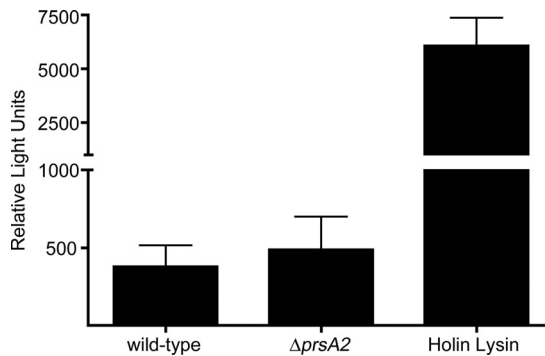

FIG. 8. The *prsA2* mutant does not show an increased sensitivity to lysis during intracellular growth. Intracellular bacterial lysis was measured using the luciferase reporter plasmid pBHE573 in infected macrophage cells. The error bars represent the standard deviations from three independent experiments. The *L. monocytogenes* strains tested include the wild type (strain DP-L5749), Δ*prsA2* mutant (DP-L5751), and a 10403S strain expressing holin/lysin (DP-L5750).

fect. This absence of full complementation can possibly be attributed to a decrease in the ability of listeriolysin O (LLO) to form pores in the absence of PrsA2 (2, 47).

DISCUSSION

L. monocytogenes relies on numerous virulence factors to facilitate its intracellular lifestyle. Two of these factors, broad-range phospholipase C (PC-PLC) and metalloprotease of *L. monocytogenes* (Mpl), contribute to vacuolar lysis. Both proteins are produced as proenzymes that are translocated across the bacterial membrane and retained at the membrane-cell wall interface until a decrease in vacuolar pH triggers Mpl autocatalysis followed by Mpl-mediated maturation of PC-PLC (28, 31, 40). This maturation process correlates with the detection of Mpl and PC-PLC in the host cell. PrsA2, a peptidyl prolyl *cis-trans* isomerase (PPIase) and posttranslocation chaperone, was identified as contributing to the activity of PC-PLC (2, 3, 47). In this study, we assessed the mechanism by which PrsA2 controls PC-PLC activity. Our data showed that, in the absence of PrsA2, the proforms of PC-PLC and Mpl are prematurely secreted across the cell wall and that a decrease in intracellular pH did not lead to maturation of either protein. This phenomenon does not appear to result from a change in
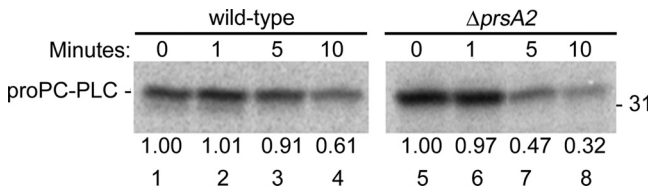

FIG. 9. PC-PLC is less stable when produced in the absence of PrsA2. Infected mouse J774 macrophage-like cells were pulse-labeled for 5 min and chased for 0, 1, 5, and 10 minutes. Secreted PC-PLC was then immunoprecipitated as described in Materials and Methods and detected by phosphorimaging. The relative amount of protein detected at each time point in comparison to the 0-min lanes was measured using ImageJ. *L. monocytogenes* strains 10403S (wild type) and DP-L5601 (Δ*prsA2* mutant) were tested. The position of the 31-kDa protein standard is indicated to the right of the gel.
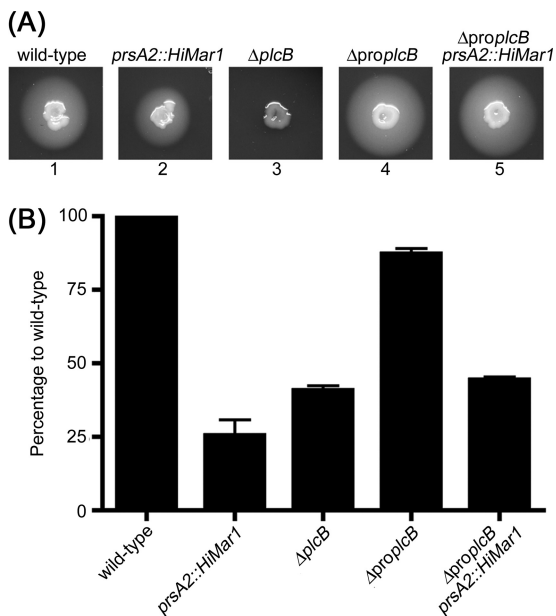
FIG. 10. Deletion of the PC-PLC propeptide restores phospholipase activity in the *prsA2* mutant. (A) Detection of PC-PLC activity on LB egg yolk agar plates. A zone of opacity indicates PC-PLC activity. *L. monocytogenes* strains 10403S (wild type) (panel 1), DP-L5596 (*prsA2::Himar1*) (panel 2), DP-L1935 (Δ*plcB* mutant) (panel 3), HEL-335 (propeptide deletion mutant [Δpro*plcB*]) (panel 4), and HEL-1405 (HEL-335 *prsA2::Himar1*) (panel 5) were tested. (B) Plaque assay to measure *L. monocytogenes* cell-to-cell spread in mouse L2 fibroblast cells. Plaque area is reported as a percentage of the wild-type plaque area. The error bars represent the standard deviations from three independent experiments. The strains tested were the same as those tested in panel A. Statistical significance was tested using ANOVA with Tukey-Kramer multiple-comparison test. All comparisons were significantly different ($P \leq 0.05$) except between strains DP-L1935 and HEL-1405.

cell wall porosity, although there is biochemical evidence that the cell wall of the *prsA2* mutant is slightly modified compared to the cell wall of the wild type. The stability of the secreted proform of PC-PLC was compromised in the absence of PrsA2, whereas the secreted Mpl zymogen was very stable. However, the PC-PLC activity defect observed in the absence of PrsA2 was rescued by deleting the propeptide of PC-PLC. We conclude that the PC-PLC activity defect observed in the absence of PrsA2 results from the premature secretion of the Mpl zymogen, possibly caused by changes in the biochemical properties of the cell wall, and from the inability of the zymogen to undergo autocatalysis postsecretion.

The ability of Mpl to localize at the membrane-cell wall interface during intracellular growth is imperative to its function (31). This fact was emphasized in this study by the observation that, in the absence of PrsA2, Mpl was prematurely secreted and did not undergo maturation upon a decrease in pH. This phenomenon is probably not due to a change in Mpl conformation, as the prematurely secreted enzyme was very stable. This was not surprising, as Mpl folding is unlikely to be assisted by PrsA2 considering that the individual propeptides of metalloproteases have been shown to act as folding chaperones (8, 25). If Mpl autocatalysis occurs when the protein localizes at the membrane-cell wall interface, then Mpl-mediated maturation of PC-PLC is likely to occur before secretion of these two proteins across the cell wall, as the confined area within the cell envelope would favor an encounter between the proform of PC-PLC and mature Mpl. Control of Mpl-mediated activation of PC-PLC is less stringent when the proteins are secreted by bacteria grown on complex medium, as some PC-PLC activity was detected around the colonies formed by the *prsA2* mutant. However, it is difficult to compare extracellular and intracellular conditions. The pH stability experienced by bacteria multiplying in the cytosol of host cells cannot be reproduced when *L. monocytogenes* is grown in complex medium. Consequently, the Mpl zymogen may be exposed to acidic conditions during translocation across the bacterial cell wall, enabling immediate autocatalysis. However, the fact that some level of PC-PLC was detected on complex medium in the absence of PrsA2 argues that PrsA2 is not required for Mpl folding. We conclude that Mpl autocatalysis is controlled by factors and/or conditions that prevail within the cell envelope and that a decrease in pH is not sufficient to trigger Mpl autocatalysis.

The results from the present study indicated that the cell wall of the *prsA2* mutant was modified as assessed by an increased sensitivity to lysozyme and a modified sensitivity to an *L. monocytogenes*-specific cell wall hydrolase. These results, along with the previous observation that the *prsA2* mutant is more sensitive to penicillin than the wild type (3), suggest that the *prsA2* mutant has a modified cell wall. It appears that biochemical differences exist in the glycan chains of peptidoglycan between the wild type and the *prsA2* mutant, as both lysozyme and phage endolysin Ply118 recognize the sugar moieties of peptidoglycan (23, 29). The resistance of *L. monocytogenes* to lysozyme is conferred by PgdA, an enzyme that deacetylates *N*-acetylglucosamine residues of peptidoglycan (19). The absence of PrsA2 may alter the activity of PgdA, thus making peptidoglycan more sensitive to lysozyme (7). Alternatively, lysozyme activity may be affected by cell wall charges. The levels of D-alanylation of teichoic and lipoteichoic acids modulate the levels of negative charges in the cell wall (33). Lysozyme is positively charged, and bacteria unable to D-alanylate teichoic acid are more susceptible to lysozyme (16, 20). The absence of PrsA2 may affect the cell wall charge and the levels of cations bound to the cell wall, which could modulate the rate of folding and secretion of the Mpl zymogen. Similarly, these differences could influence the folding and secretion of PC-PLC. Alternatively, these changes in cell wall properties could influence other proteins that contribute to maintain the Mpl zymogen and the proform of PC-PLC associated with bacteria.

The mechanism that controls the compartmentalization of PC-PLC is unknown. The proform of PC-PLC is secreted across the cell wall at a rate that is lower than the rate of translocation across the cytoplasmic membrane, enabling the protein to accumulate at the membrane-cell wall interface (40). However, when PC-PLC is synthesized without its propeptide or in the absence of PrsA2, the protein is rapidly secreted across the cell wall. Synthesis of PC-PLC in the absence of its propeptide generates an active enzyme independent of PrsA2. Therefore, neither the propeptide of PC-PLC nor PrsA2 is required for native folding of the catalytic domain, but both influence secretion of the enzyme. However, the secreted proform of PC-PLC is more stable in the presence

of PrsA2, suggesting a role for PrsA2 in folding of this enzyme. Recent work has shown that the N terminus of PrsA2 is necessary for PC-PLC activity (3). Perhaps the propeptide of PC-PLC serves to slow down protein folding and to interact with the N terminus of PrsA2. The interaction with PrsA2 would be released only once the proprotein has reached its native conformation, which would explain the low rate of protein secretion and the difference in protein stability observed between the slowly and quickly secreted forms of proPC-PLC.

In conclusion, during intracytosolic growth, *L. monocytogenes* requires PrsA2 to maintain the Mpl zymogen and the proform of PC-PLC in close proximity within the bacterial cell envelope. This condition is imperative to rapidly generate and deliver active PC-PLC in vacuoles formed upon cell-to-cell spread. We hypothesize that PrsA2 regulates the compartmentalization and activity of these proenzymes by influencing cell wall properties and by interacting with the PC-PLC propeptide. This mechanism of regulating in a spatial and temporal manner the secretion of active virulence factors is similar, in principle, to the mechanisms used by Gram-negative bacteria to deliver type III and IV secretion system effectors into eukaryotic cells.

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