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Characterization of *Listeria monocytogenes* Expressing Anthrolysin O and Phosphatidylinositol-Specific Phospholipase C from *Bacillus anthracis*

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Two virulence factors of *Listeria monocytogenes*, listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC), mediate escape of this pathogen from the phagocytic vacuole of macrophages, thereby allowing the bacterium access to the host cell cytosol for growth and spread to neighboring cells. We characterized their orthologs from *Bacillus anthracis* by expressing them in *L. monocytogenes* and characterizing their contribution to bacterial intracellular growth and cell-to-cell spread. We generated a series of *L. monocytogenes* strains expressing *B. anthracis* anthrolysin O (ALO) and PI-PLC in place of LLO and *L. monocytogenes* PI-PLC, respectively. We found that ALO was active at both acidic and neutral pH and could functionally replace LLO in mediating escape from a primary vacuole; however, ALO exerted a toxic effect on the host cell by damaging the plasma membrane. *B. anthracis* PI-PLC, unlike the *L. monocytogenes* ortholog, had high activity on glycosylphosphatidylinositol-anchored proteins. *L. monocytogenes* expressing *B. anthracis* PI-PLC showed significantly decreased efficiencies of escape from a phagosome and in cell-to-cell spread. We further compared the level of cytotoxicity to host cells by using mutant strains expressing ALO in combination either with *L. monocytogenes* PI-PLC or with *B. anthracis* PI-PLC. The results demonstrated that the mutant strain expressing the combination of ALO and *B. anthracis* PI-PLC caused less damage to host cells than the strain expressing ALO and *L. monocytogenes* PI-PLC. The present study indicates that LLO and *L. monocytogenes* PI-PLC has adapted for *L. monocytogenes* intracellular growth and virulence and suggests that ALO and *B. anthracis* PI-PLC may have a role in *B. anthracis* pathogenesis.

Listeria monocytogenes and *Bacillus anthracis* are gram-positive bacteria in the low-G+C lineage that cause rare but fatal infections of humans and animals. Although *L. monocytogenes* has been studied as a model facultative intracellular pathogen for decades, *B. anthracis* had, until recently, received less attention. Interestingly, the *B. anthracis* genome sequence has revealed the presence of a number of potential determinants of pathogenesis shared with those of *L. monocytogenes*, including a phosphatidylinositol-specific phospholipase C (PI-PLC), a phosphatidylcholine-preferring phospholipase C (PC-PLC), and listeriolysin O (LLO) (27). In *L. monocytogenes*, these determinants are integral to its intracellular growth cycle. LLO is essential for escape from a phagosome, the phospholipases contribute to that process, and all three are important for cell-to-cell spread (3, 10, 27, 34). Although *B. anthracis* is traditionally considered an extracellular pathogen, there is considerable evidence that spores germinate and grow in macrophages during the early phase of infection (8, 30). We expressed here the *B. anthracis* orthologs of *L. monocytogenes* LLO and PI-PLC in *L. monocytogenes* in order to characterize them in a well-studied model of intracellular pathogenesis.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The *L. monocytogenes* strains used in the present study are listed in Table 1. Unless otherwise specified, all bacterial strains were grown in brain heart infusion broth, and strains derived from *L. monocytogenes* Δ*dal* Δ*dat* (Lmdd) were supplemented with 100 μg of D-alanine/ml to stationary phase at 30°C. Strains containing pKSV7 were selected and maintained with ampicillin (50 μg/ml) in *Escherichia coli* and chloramphenicol (10 μg/ml) in *L. monocytogenes*.

Tissue-culture cells and growth media. The mouse macrophage-like cell line J774 and murine L2 fibroblasts were grown in Dulbecco modified Eagle medium plus 7.5% fetal bovine serum (FBS) and 1 mM L-glutamine. Bone marrow-derived macrophages obtained from C57BL/6 mice were cultured in RPMI 1640–10% FBS–1 mM L-glutamine. All were incubated at 37°C with 5% CO_2. For bacterial infections with strains derived from Lmdd, 100 μg of D-alanine/ml was added in the medium.

Construction of *L. monocytogenes* mutants. The DNA sequences for ALO (31) and *B. anthracis* PI-PLC were obtained from the genome of *B. anthracis* strain Ames from GenBank (29). Signal peptides were determined by using the SignalP server (http://www.cbs.dtu.dk/services/SignalP-2.0/). The structural genes for ALO and *B. anthracis* PI-PLC were used for the replacement constructions. The mutant constructs were generated by PCR-mediated sequence overlap extension (15) with *Pfx* High-Fidelity DNA Polymerase, resulting in in-frame gene replacements. All gene replacements were made under their counterparts' endogenous promoters and signal peptide sequences on the chromosome. Mutants were constructed by using derivatives of a shuttle vector pKSV7 for allelic exchange. DNA sequences of mutant constructs were confirmed by automated cycle sequencing. Genes replaced in mutant strains are illustrated in Fig. 1. Oligonucleotide primers used in the present study are as follows: six primers each were used to replace LLO with ALO in the *L. monocytogenes* Δ*dal* Δ*dat* and wild-type *L. monocytogenes* background, respectively, as follows: BALLO-P1 (5′-GGTCTAGAGAGAGCGCTGCTAGGTTTGT-3′; XbaI) plus BALLO-P2 (5′-GCATTACCGGCTTGTGTTTCTGCTTCAGTTTGTTGCGCAA-3′) and DP4315 (5′-ATTGTCGACCGTATTCCTGCTTCTAATTGTTG-3′; SalI) plus DP4824 (5′-CAATTGCGCAACAAACTGAAGCAGAAACACAAGCCGGTAATGC-3′)

TABLE 1. Strains used in this study

Strain	Relevant characteristic(s)[a]	Source or reference
L. monocytogenes		
Lmdd	Δ*dal* Δ*dat*; D-alanine dependent	37
HG-L2001	Lmdd expressing H86A mutant LmPI-PLC	This study
HG-L2002	Lmdd expressing BaPI-PLC instead of LmPI-PLC	This study
HG-L2003	Lmdd expressing ALO instead of LLO	This study
HG-L2004	Lmdd expressing ALO and H86A mutant LmPI-PLC	This study
HG-L2005	Lmdd expressing ALO and BaPI-PLC	This study
10403S	Wild type *L. monocytogenes*	2
DP-L4055	*L. monocytogenes* 10403S expressing PFO	7
DP-L4450	*L. monocytogenes* 10403S expressing ALO	This study
DP-L2161	*L. monocytogenes* 10403S with LLO deleted (Δ*hly*)	16
B. anthracis Sterne 7702	pXO1$^+$, pXO2$^-$; attenuated, toxin$^+$	4

[a] LmPI-PLC, *L. monocytogenes* PI-PLC; BaPI-PLC, *B. anthracis* PI-PLC.

were used for the upstream flanking sequence of the *hly* gene; BALLO-P3 (TTGCGCAACAAACTGAAGCAGAAACACAAGCCGGTAATGC-3′) with BALLO-P4 (5′-CTTAATTTTTTACTTTTACAACTAATGACTAATAGTAG CAG-3′) and DP4825 (5′-GCATTACCGGCTTGTGTTTCTGCTTCAGTTTG TTGCGCAA-3′) with DP4826 (5′-CTTCGGATCCAACTAATGACTAATAG TAGCAGTTGG; BamHI) were used for DNA sequence encoding ALO from *B. anthracis* genomic DNA; and BALLO-P5 (5′-CTGCTACTATTAGTCATTAG TTGTAAAAGTAATAAAAAATTAAG-3′) with BALLO-P6 (5′-GGGGTAC CTGCTTCGCAGGAATCTGGCA-3′; KpnI) and DP4827 (5′-AATGGATCC GTAATAAAAAATTAAGAATAAAACC; BamHI) and DP4828 (5′-ATTGG ATCCTTATCGGTCTAGAAACCACCAGAACTTAGC; BamHI and XbaI) were used for the downstream flanking sequence of the *hly* gene. For replacement of *L. monocytogenes* PI-PLC with *B. anthracis* PI-PLC, another six primers were used: P1 (5′-ACTGTCTAGATCTCGCTAATACTCGTGAGCT-3′, with a XbaI site) and P2BA (5′-GCGAATAAGTCATTAATAAGAGATTAACATA TATTATTCCTACAA-3′) were used for the upstream flanking sequence of the *plcA* gene, P7BA (5′-TTGTAGGAATAATATATGTTAATCTCTTATTAATG ACTTATTCGC-3′) and P8N (5′-CCCATTAGGCGGGAAAGCAGCTAGCT CTGTTAATGAGCT-3′) were used for the DNA sequence encoding *B. anthracis* PI-PLC from *B. anthracis* genomic DNA, and P3N (5′-AGCTCATTAACA GAGCTAGCTGCTTTCCCGCCTAATGGG-3′) and P4 (5′-ACGTGGTACC ACTGCATCTCCGTGGTATAC-3′, with a KpnI site) were used for the downstream flanking sequence of the *plcA* gene. All PCR products were directly cloned into pCR-Blunt II-TOPO (Invitrogen) and were cycle sequenced.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the subsequent Western blotting were performed as previously described (40). The primary antibodies used in the present study were a rabbit anti-*B. thuringiensis* PI-PLC for detecting *B. anthracis* PI-PLC and a rabbit α-perfringolysin O (PFO) for detecting ALO expression. The secondary antibody was a goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase.

Expression and purification of recombinant His-tagged hemolysins. Recombinant mature His-tagged LLO and PFO proteins were constructed, expressed, and purified as previously described (12). ALO was PCR amplified from *B. anthracis* chromosomal DNA with the primers 5-CGGGGGATCCGAAACAC AAGCC-3 and 5-CCGGGAATTCCCTAATGACTAATAGTAGC-3 and cloned into the BamHI and EcoRI sites of the pTrcHisA expression vector (Invitrogen), resulting in an ALO fusion protein that starts at E35 for the ALO protein.

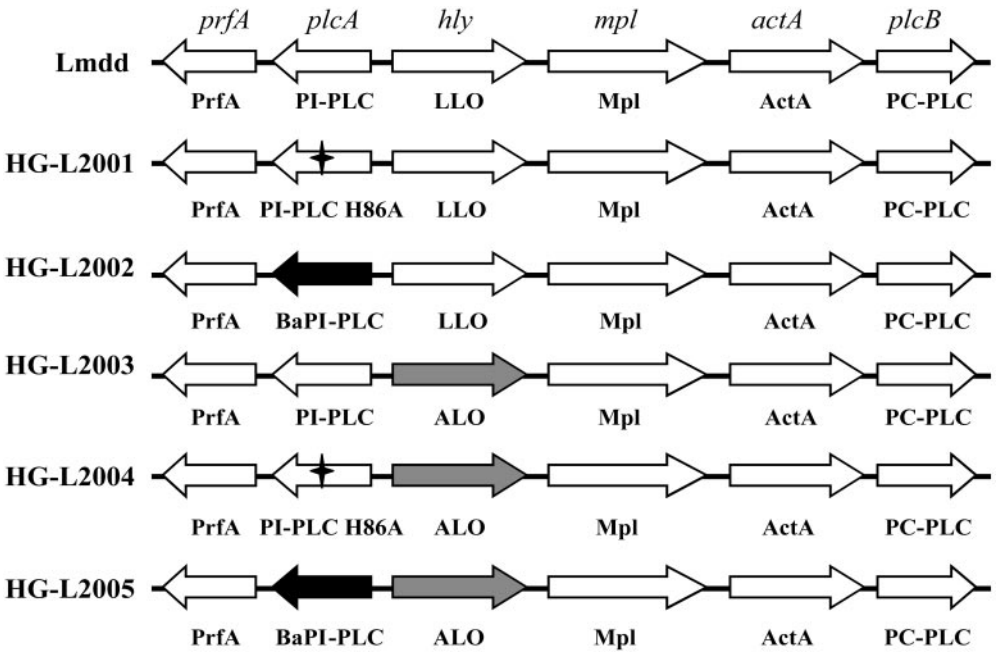

FIG. 1. Diagram of the *L. monocytogenes* PrfA regulon and the mutants constructed in the present study. The star indicates a nonfunctional H86A mutation in *L. monocytogenes* PI-PLC (1), the black arrows indicate the replacement of *L. monocytogenes* PI-PLC with *B. anthracis* PI-PLC, and the gray arrows indicate the replacement of LLO with ALO. BaPI-PLC, *B. anthracis* PI-PLC.

Recombinant ALO was expressed and purified as previously described by Shepard et al. (32) and stored in buffer (10 mM morpholineethanesulfonic acid, 300 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol buffered to pH 6.5). Purified protein concentrations were determined by the Bradford method.

Hemolytic activity. Hemolytic activity of bacterial broth cultural supernatants was measured by using sheep red blood cells as previously described (28). Hemolytic units were defined as the dilution of the sample at which 50% of the sheep red blood cells lysed. The specific activity of the recombinant His-tagged proteins was determined as described previously (12).

Diff-Quik staining. Bacteria grown in J774 host cells were visualized by using a Diff-Quik staining set (Dade Diagnostics of P. R. Inc.) after bacterial infection for 6 h according to the manufacturer's instructions.

Detection of PI-PLC activity on PI. Enzymatic activity on PI by PI-PLC was detected by using ALOA *Listeria* agar plates (Microbiology International, Frederick, MD). The size of an opaque halo surrounding a colony reflects the activity on PI. A quantitative method with [^{3}H]inositol-PI was also done as previously described (13).

Detection of PI-PLC activity on glycosyl phosphatidylinositol (GPI)-anchored proteins. Splenocytes were harvested from C57BL/6 mice. Cleavage of the GPI-anchored protein Thy1.2 on $CD4^+$ and $CD8^+$ T cells was measured by fluorescence-activated cell sorting (FACS). A total of 10 μl from overnight bacterial supernatant was mixed with 10^6 splenocytes in a well of a 96-well plate and incubated at 37°C for 1 h. Cells were then stained in phosphate-buffered saline (PBS), 1% bovine serum albumin with MAb to CD4, CD8, and Thy1.2. The cells were washed several times with PBS, 1% bovine serum albumin, fixed with 2% paraformaldehyde, and analyzed with a FACSCalibur (Becton Dickinson), and data were analyzed by using FlowJo (version 3.7; TreeStar, Inc.).

Escape from the primary phagocytic vacuole. The escape of *L. monocytogenes* from the primary vacuole was determined by measuring the percentage of bacteria coated with polymerized actin filaments (stained with Alexa Fluor 568 phalloidin) in the cytosol (16). Briefly, J774 cells were plated and infected by using fluorescein isothiocyanate-labeled bacteria (38) for 90 min, and the ratio of escaped bacteria (red) over the total bacteria (green) was determined based on microscopy with appropriate filters.

Intracellular growth curve. Stationary-phase bacterial cultures were washed and used to infect J774 macrophages on 12 mm glass coverslips at 37°C. After 1 h of infection, 50 μg of gentamicin/ml was added to the culture medium to kill the extracellular bacteria when needed. CFU per coverslip were determined by lysing host cells in sterile water and plating on brain heart infusion agar plates (34, 36).

Plaque formation. Plaque formation assays with murine L2 fibroblasts were performed as previously described (36). Briefly, overnight murine L2 fibroblasts cultured in six-well tissue culture plates were infected with *L. monocytogenes* for 60 min. The monolayer was then washed with PBS and covered with 2 ml of 1% Dulbecco modified Eagle medium-containing agarose with 10 μg of gentamicin/ml. After incubation at 37°C for 3 days, a second overlay of agarose with Neutral Red was added to allow visualization of plaques. The diameters of plaques were measured and compared to that of the parental strain, Lmdd.

LDH release assay. Lactate dehydrogenase (LDH) release from J774 host cells after bacterial infection was monitored by using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega) according to the manufacturer's instructions. Briefly, J774 cells were infected with *L. monocytogenes* strains at a multiplicity of infection, resulting in at least one bacterium per host cell in a 96-well plate for 5 h, and the supernatant from each well was taken for the LDH activity assay. This assay was performed in the absence of gentamicin to favor host cell lysis over rapid bacterial death (7).

FACS analysis of propidium iodide uptake. After bacterial infection, the cytotoxicity level was detected by staining cellular DNA with the fluorescent dye propidium iodide (Molecular Probes) (11, 12). Briefly, monolayers of bone marrow-derived macrophages from C57BL/6 mice, obtained as previously described (36), were infected with *L. monocytogenes* strains at a multiplicity of infection of one for 30 min and then washed with PBS and incubated until 2 h postinfection at 37°C and 5% CO_2. No gentamicin was added in this experiment to avoid the adverse effect of gentamicin on the most cytotoxic strains (13). The bone marrow-derived macrophages were then removed from the petri dish, washed with precooled PBS–10% FBS, stained with propidium iodide, and analyzed by FACS.

RESULTS

Construction, expression, and characterization of ALO and *B. anthracis* PI-PLC in *L. monocytogenes*. Five strains of *L. monocytogenes* were constructed in which wild-type LLO and PI-PLC were replaced individually or in combination as illustrated in Fig. 1. For safety reasons, we primarily used a *L. monocytogenes* Δ*dal* Δ*dat* strain (Lmdd), which requires D-alanine for growth, as the parental strain (37). The expression of ALO and *B. anthracis* PI-PLC were confirmed from the bacterial culture supernatants by Western blotting with PFO and *B. thuringiensis* PI-PLC antibodies, respectively (data not shown).

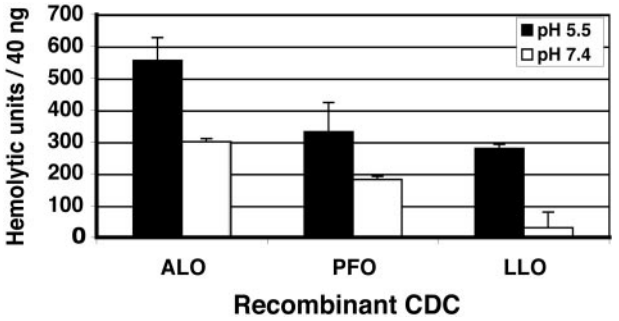

FIG. 2. Specific hemolytic activities of recombinant cholesterol-dependent cytolysins (CDC) on sheep red blood cells at the indicated pH. Values represent the mean ± the SD of three independent experiments.

We first analyzed the hemolytic activity of supernatants from the ALO-expressing *L. monocytogenes* strain. Supernatants from the strain expressing ALO showed 5 to 10-fold higher hemolytic activities at pH 7.4 than those from Lmdd (data not shown). We then compared hemolytic activity using purified proteins. Recombinant ALO showed greater hemolytic specific activity than recombinant LLO and PFO (Fig. 2). We compared the activities of recombinant ALO, PFO, and LLO at pH 5.5 and 7.4. As observed previously, the ratio of activities (pH 5.5/pH 7.4) for LLO is high, 9.0, whereas that of PFO is much lower, 1.8. The ratio of ALO is similar to that of PFO, 1.8, indicating that, unlike LLO, ALO does not have an activity optimum at an acidic pH (2).

To visualize any difference in PI-PLC activities, we streaked *L. monocytogenes* strains on an ALOA *Listeria* agar plate with D-alanine and incubated them at 37°C overnight. A nonfunctional *L. monocytogenes* PI-PLC mutant with one amino acid change at its active site (H86A) was used as the negative control (1). The activities of *B. anthracis* PI-PLC and *L. monocytogenes* PI-PLC appeared to be similar (Fig. 3A). A quantitative assay with [^{3}H]inositol-PI showed the same result that *B. anthracis* PI-PLC has almost the same activity on PI as *L. monocytogenes* PI-PLC (Fig. 3B). To compare their activities on GPI-anchored proteins, we measured the cleavage of Thy1 (CD90) on murine T cells. *B. anthracis* PI-PLC in supernatants of strain HG-L2002 showed strong activity, whereas *L. monocytogenes* PI-PLC showed no activity compared to a negative control (Fig. 3C). Supernatants from HG-L2005, expressing both ALO and *B. anthracis* PI-PLC, showed similar strong activity on Thy1 (data not shown).

Intracellular growth and cell-to-cell spread of *L. monocytogenes* strains. The second major issue addressed in our studies was whether the heterologous expression of ALO and *B. an-*

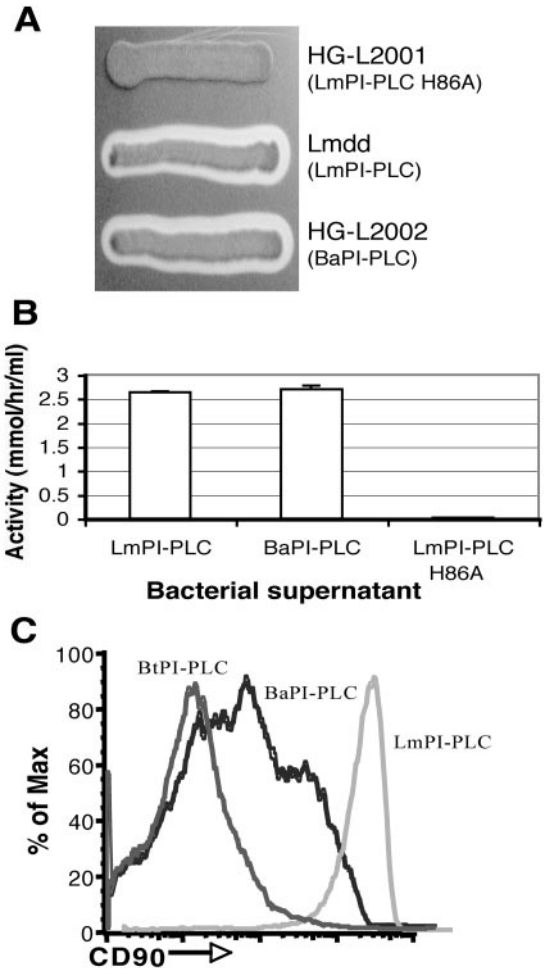

FIG. 3. Detection of PI-PLC activities. (A) Activity of indicated strains on PI using ALOA *Listeria* agar plates; (B) supernatant activity on PI using a quantitative [^{3}H]inositol-PI method (13). For *L. monocytogenes* PI-PLC (LmPI-PLC) and *B. anthracis* PI-PLC (BaPI-PLC), the P value was >0.1 as determined by unpaired t test of three experiments. There is no activity detected when an inactive mutant form of *L. monocytogenes* PI-PLC, H86A, was used. (C) Activity on the GPI-anchored protein CD90 as determined by FACS. Left, pure *B. thuringiensis* PI-PLC (BtPI-PLC; 6 μg/ml); middle, supernatant of strain HG-L2002 (*B. anthracis* PI-PLC [BaPI-PLC]); right, supernatant of strain Lmdd (*L. monocytogenes* PI-PLC [LmPI-PLC]). In several separate experiments the negative control of untreated cells overlapped with the Lmdd curve (not shown).

thracis PI-PLC affected the pathogenesis of *L. monocytogenes*. To examine the effects of heterologous expression of *B. anthracis* proteins on intracellular growth, we measured the ability of these strains to escape from a primary vacuole of the J774 macrophage-like cell, their intracellular growth, and their ability to spread from cell to cell.

To determine the efficiencies of escape by different *L. monocytogenes* strains, we measured the fraction of bacteria stained with fluorescent phalloidin, which detects polymerized actin surrounding bacteria in the cytosol (16). At 90 min after infection, all mutant strains were found to be defective in escape compared to the Lmdd strain (Table 2). Consistent with previous observations (1), the strain expressing a full-length inactive *L. monocytogenes* PI-PLC performed like a *plcA* in-frame deletion strain (3). Escape of the *B. anthracis* PI-PLC-expressing strain was significantly less than either the Lmdd strain or the inactive mutant PI-PLC strain, indicating that *B. anthracis* PI-PLC could not functionally replace *L. monocytogenes* PI-PLC, although they have almost the same activities on PI (Fig. 3A and B). Escape of all of the ALO-expressing strains were significantly lower than the Lmdd strain, as has been observed with a PFO-expressing strain (16).

TABLE 2. Measurements of *L. monocytogenes* phagosomal escape and cell-to-cell spread

Strain	% Phagosomal escape[a] ± SD	Plaque size[b] (%) ± SD
Lmdd	65 ± 9	100
HG-L2001	47 ± 9*	91 ± 3.4†
HG-L2002	37 ± 10†‡	61 ± 4.6†
HG-L2003	39 ± 9†	0
HG-L2004	28 ± 10†	0
HG-L2005	36 ± 13†	0

[a] That is, the percentage of total intracellular bacteria staining with Alexa Fluor 568 phalloidin at 90 min after infection of J774 cells. Two-tailed P values of unpaired t tests are indicated as follows: *, $P < 0.05$; †, $P < 0.001$ compared to values obtained with the Lmdd strain; ‡, $P < 0.05$ compared to values obtained with-HG-L2001. At least 200 bacterium-associated macrophages were counted for each of three experiments.

[b] That is, the plaque size relative to that of strain Lmdd. A minimum of 10 plaques per strain per experiment were measured. Values are means of three independent experiments. Two-tailed P values of unpaired t tests are classified as follows: †, $P < 0.001$ compared to values obtained with the Lmdd strain.

Subsequent to escape from a primary vacuole, *L. monocytogenes* replicates rapidly in the cytosol (5, 24). We examined the ability of mutant strains to grow in the macrophage-like cell line J774. In this assay, gentamicin (50 μg/ml) was added to the culture medium 1 h after bacterial infection in order to kill extracellular bacteria. The results demonstrated that strains with the heterologous expression of ALO showed a 1,000-fold decrease in CFU at 8 h postinfection, suggesting that ALO-expressing mutant strains were capable of permeabilizing the host cell membrane and allowing gentamicin to gain entry to intracellular bacteria (Fig. 4A). J774 cells were then infected with Lmdd expressing ALO in media containing gentamicin (50 μg/ml) and examined by light microscopy. A large number of bacteria were seen in cells infected with the parental strain for 6 h, whereas only few bacteria could be found in cells infected with the ALO-expressing strain (Fig. 4B). The decrease in CFU and the lack of bacterial cell division suggest that ALO causes membrane damage in the host cell, permitting the entry of extracellular gentamicin, which kills intracellular bacteria. The other ALO-expressing strains, HG-L2004 and HG-L2005, showed similar results (data not shown). We also inserted the gene for ALO into the wild-type strain 10403S. When growth curves were generated in the presence of gentamicin, results were similar to those obtained with strain HG-L2003 and *L. monocytogenes* expressing PFO (Fig. 4C and E). An additional experiment was done in which gentamicin, 50 μg/ml, was added for only a short pulse of half an hour during the early bacterial infection. In this case, the growth of the strains expressing ALO or PFO was similar to that of the wild-type 10403S (Fig. 4D). However, when observed under the microscope at 6 h postinfection, it appeared that the in-

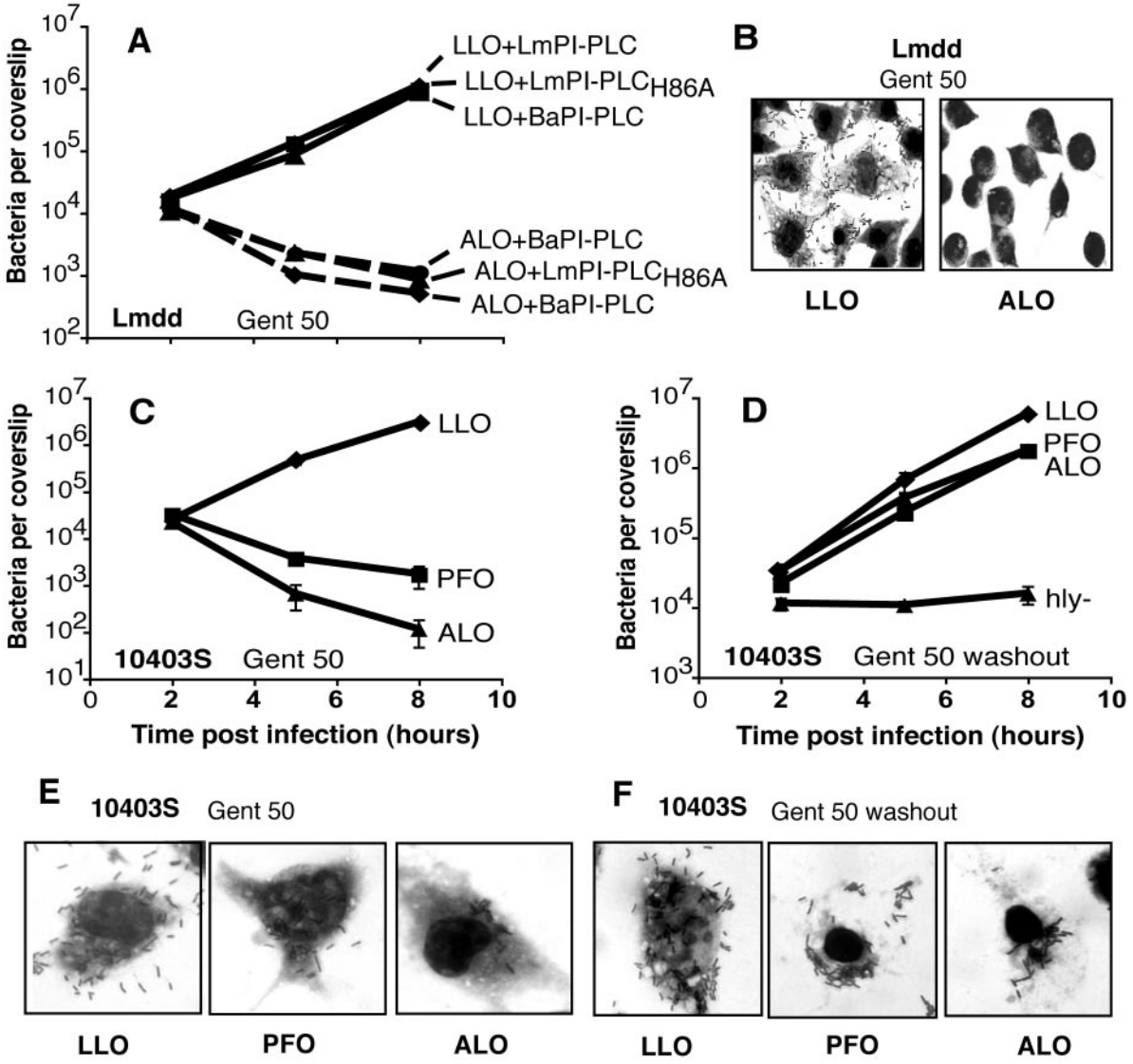

FIG. 4. Intracellular growth in J774 murine macrophage-like cells. The data shown are representative of at least three experiments. (A) Growth of Lmdd strains. (B) Light micrograph of J774 cells 6 h postinfection with Lmdd or Lmdd expressing ALO. For panels A and B, gentamicin at 50 μg/ml was added at 1 h postinfection for the duration of the experiment. (C and D) Growth of wild-type *L. monocytogenes* expressing LLO (10403S), PFO (DP-L4055), or ALO (DP-L4450). Gentamicin at 50 μg/ml was added at 1 h postinfection for the duration of the experiment (C) or for a short pulse from 1 to 1.5 h postinfection (D). *L. monocytogenes* with the LLO gene deleted (Δ*hly*, DP-L2161) controls for killing of extracellular bacteria during the gentamicin pulse. (E and F) Light micrographs of J774 cells 6 h postinfection with WT strain 10403S or derivatives expressing either PFO or ALO. Gentamicin at 50 μg/ml was added at 1 h postinfection for the duration of the experiment (E) or for a short pulse from 1 to 1.5 h postinfection (F). LmPI-PLC, *L. monocytogenes* PI-PLC; BaPI-PLC, *B. anthracis* PI-PLC.

fected host cells had died and the bacteria were mostly extracellular (Fig. 4F). In contrast, strains expressing inactive *L. monocytogenes* PI-PLC or *B. anthracis* PI-PLC along with LLO showed almost the same rate of intracellular growth as wild-type Lmdd (Fig. 4A). Thus, heterologous expression of *B. anthracis* PI-PLC does not alter the early growth of *L. monocytogenes* in J774 cells.

To further study the effects on *L. monocytogenes* pathogenesis of heterologous expression of *B. anthracis* virulence factors, we performed a plaque assay using L2 fibroblast monolayers. The plaque size reflects a bacterial strain's ability to spread from cell to cell and escape from the secondary double-membrane vacuole formed in adjacent cells. Consistent with the results presented in Fig. 4, all ALO-expressing strains showed no detectable plaques on L2 monolayers after 3 days in the presence of gentamicin (Table 2). This is consistent with the rapid killing by gentamicin of ALO-expressing strains in the primary cell after infection (Fig. 4). The strain with heterologous expression of *B. anthracis* PI-PLC formed plaques that were considerably smaller than either the Lmdd strain or the inactive PI-PLC strain (Table 2). Since expression of *B. anthracis* PI-PLC did not affect the early intracellular growth of *L. monocytogenes*, the reduction of plaque size indicates a defect in cell-to-cell spread.

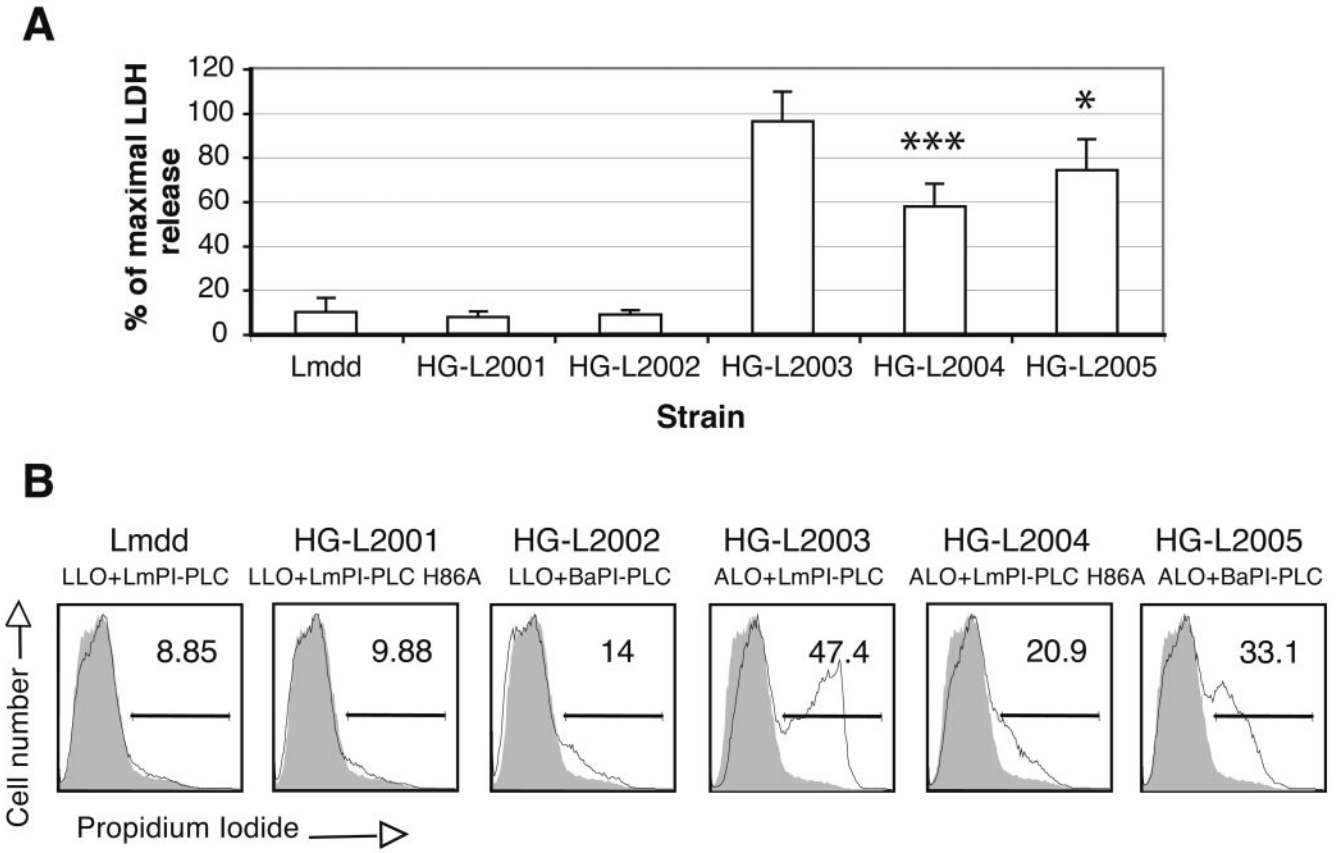

FIG. 5. Evaluation of the integrity of the host cell plasma membrane after *L. monocytogenes* infections. (A) LDH release by infected J774 cells. The release of LDH after infection of J774 cells by each strain was compared to total LDH determined after lysis with detergent. Values represent the mean ± the SD of three independent experiments. ✱, $P < 0.05$; ✱✱✱, $P < 0.001$ (as determined by unpaired *t* test compared to strain HG-L2003). (B) Cellular DNA staining by propidium iodide using mouse bone marrow-derived macrophages. The gray histogram represents the uninfected cells as negative control. The percentage of cells that were stained with propidium iodide is indicated. These data are representative of three experiments.

Toxicity of *L. monocytogenes* strains expressing ALO. The third major issue to be addressed by the present study was whether PI-PLC affects the permeabilization of the host cell plasma membrane by ALO. Given the ability of ALO to permeabilize the host cell membrane and allow entry of gentamicin, which kills the infecting bacteria, we compared the effects on cytotoxicity of expression of *B. anthracis* PI-PLC and *L. monocytogenes* PI-PLC in strains expressing ALO.

To measure differences in cytotoxicity, we first monitored the release of LDH from the cytosol of J774 cells into the tissue culture medium. Infection with the Lmdd bacteria resulted in ca. 10% LDH release during a 5-h incubation. The same was true for the other two mutant strains expressing LLO (Fig. 5A). Conversely, mutant strains expressing ALO produced much higher release of LDH. ALO in combination with *L. monocytogenes* PI-PLC exhibited the highest release, which was 96% of the maximal release of LDH. ALO with *B. anthracis* PI-PLC resulted in 74% of the maximal LDH release, and ALO in combination with inactive *L. monocytogenes* PI-PLC showed the lowest release, 58% (Fig. 5A).

We further measured cytotoxicity by examining membrane integrity using the membrane-impermeant fluorescent dye propidium iodide (11, 12). This dye binds cellular DNA when the membrane is damaged and increases the fluorescence of host cells, which in these experiments were bone marrow-derived macrophages. The data obtained by FACS agreed with the LDH release results, showing that ALO with *L. monocytogenes* PI-PLC displayed the highest level of fluorescence, ALO with *B. anthracis* PI-PLC displayed the medium level of fluorescence, and ALO with inactive *L. monocytogenes* PI-PLC displayed the lowest level of fluorescence (Fig. 5B).

In summary, with both assays the highest cytotoxicity was observed upon infection with the strain expressing ALO and *L. monocytogenes* PI-PLC. Less cytotoxicity was observed upon infection with the strain expressing ALO and *B. anthracis* PI-PLC. The least cytotoxicity was seen when ALO was expressed with an inactive form of *L. monocytogenes* PI-PLC.

DISCUSSION

LLO and PI-PLC are two critical virulence factors that are largely responsible for mediating the escape of *L. monocytogenes* from host cell vacuoles (3, 6, 21, 28, 34). What roles their orthologs may play in the pathogenesis of *B. anthracis* is unknown. Most studies on the pathogenesis of *B. anthracis* have focused on the terminal stages of the disease in which anthrax

toxin plays the dominant role. The recent completion of the *B. anthracis* genome sequence has revealed a number of proteins that are orthologous to known virulence factors of *L. monocytogenes*, including ALO and *B. anthracis* PI-PLC (29). In the present study, we characterized the properties of ALO and *B. anthracis* PI-PLC expressed in *L. monocytogenes* and investigated their effects on intracellular growth and cell-to-cell spread by using the well-established *L. monocytogenes* pathogenesis system. The results of our studies indicate that ALO can functionally replace LLO in mediating escape of *L. monocytogenes* from the primary vacuole; however, it exerts a toxic effect on the host cell (Table 2 and Fig. 4). Our results also indicate that expression of *B. anthracis* PI-PLC, which has strong activity on GPI-anchored proteins (Fig. 3C), hampers *L. monocytogenes* escape from a vacuole and reduces its cell-to-cell spread (Table 2).

LLO has uniquely evolved to decrease its toxicity in the host through having much lower activity at pH 7.4 than at the acidic pH of the phagosome (11, 12) and by having a PEST-like N-terminal sequence, which results in very low LLO levels when *L. monocytogenes* is growing in the host cell cytosol (7). ALO, which has 87% similarity to PFO, but only 64% similarity to LLO (18), does not have a PEST-like sequence, and its activity at pH 7.4 is almost as high as at pH 5.5 (Fig. 2). Expression of ALO by *L. monocytogenes* resulted in strong toxicity to both J774 cells and murine bone marrow-derived macrophages, as evidenced by permeabilization of infected cells to gentamicin (Fig. 4) and propidium iodide (Fig. 5B), and by the release of LDH from infected cells (Fig. 5A). When PFO was expressed by *L. monocytogenes*, similar toxicity was observed (Fig. 4E and F) (16). Mutations in PFO that altered its pH optimum to resemble that of LLO resulted in much less toxicity to the host cell (17). Mutants of LLO that increase its activity at neutral pH are more toxic (12). Therefore, the data suggest that ALO is functionally closer to PFO than LLO.

B. anthracis PI-PLC is almost identical to the well-characterized PI-PLCs from *B. cereus* and *B. thuringiensis* (14) and, like them, it is active on both PI and GPI-anchored proteins (Fig. 3 and unpublished data). *L. monocytogenes* expressing *B. anthracis* PI-PLC was able to form plaques in L2 monolayers, but the plaque size was significantly reduced compared to both strain Lmdd and a strain expressing inactive *L. monocytogenes* PI-PLC (Table 2). A comparison of their crystal structures has shown that *L. monocytogenes* PI-PLC and *B. cereus* PI-PLC have similar molecular structures; however, *B. cereus* PI-PLC has an extra β-strand (Vb) which is thought to be needed for recognition of GPI anchors (9, 23). *B. anthracis* PI-PLC has 97% similarity to *B. cereus* PI-PLC, and both have exactly the same Vb β-strand amino acid sequence. As expected, supernatants from *L. monocytogenes* expressing either *B. cereus* PI-PLC or *B. anthracis* PI-PLC have similar activities on the GPI-anchored protein Thy1 (unpublished data). In another study, we showed that expression of *B. cereus* PI-PLC inhibits escape of *L. monocytogenes* from a primary vacuole, blocks cell-to-cell spread, and reduces virulence in mice. We hypothesized that *L. monocytogenes* PI-PLC has evolved for intracellular growth and virulence by its greatly reduced activity on GPI-anchored proteins through the absence of the Vb β-strand. We speculate that cleavage of GPI-anchored proteins on the cell surface or more likely in the vacuole hampers escape and cell-to-cell spread of *L. monocytogenes*. The cleavage of GPI-anchored proteins by *B. anthracis* PI-PLC could influence the normal function of LLO directly or through host cell signals. At this stage, we also do not know which GPI-anchored proteins are cleaved by *B. anthracis* PI-PLC. Future studies will help to shed light on these questions.

The study of *L. monocytogenes* PI-PLC has been focused on its role in escape of the bacterium from the primary vacuole and its synergistic effects with LLO and PC-PLC during cell-to-cell spread (1, 3, 34). The abilities of *L. monocytogenes* PI-PLC to cleave PI, produce diacylglycerol, and activate protein kinase C isoforms in host cells appear to be important in its early interactions with the macrophages (38, 39). In contrast, *B. anthracis* PI-PLC does not complement *L. monocytogenes* PI-PLC in escape from the primary phagocytic vacuole; indeed, it appears to be inhibitory (Table 2).

In the present study, we used the combination of ALO with different PI-PLCs to investigate potential contributions of PI-PLC to host cell membrane damage. This method proved to be useful for evaluating the role of PI-PLC in its interplay with ALO. We demonstrated that, in combination with ALO, *L. monocytogenes* PI-PLC resulted in greater host membrane damage than *B. anthracis* PI-PLC. An early finding in the study of anthrax was an alkaline phosphatasemia produced during experimental infections of animals with *B. anthracis*. This was later determined to result from the cleavage of GPI-linked alkaline phosphatase by a bacterial activity (19, 35), which we have now characterized. Thus, cleavage of GPI anchors is manifested during *B. anthracis* infection. Its role, if any, in the pathogenesis of *B. anthracis*, is yet to be determined.

B. anthracis is hemolytic to human red blood cells, especially under anaerobic conditions (18). Therefore, it is possible that the expression of cytolytic genes of *B. anthracis* is induced under certain environmental conditions. The genes for ALO and PI-PLC, along with that for PC-PLC appear to be upregulated early during macrophage infection by germinated *B. anthracis* spores (18). Recent studies have suggested that the regulation of virulence genes in *B. anthracis*, either by a truncated pleiotropic transcriptional regulator PlcR or by the transactivator AtxA, is complex (22, 26, 33). Early *B. anthracis* escape from macrophage by lysis of the cell is regulated by AtxA but does not require the toxin genes expressed from pXO1 (8). Taken together, the results we have presented here suggest a role for ALO and *B. anthracis* PI-PLC in *B. anthracis* intracellular infection.

ALO has recently been shown to be an agonist for Toll-like receptor 4 (TLR4) (25). It is intriguing to note that CD14, a GPI-anchored protein, serves as a coreceptor of TLR4 (20). This suggests a site at which ALO and *B. anthracis* PI-PLC may function together during *B. anthracis* infections. To further examine the precise functions of putative *B. anthracis* virulence factors, it will be necessary to generate defined mutant strains and test their roles in pathogenesis in both tissue culture and animal models of infection.

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