The Metalloprotease of *Listeria monocytogenes* Controls Cell Wall Translocation of the Broad-Range Phospholipase C

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*Listeria monocytogenes* is a gram-positive bacterial pathogen that multiplies in the cytosol of host cells and spreads directly from cell to cell. During cell-to-cell spread, bacteria become temporarily confined to secondary vacuoles. The broad-range phospholipase C (PC-PLC) of *L. monocytogenes* contributes to bacterial escape from secondary vacuoles. PC-PLC requires cleavage of an N-terminal propeptide for activation, and Mpl, a metalloprotease of *Listeria*, is involved in the proteolytic activation of PC-PLC. Previously, we showed that cell wall translocation of PC-PLC is inefficient, resulting in accumulation of PC-PLC at the membrane-cell wall interface. In infected cells, rapid cell wall translocation of PC-PLC is triggered by a decrease in pH and correlates with cleavage of the propeptide in an Mpl-dependent manner. To address the role of the propeptide and of Mpl in cell wall translocation of PC-PLC, we generated a cleavage site mutant and a propeptide deletion mutant. The intracellular behavior of these mutants was assessed in pulse-chase experiments. We observed efficient translocation of the proform of the PC-PLC cleavage site mutant in a manner that was pH sensitive and Mpl dependent. However, the propeptide deletion mutant was efficiently translocated into host cells independent of Mpl and pH. Overall, these results suggest that Mpl regulates PC-PLC translocation across the bacterial cell wall in a manner that is dependent on the presence of the propeptide but independent of propeptide cleavage. In addition, similarly to Mpl-mediated cleavage of PC-PLC propeptide, Mpl-mediated translocation of PC-PLC across the bacterial cell wall is pH sensitive.

*Listeria monocytogenes* is an opportunistic bacterial pathogen that has the ability to multiply in the cytosol of eukaryotic cells (9, 32) and spread to neighboring cells without entering the extracellular milieu. Successful infection by *L. monocytogenes* is dependent on the bacterium’s ability to escape membrane vacuoles formed upon initial entry into a host cell and upon direct cell-to-cell spread. Among the factors involved in bacterial escape from these vacuoles is a secreted broad-range phospholipase C (PC-PLC) (Fig. 1a) (28, 34), whose activity requires proteolytic cleavage of a 24-amino-acid N-terminal propeptide (24, 25). A metalloprotease of *L. monocytogenes* (Mpl) is associated with the proteolytic activation of PC-PLC (19, 25). During intracellular infection, PC-PLC activation is dependent on cell-to-cell spread and vacuolar acidification (19), indicating that cleavage of the propeptide occurs specifically in acidified vacuoles. We are interested in defining the mechanism regulating PC-PLC activity during intracellular infection.

A number of prokaryotic and eukaryotic proteins are synthesized as preproteins, in which the signal peptide is followed by a propeptide whose proteolytic cleavage is required to generate the mature protein (5, 27). There are two classes of propeptides, class I and II. Class I propeptides generally function as intramolecular chaperones, facilitating folding of the mature protein. In some instances the propeptide can function in trans, but native conformation cannot be reached without the propeptide (21, 36). Class I propeptides are primarily associated with proteases. Class II propeptides exert their biological function by a variety of mechanisms, including oligomerization, intermolecular interactions, maturation, sorting, folding, and secretion (27). Among others are the propeptide of *Bacillus amyloliquefaciens* small RNase barnase, which decreases the rate of protein folding in the presence of GroEL (11), the propeptides of *Staphylococcus aureus* nuclease and of *Rhizopus oryzae* lipase, which increase the rate of protein folding and secretion (3, 17), and that of human myeloperoxidase, which is required for the maturation process and sorting of the protein to azurophil granules of neutrophils (1).

Studies have shown that processing of the PC-PLC propeptide is a prerequisite to enzymatic activity (25), but the mechanism by which the propeptide prevents activity is unknown. The propeptide could interact with the phospholipase active site or interfere with folding of the catalytic domain. In a recent study, we showed that the proform of PC-PLC does not translocate very efficiently across the bacterial cell wall and that processing of the PC-PLC propeptide correlates with a rapid increase in the rate of PC-PLC translocation (20, 30). This observation is consistent with a role of the propeptide in protein folding, as there are examples of proteins whose rate of folding correlates with their rate of translocation across the cell wall (14, 33, 35). Alternatively, intermolecular interactions involving the prodomain could prevent translocation of PC-PLC across the cell wall.

Mpl is predicted to be a 55-kDa secreted protein composed of a 20-kDa propeptide and a 35-kDa catalytic domain (22). Like PC-PLC, Mpl is bacterium associated and secreted (30). In infected mammalian cells, rapid translocation of bacteria-
associated PC-PLC is triggered by a decrease in pH in an Mpl-dependent manner. Therefore, Mpl-mediated processing of the PC-PLC propeptide might control translocation of PC-PLC across the bacterial cell wall. Alternatively, Mpl may target secondary molecules whose processing or degradation is the prerequisite to the efficient translocation of PC-PLC across the cell wall. Moreover, Mpl may carry a dual function as a chaperone and a protease contributing to PC-PLC folding and proteolytic activation. DegP, also known as HtrA, is a good example of a bacterial extracytoplasmic protein carrying such a dual function (2, 15, 31).

The present study investigates the role of the PC-PLC propeptide and of Mpl in the mechanism regulating translocation of PC-PLC across the bacterial cell wall. To address this point, we created a PC-PLC cleavage site mutant (PC-PLC S51D S53N) and a prodomain deletion mutant (PC-PLC Δpro). The intracellular behaviors of the mutated PC-PLC proteins as a function of pH and of Mpl were investigated. Our results indicate that Mpl contributes to the translocation of PC-PLC independently of cleavage of the propeptide and that, in absence of the propeptide, PC-PLC translocation across the bacterial cell wall is no longer regulated by Mpl and pH.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Bacterial strains and relevant genotypes are listed in Table 1. For the in vitro experiments, expression of the PrfA-regulated genes was induced by growing bacteria in Luria-Bertani (LB) broth with 50 mM morpholinepropanesulfonic acid (MOPS) adjusted to pH 7.3, 25 mM glucose-1-phosphate (G1P), and 0.2% activated charcoal (LB-MOPS-G1P) (26, 30). For J774 infections, bacteria were grown overnight at 30°C in brain heart infusion broth.

**Construction of plcB Δpro.** An internal in-frame deletion was created in the plcB gene to delete the propeptide coding sequence (Fig. 1b). A 672-bp fragment containing the 5' end of the plcB gene (coding for the signal sequence) and upstream region was amplified by PCR with primers Marq-46 and Marq-123 (Table 2), using 10403S genomic DNA as a template. A second fragment of 1,300 bp containing sequence coding for the catalytic domain of PC-PLC and downstream sequence was amplified by PCR with primers Marq-124 and Marq-107 (Table 2). PCR fragments were purified, digested with AvaIII, and ligated. Ligation product was used as template for PCR amplification of plcBΔpro using flanking primers Marq-46 and Marq-107. The resulting product (1,938 bp) was digested with HindIII and XbaI and cloned into the shuttle vector pKSV7 (29), creating pHEL-332. pHEL-332 was electroporated into strains 10403S and the mpl mutant (DP-L2296), and the mutation was introduced into the chromosome of these strains by allelic exchange as previously described (6), creating HEL-333 and HEL-335 (Table 1).

**Construction of plcB cleavage site mutant.** The plcB cleavage site mutant was generated by random mutagenesis. As described in more detail below, a degenerate primer with the sequence RRYTGGRRY was used, which would put one of four amino acids (D, N, S, and G) at S51 and S53 of the plcB open reading frame. D, N, and G are not generally favorable for protease activity of the Mpl family (23).

**TABLE 1. L. monocytogenes strains and genotypes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotypea</th>
<th>Source of strain or reference</th>
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<tr>
<td>10403S</td>
<td>Wild type</td>
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<tr>
<td>DP-L1935</td>
<td>ΔplcB</td>
<td>28</td>
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<tr>
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<td>ΔplcBΔmpl</td>
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<td>38</td>
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<td>HEL-333</td>
<td>plcBΔpro</td>
<td>This study</td>
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<tr>
<td>HEL-335</td>
<td>plcBΔpro</td>
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<tr>
<td>HEL-337</td>
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<tr>
<td>HEL-338</td>
<td>plcB S51D S53NΔmpl</td>
<td>This study</td>
</tr>
</tbody>
</table>

a All mutants are derivatives of 10403S.

b Gene fusion of L. monocytogenes prepro-PC-PLC plcB sequence and B. cereus catalytic domain of plc sequence.
To construct PC-PLC cleavage site mutants, three separate fragments were amplified by PCR using purified 10403S genomic DNA as a template. The hly promoter region (621 bp) was amplified with primers Marq-122 and Marq-106 (Table 2); the 5′ end of the plcB gene, containing the signal sequence and propeptide (170 bp) was amplified with primers Marq-105 and Ncol (Table 2); and the 3′ end of plcB (1,300 bp) was amplified with primer Marq-101, which contains degenerate codons for the cleavage site mutants, and primer Marq-107 (Table 2). The hly promoter fragment was digested with BamHI and Ncol, the plcB 5′ fragment was digested with BspHI and Hind III, and the plcB 3′ fragment was digested with HindIII and KpnI. All three fragments were sequentially ligated together and cloned into pPL2. pPL2 is an *L. monocytogenes* phage-specific integration vector (16). Resulting constructs were conjugated into *L. monocytogenes* cells containing bactopET and Western immunoblot assays were performed for detection of PC-PLC from bacterial supernatants. One mutant was selected for its defect in PC-PLC processing.

**Detection of phospholipase activity.** LB agar was supplemented with 0.2% activated charcoal granules before autoclav ing. The medium was cooled down to 55°C before adding glucose-1-phosphate (25 mM), egg yolk (1.25%), and streptomycin (200 μg/ml). LB-G1P-EY plates were poured without the charcoal. Incorporated plates were incubated at 37°C, and PC-PLC activity was detected by the formation of a zone of opacity in the overlay.

**Western immunoblotting.** Western immunoblot assays were performed as described previously (30). Primary antibodies used were affinity-purified rabbit anti-PC-PLC (1/5,000) and affinity-purified rat anti-PC-PLC propeptide (1/1,000). Secondary antibodies used were alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) and anti-rat IgG (1/25,000).

**Generation of anti-propeptide antibody and affinity purification.** An 18-mer peptide (CDEYLTPAAPHDIDSKL), encompassing three-fourths of the PC-PLC propeptide (CDEYLQTPAAPHDIDSKL), was synthesized (Protein Chemistry Laboratory of the Medical School of the University of Pennsylvania) and conjugated to keyhole limpet hemocyanin. Generation of the rat immune serum was contracted by Cocalico Biologicals, Inc. For affinity purification of antibodies, the propeptide was conjugated to SulfoLink coupling gel (Pierce Biotechnology, Inc.), and specific antibodies were purified according to the manufacturer’s instructions.

**Proteolytic digests.** To investigate the folding of secreted PC-PLC, bacterial culture supernatants were treated with different endopeptidases prior to Western immunoblotting. Briefly, *L. monocytogenes* wild-type and mutant strains were grown in LB-MOPS-G1P at 37°C without shaking to an optical density of 600 nm of 1.2 to 1.4. Bacterial cells were pelleted by centrifugation at 8,820 × g for 10 min at 4°C. Supernatants were collected and treated with 200 μg of trypsin/ml for 15 min at 25°C, 50 μg of chymotrypsin/ml for 15 min at 25°C, 10 μg of thermolysin/ml for 18 h at 37°C, or 2 U of glutamyl endopeptidase (V8)/ml for 18 h at 37°C. Endopeptidase concentrations and incubation times used in these reactions were optimized experimentally. Digested products were precipitated on ice by adding 10% trichloroacetic acid. Precipitates were pelleted by centrifugation at 20,000 × g at 4°C for 10 min and washed with acetone. Precipitates were solubilized in 2× sample buffer (4% SDS, 20% glycerol, 125 mM Tris-HCl [pH 6.8], 10% β-mercaptoethanol, bromophenol blue) and boiled for 5 min. Proteins from an equivalent of 250 μl of bacterial supernatant were resolved by electrophoresis on a SDS-11% polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane by using a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, Calif.). PC-PLC was detected by Western immunoblotting.

**RESULTS**

Active PC-PLC is generated independently of the propeptide. Protein peptidases may function as intramolecular chaperones and in some instances are required for a protein to reach its native conformation. To test whether the propeptide of PC-PLC is required for protein folding, we created a mutant in which the prodomain was deleted (*plcBΔpro*) (Fig. 1b). The *plcBΔpro* strain was tested for phospholipase activity on egg yolk plates and showed a strong zone of opacity, which is

<table>
<thead>
<tr>
<th>Marq no.</th>
<th>Nucleotide sequence (5′-3′) with underlined restriction sites</th>
<th>Characteristics</th>
</tr>
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<td>46</td>
<td>AATAAGCCGACCCAGCTTGTAAGTGAATGACATAAATA</td>
<td>plcB HindIII fwd</td>
</tr>
<tr>
<td>101</td>
<td>CCACATGACCTTRRTGRGGYCGCGATTAC</td>
<td>plcB cleavage HindIII fwd</td>
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<td>102</td>
<td>GCAGCCACTAAGCTATGTTAGGGATAATTGCTG</td>
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<tr>
<td>105</td>
<td>GTGTAATGCTAGTTAGTTAAGGATTTGTGTTTT</td>
<td>plcB signal peptide BspHI fwd</td>
</tr>
<tr>
<td>106</td>
<td>CTATGGCTAGCTTTTCATTGTTTTTACTCTCC</td>
<td>hly P Ncol rev</td>
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<tr>
<td>107</td>
<td>TTACCTAGACATGCTGGCATCCTTGT</td>
<td>plcB XbaI and KpnI rev</td>
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<tr>
<td>115</td>
<td>TTTAGATTCTGGCTCTGTTTTAAAATGTTGTG</td>
<td>hly P BamHI fwd</td>
</tr>
<tr>
<td>122</td>
<td>GCTAAGTGGATGTCACATGCTATTGCTTTTATA</td>
<td>plcB Apro AvalIII fwd</td>
</tr>
<tr>
<td>124</td>
<td>CACCATACAATATGCTATGCTAGCGGATAC</td>
<td>plcB S51D S53N fwd</td>
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<tr>
<td>152</td>
<td>GTTATCCCGTTTTCAACATGTTTATG</td>
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proteolysis was observed for the proform of PC-PLC (Fig. 3, upper panel, and data not shown). Trypsin, chymotrypsin, and thermolysin digests generated one major product that reacted with the affinity-purified PC-PLC antibody and comigrated with the active form of PC-PLC. It was further determined that the N terminus of the proform of PC-PLC was being cleaved by these three peptidases, as the PC-PLC product generated was not recognized by a propeptide-specific antibody (Fig. 3, bottom panel, and data not shown). The proform of PC-PLC was also partially sensitive to proteolysis by glutamyl endopeptidase. The product generated comigrated with the active form of PC-PLC and did not react with the propeptide-specific antibody (data not shown). Interestingly, cleavage of PC-PLC propeptide by these peptidases did not generate active PC-PLC, as tested by the egg yolk overlay assay (data not shown). According to structural studies on phospholipase C from B. cereus, a PC-PLC ortholog, the first amino acid residue of the PC-PLC catalytic domain (W52) would be an important zinc-coordinating residue (12). Perhaps specific cleavage between S51 and W52 is essential to generate an active enzyme.

The active form of PC-PLC (PC-PLCΔpro) was completely resistant to proteolysis by all four peptidases and the protein maintained its full activity, indicating that none of the putative cleavage sites are accessible on active PC-PLC (Fig. 3, upper panel, and data not shown). Overall, these results suggest that the catalytic domain of PC-PLC reaches native conformation independent of the propeptide. The propeptide may interfere with folding of the catalytic domain, hampering translocation of PC-PLC across the cell wall. We performed proteolytic digests of the secreted pro and active forms of PC-PLC to investigate their structure. Trypsin, chymotrypsin, and glutamyl endopeptidase (V8), which are serine peptidases, and the zinc-dependent peptidase thermolysin were used for proteolytic digests. There are over 30 putative sites for each one of these peptidases in the pro and active forms of PC-PLC (37). Partial proteolysis was observed for the proform of PC-PLC (Fig. 3, upper panel, and data not shown). Trypsin, chymotrypsin, and thermolysin digests generated one major product that reacted with the affinity-purified PC-PLC antibody and comigrated with the active form of PC-PLC. It was further determined that the N terminus of the proform of PC-PLC was being cleaved by these three peptidases, as the PC-PLC product generated was not recognized by a propeptide-specific antibody (Fig. 3, bottom panel, and data not shown). The proform of PC-PLC was also partially sensitive to proteolysis by glutamyl endopeptidase. The product generated comigrated with the active form of PC-PLC and did not react with the propeptide-specific antibody (data not shown). Interestingly, cleavage of PC-PLC propeptide by these peptidases did not generate active PC-PLC, as tested by the egg yolk overlay assay (data not shown). According to structural studies on phospholipase C from B. cereus, a PC-PLC ortholog, the first amino acid residue of the PC-PLC catalytic domain (W52) would be an important zinc-coordinating residue (12). Perhaps specific cleavage between S51 and W52 is essential to generate an active enzyme.

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Efficient translocation of PC-PLC across the bacterial cell wall is independent of propeptide cleavage but dependent of Mpl. Rapid translocation of PC-PLC across the bacterial cell wall occurs in response to a decrease in pH and correlates with processing of the PC-PLC propeptide in an Mpl-dependent manner (20, 30). To investigate the specific role of the propeptide in PC-PLC translocation across the cell wall, we created a PC-PLC cleavage site mutant. The cleavage site between the pro and catalytic domains of PC-PLC is K49-L50-S51//W52-S53N. For PC-PLC, efficient translocation correlates with proteolytic activation, and the propeptide may interfere with folding of the catalytic domain, hampering translocation of PC-PLC across the cell wall. We performed proteolytic digests of the secreted pro and active forms of PC-PLC to investigate their structure. Trypsin, chymotrypsin, and glutamyl endopeptidase (V8), which are serine peptidases, and the zinc-dependent peptidase thermolysin were used for proteolytic digests. There are over 30 putative sites for each one of these peptidases in the pro and active forms of PC-PLC (37). Partial proteolysis was observed for the proform of PC-PLC (Fig. 3, upper panel, and data not shown). Trypsin, chymotrypsin, and thermolysin digests generated one major product that reacted with the affinity-purified PC-PLC antibody and comigrated with the active form of PC-PLC. It was further determined that the N terminus of the proform of PC-PLC was being cleaved by these three peptidases, as the PC-PLC product generated was not recognized by a propeptide-specific antibody (Fig. 3, bottom panel, and data not shown). The proform of PC-PLC was also partially sensitive to proteolysis by glutamyl endopeptidase. The product generated comigrated with the active form of PC-PLC and did not react with the propeptide-specific antibody (data not shown). Interestingly, cleavage of PC-PLC propeptide by these peptidases did not generate active PC-PLC, as tested by the egg yolk overlay assay (data not shown). According to structural studies on phospholipase C from B. cereus, a PC-PLC ortholog, the first amino acid residue of the PC-PLC catalytic domain (W52) would be an important zinc-coordinating residue (12). Perhaps specific cleavage between S51 and W52 is essential to generate an active enzyme.

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S53-A54 (Fig. 1b). The catalytic domain of the *B. cereus* ortholog also starts with WSA, and the crystal structure indicates that the tryptophan residue is one of the nine zinc-coordinating residues (12). Site-directed mutagenesis was used to mutate the last amino acid residue of the propeptide (S51) and the second amino acid residue of the catalytic domain (S53). The resulting mutant, PC-PLC S51D S53N (Fig. 1b), was negative for PC-PLC activity when grown on egg yolk semisolid medium (Fig. 2), although a small amount of processed PC-PLC was detectable from infected cells (see below). PC-PLC S51D S53N behaved similarly to PC-PLC when subjected to trypsin, chymotrypsin, thermolysin, and glutamyl endopeptidase activities, suggesting that the double mutation did not affect PC-PLC native conformation (Fig. 3 and data not shown).

Pulse-chase experiments were performed to follow translocation of PC-PLC S51D S53N across the bacterial cell wall as a function of pH. Infected cells were pulse-labeled with [35S]Met and then chased in a potassium-based buffer at either pH 7.3 or 6.5 in the presence of nigericin as described in Materials and Methods. When indicated, samples were treated for an additional 5 min with nigericin in buffer of pH 7.3 or 6.5. PC-PLC was immunoprecipitated from bacterial lysates (bacteria-associated) and host cell lysates (secreted), and samples were fractionated by SDS-PAGE. The gel was scanned with a phosphorimager. Proform and processed forms of PC-PLC migrated as indicated on the left side of the figure.

Next, the requirement for Mpl for translocation of PC-PLC across the bacterial cell wall was evaluated as a function of pH and Mpl. At pH 7.3, PC-PLC remained largely bacterium-associated (7.9% and 73.8% translocated, respectively (Table 3 and Fig. 4, upper panel). At pH 6.5, PC-PLC was activated and translocation increased by 9.3-fold (73.8% translocated), whereas 55.9% of PC-PLC was immunoprecipitated from the host cell lysate and host cell lysates (secreted), and samples were fractionated by SDS-PAGE. The gel was scanned with a phosphorimager. Proform and processed forms of PC-PLC migrated as indicated on the left side of the figure.

PC-PLCΔpro is efficiently translocated across the bacterial cell wall at physiological pH. To investigate the specific role of the PC-PLC propeptide in protein translocation, the intracellular behavior of PC-PLCΔpro was evaluated as a function of pH and Mpl. At pH 7.3, PC-PLC remained largely bacterium-associated (7.9% translocated), whereas 64.4% of PC-PLCΔpro was immunoprecipitated from the host cell lysate (Table 3 and Fig. 4, lower panel). At pH 6.5, PC-PLC was activated and translocation increased by 9.3-fold (73.8% translocated), whereas PC-PLCΔpro translocation only increased by 1.3-fold (82.8% translocated) (Table 3 and Fig. 5, upper panel). These results indicate that in the absence of a propeptide, translocation of PC-PLC across the bacterial cell wall is relatively insensitive to pH.

Next, the requirement for Mpl for translocation of PC-PLCΔpro across the bacterial cell wall was evaluated. At pH 7.3 and in the absence of Mpl, PC-PLC remained largely bacterium-associated (6.5% translocated), whereas 55.9% of PC-PLCΔpro was immunoprecipitated from the host cell lysate (Table 3 and Fig. 5, lower panel). At pH 6.5 and in the absence of Mpl, PC-PLC remained largely bacterium-associated (15.1% translocated), whereas 73.5% of PC-PLCΔpro was immunoprecipitated from the host cell lysate (Table 3 and Fig. 5, lower panel). Although the small changes in protein translocation at

### TABLE 3. Quantification of PC-PLC translocation across the bacterial cell wall

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Translocation at pH 7.3</th>
<th>Translocation at pH 6.5</th>
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<tr>
<td>Wild type</td>
<td>7.9 ± 0.5</td>
<td>73.8 ± 2.9</td>
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<tr>
<td><em>plcB S51D S53N</em></td>
<td>9.4 (7.2, 11.6)</td>
<td>79.2 (72.7, 85.8)</td>
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<td>Δmpl <em>plcB S51D S53N</em></td>
<td>11.0 (8.0, 14.0)</td>
<td>18.6 (14.0, 23.3)</td>
</tr>
<tr>
<td><em>plcBΔpro</em></td>
<td>64.4 ± 9.3</td>
<td>82.8 ± 7.7</td>
</tr>
<tr>
<td>Δmpl</td>
<td>6.5 ± 4.5</td>
<td>15.1 ± 3.6</td>
</tr>
<tr>
<td>Δmpl <em>plcBΔpro</em></td>
<td>55.9 ± 22.0</td>
<td>73.5 ± 12.6</td>
</tr>
</tbody>
</table>

* Mean percent ± standard deviation of PC-PLC immunoprecipitated from lysates of infected cells, determined by quantitative analysis of phosphorimagers from three independent experiments, except for the cleavage site mutants for which two independent experiments were performed. Results from each individual experiment performed with the cleavage site mutants are shown in parentheses.  

* P values (*, P < 0.0001) for comparison of PC-PLC translocation at either pH 7.3 or 6.5 by two-sample t test. Statistical analyses were not performed on samples with less than three replicates.
proteins from bacterial and host cell lysates, respectively. We observed that PC-PLC and PLCBC were present in similar amounts in the combined cell wall-associated and secreted fractions (Fig. 6). However, at physiological pH, the proform of the chimera was more efficiently translocated across the bacterial cell wall of *L. monocytogenes* than that of PC-PLC (62% translocation for PLCBC and 5% for PC-PLC) (Fig. 6). These results indicate that the PC-PLC propeptide does not have an intrinsic ability to regulate protein translocation across the bacterial cell wall, but rather the ability of the PC-PLC propeptide to regulate protein translocation is influenced by the nature of the protein to which it is bound.

**DISCUSSION**

*L. monocytogenes* has the remarkable ability to maintain a pool of PC-PLC at its membrane-cell wall interface and to promptly release a bolus of active PC-PLC within vacuoles formed in the process of cell-to-cell spread (19, 20, 30). Efficient translocation of PC-PLC across the bacterial cell wall occurs upon a decrease in pH and correlates with the proteolytic cleavage of an N-terminal propeptide in an Mpl-dependent manner. In the absence of Mpl, bacteria-associated PC-PLC remains in its proform and translocation across the bacterial cell wall is very inefficient independent of pH. In this study, we investigated the role of the propeptide and of Mpl in the mechanism regulating translocation of PC-PLC across the bacterial cell wall. Our results revealed that Mpl regulates PC-PLC translocation across the bacterial cell wall in a manner that is dependent on the presence of the propeptide, but independent of propeptide cleavage. In addition, similar to Mpl-mediated cleavage of the PC-PLC propeptide, Mpl-mediated translocation of PC-PLC across the bacterial cell wall is regulated by pH. Therefore, both Mpl functions are pH sensitive and dependent on the PC-PLC propeptide.

A cleavage site mutant (PC-PLC S51D S53N) was created to determine whether Mpl possesses a biological function that is independent of cleavage of the PC-PLC propeptide. PC-PLC S51D S53N was processed upon a decrease in intracellular pH, although to a much lesser extent than wild-type PC-PLC. However, in absence of Mpl, processing of PC-PLC S51D S53N was undetectable. These results suggest that Mpl is responsible for the proteolytic activation of PC-PLC S51D S53N, even though D and N are not generally favorable for protease activity of the Mpl family (23).

Proteolytic digests of PC-PLC and PC-PLC S51D S53N suggested that the proteins are structurally identical. Similarly to
PC-PLC, PC-PLC S51D S53N remained largely bacteria-associated at physiological pH. At acid pH, PC-PLC S51D S53N was as efficiently translocated across the bacterial cell wall as PC-PLC, even though 70% of the translocated mutant protein was in its proform. These results indicate that Mpl carries a pH-sensitive function that is independent of cleavage of the PC-PLC propeptide and that contributes to the regulation of PC-PLC translocation across the bacterial cell wall.

A prodomain deletion mutant was created to address the role of the propeptide in translocation of PC-PLC across the bacterial cell wall. This mutant makes constitutively active PC-PLC, indicating that the PC-PLC propeptide is not required for folding of the mature protein, suggesting that it is a class II propeptide (27). Class II propeptides are associated with a variety of biological functions, including intercellular interactions, sorting, and secretion. Remarkably, PC-PLC apoprotein was very efficiently translocated across the bacterial cell wall at physiological pH independent of Mpl, a behavior that is contrary to that of PC-PLC. This result suggests that the PC-PLC propeptide is a regulatory element, as the propeptide enables the formation of a pool of PC-PLC at the membrane-cell wall interface and prevents premature PC-PLC activity. However, the propeptide does not regulate translocation of the PLC domain chimera, even though it regulates its activity (38). It seems that the sorting function of the propeptide is influenced by the nature of the protein to which it is bound.

Efficient translocation of PC-PLC across the bacterial cell wall and cleavage of the PC-PLC propeptide are both mediated by Mpl upon a decrease in pH. Mpl is active at pH values ranging from 5 to 9 (7), suggesting that Mpl proteolytic activity per se is unlikely to be the pH-limiting factor. However, similar to PC-PLC, Mpl is made as a proenzyme whose activation requires cleavage of an N-terminal propeptide, so proteolytic activation of Mpl could be the pH-sensitive step. It is possible that Mpl functions as a chaperone in its proform, preventing PC-PLC to translocate across the bacterial cell wall and perhaps contributing to PC-PLC folding. DegP, also known as HtrA, is an example of a protein carrying a dual function as a chaperone and a protease (2, 15, 31). If Mpl contributes to PC-PLC folding, PC-PLC translocation across the bacterial cell wall would be impaired in the absence of Mpl as a result of PC-PLC misfolding. Alternatively, Mpl may target molecules other than PC-PLC whose processing or degradation is the prerequisite for the efficient translocation of PC-PLC across the cell wall. Future studies will assess the requirement for Mpl proteolytic activity in the mechanism regulating PC-PLC translocation across the bacterial cell wall.

In conclusion, results from this study and previous studies indicate that Mpl contributes to the regulation of PC-PLC activity by two independent mechanisms, both of which are pH sensitive. First, Mpl regulates cell wall translocation of PC-PLC. Second, Mpl regulates the proteolytic activation of PC-PLC. Importantly, both functions require the presence of the PC-PLC propeptide, because if the propeptide is absent, then PC-PLC translocation and activity are no longer regulated by Mpl and pH. These studies emphasize the complexity of protein translocation across the cell wall of gram-positive bacteria and the ability of a bacterial pathogen to regulate in a spatial and temporal manner the activity of an important virulence factor.

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