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The Two Distinct Phospholipases C of *Listeria monocytogenes* Have Overlapping Roles in Escape from a Vacuole and Cell-to-Cell Spread

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Listeria monocytogenes **secretes two distinct phospholipases C, a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-range phospholipase C (PC-PLC). In this study, single in-frame deletion mutants with mutations in each PLC and a double mutant lacking both PLCs were characterized with regard to virulence in mice, escape from a primary vacuole, and cell-to-cell spread in cell culture. The mutant lacking PI-PLC, previously shown to be twofold less virulent than the wild type in mice, had a minor defect in escape from a primary vacuole but was not notably affected in cell-to-cell spread. The mutant lacking PC-PLC was 20-fold less virulent in mice and was defective in cell-to-cell spread but had no measurable defect in escape from a primary vacuole. The mutant lacking both PLCs was 500-fold less virulent in mice and was severely diminished in its ability to escape from the primary vacuole and to spread cell to cell. Cellular levels of diacylglycerol and ceramide, products of PLC activity, accumulated beginning 3 to 4 h after infection of cells with wild-type bacteria. The bacterial PLCs were partially responsible for this activity, since cells infected with the mutant lacking both PLCs had a reduced increase in diacylglycerol and no increase in ceramide. Elevation of diacylglycerol in the absence of bacterial PLCs indicated that host cell phospholipase(s) was activated during infection. The results of this study were consistent with the two bacterial PLCs having overlapping functions throughout the course of intracellular infection. Furthermore, the PC-PLC, and possibly PI-PLC, appeared to be enzymatically active intracellularly.**

Listeria monocytogenes is a facultative intracellular pathogen of humans and animals. Subsequent to internalization, a significant proportion of invading bacteria escape from a vacuole, grow rapidly, and are propelled through the cytoplasm by inducing the polymerization of host actin (33, 45). Bacteria spread from cell to cell by inducing formation of pseudopod-like structures, each containing a bacterium at its tip, which are apparently internalized by neighboring host cells. At this stage, bacteria can be found in double-membrane vacuoles from which they escape to repeat the cycle (7, 33, 45).

Several contiguous genes required for *L. monocytogenes* pathogenesis are organized on the chromosome in the following order: *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB* (35). PrfA is a transcription factor required for expression of the other genes in the regulon. ActA is a surface protein required for polymerization of host cell actin. Three of the other genes encode proteins that interact in different ways with host cell membranes. Listeriolysin O (LLO), encoded by *hly*, is a member of a family of sulfhydryl-activated, cholesterol-requiring, pore-forming cytolysins. Other members of this family, including streptolysin O and perfringolysin O, are known to permeabilize membranes by aggregating within the bilayer and creating large pores (1). Mutants with mutations in LLO are unable to escape from a macrophage primary phagocytic vesicle, do not spread from cell to cell, and are completely avirulent (12, 27, 36).

The other two membrane-interactive proteins are phospholipases of the C type (PLCs), which cleave the polar head group of phospholipids at the glycerol-to-phosphorus bond, releasing diacylglycerol and a water-soluble phosphate ester. Although there was early evidence for lecithinase activity associated with *L. monocytogenes* (2, 11), the first gene to be cloned was *plcA*, encoding a phosphatidylinositol-specific PLC (PI-PLC) (5, 28, 31). This protein, which shares sequence homology with PI-PLCs from other gram-positive bacteria (8, 19), has been purified to homogeneity and characterized biochemically (16). It is highly specific for phosphatidylinositol (PI) and, unlike other members of this family, exhibits very low activity on glycosyl-PI-anchored membrane proteins (13). An in-frame deletion mutation of *plcA* results in a small decrease in virulence in mice, impaired growth in the mouse liver, and less efficient escape from a primary phagocytic vacuole compared with that of the wild type (6).

The second PLC, encoded by *plcB* (47), is a broad-range enzyme which cleaves phosphatidylcholine (PC); it will be referred to as PC-PLC. The highly purified enzyme cleaves a variety of phospholipids, including sphingomyelin in detergent micelles, liposomes, and biological membranes (14, 15). Its ability to completely hydrolyze the major phospholipids of a mammalian membrane in isotonic media was demonstrated with erythrocyte ghosts (15). An insertion mutation in *plcB* was found previously to lead to a decrease in plaque size, suggesting a partial defect in cell-to-cell spread or intracellular growth. Additionally, the mutant strain was observed to accumulate in double-membrane vesicles which form as a consequence of cell-to-cell spread (47). Thus, PC-PLC appears to be partially responsible for gaining access to the cytoplasm of newly infected cells after a cell-to-cell spreading event. Because the defect in cell-to-cell spread was partial, additional

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bacterial factors probably contribute to this process. In broth culture, a metalloprotease encoded by the *L. monocytogenes* gene *mpl* is required to process the inactive 33-kDa precursor form of PC-PLC to the mature, active 28-kDa form (9, 32).

This study was undertaken to explore the unique and possibly overlapping functions of the two PLCs with respect to virulence, escape from a vacuole, growth in the cytoplasm, and cell-to-cell spread. To clarify their roles in these processes, we have generated deletion mutations in the genes that encode these proteins and examined their effects upon cellular infections, the turnover of phospholipids in infected cells, and the generation of the products of phospholipid hydrolysis, diacylglycerol and ceramide.

(Some of these results were reported previously at the GPI-Anchor Proteins: Structure and Function—1994 meeting, Angra dos Reis, Rio de Janeiro Brasil [37].)

MATERIALS AND METHODS

Construction of *L. monocytogenes* Δ*plcB* mutant strains. The *Escherichia coli* host for all plasmid constructs was DH5α (Bethesda Research Laboratories Life Technologies, Inc.). Derivatives of pKSV7 (43) were maintained in *E. coli* by growth in the presence of 50 μg of ampicillin per ml of medium. Two fragments of the *L. monocytogenes plcB* gene were amplified from 10403S chromosomal DNA by the PCR and ligated sequentially into pKSV7. The first fragment encoded bases 2097 to 2498 (47), which included a 3′ coding sequence of the *actA* gene, and was ligated into pKSV7 by use of *Bam*HI- and *Eco*RI-generated DNA ends to generate pDP1870. Primers used were 5′-GGGAATTCTTCTCTTTA GGGGCGTTTAT-3′ (primer 1859) and 5′-GGGGATCCCGCATCATATATT CCTTGAG-3′ (primer 1860). The second fragment encoded bases 2774 to 3173 (47), which included a 5′ coding sequence of the ORFX gene (47), and was ligated into pDP1870 by use of *Bam*HI- and *Hin*dIII-generated DNA ends to generate pDP1888. Primers used were 5′-GGGGATCCAATTACGTAGATAC CATTAAAC-3′ (primer 1861) and 5′-GGAAGCTTCGATTACTAACACTAC AAAGA-3′ (primer 1862). This resulted in a Δ*plcB* allele with a 276-bp in-frame deletion.

L. monocytogenes was transformed with pDP1888. Allelic exchange of the wild-type chromosomal *plcB* allele with the pDP1888 Δ*plcB* allele was performed as described previously (6). Strain DP-L1935 (Δ*plcB*) was derived from *L. monocytogenes* 10403S. Strain DP-L1936 (Δ*plcA* Δ*plcB*) was derived from DP-L1552 (Δ*plcA*) (6).

Complementation of *L. monocytogenes* Δ*plcB* mutant strains. The *L. monocytogenes plcB* gene was amplified from 10403S chromosomal DNA by PCR and ligated into pKSV7 by use of *Eco*RI- and *Hin*dIII-generated DNA ends to generate pDP2120. The primers used were primers 1859 and 1862 (see above). Allelic exchange was used to replace the Δ*plcB* alleles of strains DP-L1935 and DP-L1936 with the wild-type pDP2120 *plcB* allele as described above.

For DP-L1935 and DP-L1936, wild-type revertants were screened for by plaque assay. All wild-type revertant plaques examined were found to be genotypically wild type or merodiploids possessing both the Δ*plcB* and wild-type *plcB* alleles. Strains DP-L2167 (derived from DP-L1935) and DP-L2168 (derived from DP-L1936) were examples of genotypic wild-type strains isolated in this way. No wild-type-size plaques were ever observed with strains DP-L1935 and DP-L1936 prior to complementation.

Plaque and virulence assays. The formation of plaques by *L. monocytogenes* in the mouse fibroblast L2 cell line was done as described previously (44). Plaque size was measured by projecting neutral red-stained monolayers with an overhead projector directed at a wall 90° to the light path. For each sample, the diameters of 10 to 20 plaques were measured with a ruler, and the results were averaged. Wild-type-derived plaques were always included as a standard against which measured plaque diameters were normalized. Repeated measurements were averaged, and their standard deviations were calculated. Fifty percent lethal dose (LD_{50}) values of bacterial strains following intravenous injection in BALB/c mice were determined as described previously (36).

Tissue culture growth assays. Intracellular growth assays in J774 cells were done as described previously (44), with some modifications. Bacteria were grown overnight in brain heart infusion broth at 30°C, washed once in phosphate-buffered saline (PBS), and used to infect monolayers of J774 cells at 2.5×10^4 to 5.0×10^5 bacteria per ml. At 30 min postinfection, the monolayers were washed three times with PBS. Gentamicin sulfate was added to a final concentration of 50 μg/ml at 1 h postinfection. At each subsequent time point (2, 7, 12, 21, and 31 h postinfection), the number of viable bacteria was determined by depositing coverslips, in triplicate, into 15-ml conical tubes containing 5 ml of distilled water. After vigorous vortex mixing for 15 s, dilutions were plated onto Luria-Bertani (LB) agar (23, 36).

The human epithelial cell line Henle 407 was propagated in Dulbecco's modified Eagle's medium containing 7.5% fetal calf serum. Henle 407 cells were seeded on glass coverslips in 100-mm-diameter dishes 2 days before infection. Bacteria were grown overnight in brain heart infusion broth at 30°C and washed once in PBS. The cells were infected (time [t] = 0) with 2×10^6 bacteria per ml, which resulted in infection of approximately 5% of the cells. At t = 1 h, the cells were washed three times with PBS, and prewarmed culture medium was added. At that time, half of the coverslips from each dish were transferred to a duplicate series of 100-mm-diameter dishes containing prewarmed culture medium. At t = 1.5 h, gentamicin was added to all dishes at a final concentration of 50 μg/ml, and cytochalasin D (Sigma) was added to the duplicate set of dishes at a final concentration of 0.25 μg/ml. At 2.5, 5.5, 8.5, 11.5, and 14.5 h postinfection, the number of intracellular bacteria was determined in triplicate as described above (23, 36).

Detection of intracytoplasmic bacteria by staining with phalloidin. The percentage of bacteria which had escaped into the host cell cytoplasm in mouse bone marrow macrophages 90 min after infection was determined as described previously (24). Briefly, the total number of intracellular bacteria was obtained by labelling bacteria with polyclonal anti-*Listeria* antibody (Listeria O Antiserum Poly; Difco) and then with fluorescein isothiocyanate-goat anti-rabbit immunoglobulin G. The presence of bacteria in the cytoplasm was then inferred by colabelling with tetramethyl rhodamine isothiocyanate-phalloidin, since only bacteria in the cytoplasm are coated with F-actin (7).

Infection of cells prelabelled with [U-^{14}C]glycerol. J774 cells were plated, 10^6 cells per 35-mm-diameter dish, in 2 ml of Dulbecco's modified Eagle's medium plus 5% fetal calf serum and allowed to adhere at 37°C. [U-^{14}C]glycerol (Amersham; 4.0 μCi, 161 μCi/μmol) was added after removal of 0.5 ml of medium. After overnight growth, the labelled medium was removed and replaced with 2 ml of prewarmed growth medium. Cells were infected (t = 0 h) with 4 μl of washed overnight cultures of *L. monocytogenes* which had been resuspended in PBS (pH 7.4) to the original culture volume so that the multiplicity of infection for the wild type was 2 to 4 bacteria per cell. Control dishes were mock-infected with PBS (pH 7.4). At t = 0.5 h, gentamicin was added to give a final concentration of 50 μg/ml. At t = 4 and 5 h, three dishes each of infected and control cells were harvested by removing the medium and adding 1 ml of ice-cold methanol. The cells were scraped and transferred to glass tubes in an ice bath with two additional washes of 0.25 ml of cold methanol. Lipids were then extracted with mixtures of chloroform and methanol (3), with the addition of 10 μg of diolein per tube. The lower chloroform-rich phase was transferred to a clean glass tube, and the upper phase was reextracted with 1 ml of chloroform. The combined chloroform phases were dried under a stream of nitrogen gas, and the lipids were separated on preactivated, channeled, soft silica gel G thin-layer plates with a preadsorbent zone (Analtech) by chromatography in petroleum ether (boiling point, 30 to 60°C)-diethyl ether-acetic acid (70:30:1, vol/vol/vol). Diolein standards were included in separate lanes. The plates were dried, and radioactivity was scanned on a Berthold linear analyzer. The radioactive peaks were integrated, and the results are reported as counts per minute in diacylglycerol as a percentage of total lipid counts per minute.

Assays for diacylglycerol and ceramide. To determine diacylglycerol and ceramide levels in infected cells, infections were carried out as described above, but cells were not prelabelled. To achieve comparable bacterial cell numbers, the volume of suspension used for infection was doubled for the *actA* and *plcA plcB* mutants. At 4 and 5 h after infection, three dishes each of infected and control cells were harvested, and lipids were extracted as described above except that no carrier diolein was added. Usually, one-third of the lipid extract from each dish was assayed by the diacylglycerol kinase method (39, 46) with a kit purchased from Amersham Corp. The procedure was modified as described by Wolf et al. (49), with the following further modifications. The assays were performed in acid-washed borosilicate glass tubes (13 by 100 mm) with Teflon-lined screw caps. The dried lipids were suspended in the detergent solution by sonication in a Branson 2200 water bath sonicator for 20 min after vortex mixing for 1 min. [γ-^{32}P]ATP (Dupont, NEN Research Products; 50 nmol, 2 μCi) was used for each assay. Before extraction of the incubation products, 10 μg each of unlabelled phosphatidic acid and ceramide-P were added to each tube. After extraction with chloroform-methanol (1:2, vol/vol) followed by centrifugation as recommended by the manufacturer, 1 ml of chloroform and 1 ml of 1% perchloric acid were used to complete the extraction. Standard diacylglycerol and ceramide curves were obtained for each experiment. They were plotted by use of Sigma Plot software, and linear regressions were calculated. The levels of diacylglycerol and ceramide per culture dish were quantified from these plots. Bacterial counts per dish at the time of harvest were obtained essentially as described previously (23).

Preparation of ceramide phosphate. Phospholipase D (type VI; Sigma), 0.15 mg, was incubated with sphingomyelin, 0.7 mg, in a 1-ml suspension containing 40 mM Tris-HCl (pH 8) and 20 mM $CaCl_2$ to which 0.2 ml of ethyl ether was added. The mixture was vortex mixed and shaken at 25°C for 3.5 h. Ether was added at 2 h to replace that lost through evaporation (20). The reaction was stopped by the addition of 0.2 ml of 100 mM HCl, and the ether was removed in a stream of nitrogen gas. The product was extracted by the method of Bligh and Dyer (3).

TABLE 1. Bacterial strains and properties

Strain (genotype)	LD_{50}[a]	% Plaque size[b] (mean ± SD)	% Bacteria staining with phalloidin at 1.5 h (mean ± SD)[c]
10403S (wild type)	1×10^4 to 3×10^4	100	67.3 ± 9.0
DP-L1552 (Δ*plcA*)	3.0×10^4	89 ± 4.3 (n = 7)	49.3 ± 6.4[d]
DP-L1935 (Δ*plcB*)	2.0×10^5	66 ± 6.2 (n = 13)	73.0 ± 5.6
DP-L1936 (Δ*plcA* Δ*plcB*)	5.0×10^6	32 ± 5.3 (n = 10)	34.0 ± 3.6[e]
DP-L2161 (Δ*hly*)	2×10^9		0

[a] LD_{50} values were determined after intravenous infection of BALB/c mice. Data for strain 10403S are from references 6 and 36, data for strain DP-L1552 are from reference 6, and data for strain DP-L2161 are from reference 24.
[b] Relative to plaque size of strain 10403S.
[c] Three separate experiments were done with each strain.
[d] $P < 0.05$ by unpaired t test compared with wild type.
[e] $P < 0.01$ compared with wild type and $P = 0.02$ compared with DP-L1552 by unpaired t test.

RESULTS

Plaque formation and virulence of mutants. In addition to the previously described strain containing an in-frame *plcA* deletion, we constructed strains containing an in-frame deletion in *plcB* and a double Δ*plcA* Δ*plcB* mutation. These strains were evaluated for their virulence in mice and their ability to escape from a primary vacuole and to spread from cell to cell. An in-frame deletion within *plcB* in strain DP-L1935 resulted in a 10- to 20-fold increase in LD_{50} and a 34% decrease in plaque size. Also, as previously shown, an in-frame deletion in *plcA* produced an approximately two- to threefold increase in LD_{50} (6) and a 10% decrease in plaque size (Table 1 and Fig. 1). Notably, combination of the two mutations in strain DP-L1936 resulted in an approximately 500-fold increase in mouse LD_{50} and a 68% decrease in plaque size. Complementation of mutant strains with the wild-type *plcB* allele fully restored the original plaque size (Fig. 1, strains DP-L2167 and DP-L2168).

Escape from the primary phagocytic vacuole. The initial intracellular growth rate of *L. monocytogenes* reflects both its ability to escape from the primary vacuole and its subsequent rate of growth in the cytoplasm. To separate these two components, we measured the ability of the phospholipase mutants to escape from the primary phagocytic vacuole of bone marrow macrophages, in which we had previously shown the Δ*plcA* strain to have a defect (6). The capacity to nucleate actin permits the enumeration of *L. monocytogenes* in the cytoplasmic compartment by staining with fluorescent phalloidin (Table 1). At 90 min after infection, equal proportions of the wild type and the strain lacking PC-PLC stained with phalloidin. In contrast, escape of the strain lacking PI-PLC appeared to be delayed, consistent with previous results obtained by electron microscopy (6). The double phospholipase mutant was most defective in escape from the vacuole; the fraction of bacteria staining with fluorescent phalloidin was decreased by approximately 50%.

Growth in Henle 407 cells in the absence or presence of cell-to-cell spread. The ability of *L. monocytogenes* strains to grow and spread from cell to cell was also studied in the human epithelial cell line Henle 407, which, unlike macrophages, supports the growth of LLO mutants (36). In these cells, the intracellular growth rates of the strain lacking PC-PLC and the double phospholipase mutant, but not that of the strain lacking PI-PLC alone, decreased beginning approximately 6 h postinfection (Fig. 2). On the basis of the findings of Vazquez-Boland et al. (47), we hypothesized that during cell-to-cell spread, bacteria lacking PC-PLC were being trapped in double-membrane vacuoles where nutrients are presumably limiting for growth. Consequently, preventing cell-to-cell spread with cytochalasin D, an inhibitor of actin polymerization (7, 45), should prevent bacterial entrapment and facilitate growth. Consistent with this hypothesis, in the presence of cytochalasin D, the growth rates of the strain lacking PC-PLC and the double phospholipase mutant were comparable to that of the wild type (Fig. 2). In the presence of cytochalasin D, all strains experienced a decline in viable bacteria beginning at 8 h after infection (Fig. 2), which we attribute to the onset of host cell death resulting in permeabilization to gentamicin (4).

Long-term persistence of *L. monocytogenes* strains in J774 cells. The results of plaquing experiments and growth studies with Henle 407 cells presented in the previous sections were consistent with the hypothesis that Δ*plcB* and Δ*plcA* Δ*plcB* mutants were defective in cell-to-cell spread. We also compared the growth of the phospholipase mutant strains in J774 cells with that of the wild type and a Δ*actA* mutant, which is absolutely incapable of cell-to-cell spread (Fig. 3). During the first 7 h of infection, growth of the wild type and the single Δ*plcA* and Δ*plcB* mutant strains was essentially identical, while that of the double Δ*plcA* Δ*plcB* mutant strain was approximately 30% slower. Growth of the Δ*actA* mutant was reproducibly more rapid during this initial period. After 7 h, viable cell numbers declined for all strains. The decline in cell number was slowest for the wild type, while that of the Δ*actA* mutant was most precipitate, resulting in survival of less than 0.1% of the bacteria which were viable at 7 h. The decline in viable cell count for the phospholipase mutants was in the order DP-L1936 (Δ*plcA* Δ*plcB*) > DP-L1935 (Δ*plcB*) > DP-L1552 (Δ*plcA*). These results are also consistent with a spreading defect in Δ*plcB* mutant strains.

Lipid turnover during *L. monocytogenes* infections. One potential consequence of PLC expression in mammalian cells by *L. monocytogenes* is enhanced turnover of host lipid and increased cellular levels of products of phospholipid hydrolysis, including diacylglycerol and ceramide (25, 29, 34). To assess the effects of wild-type infection on host lipid turnover, monolayers of J774 cells were prelabelled with [U-^{14}C]glycerol and harvested at intervals after infection. These experiments showed a significant increase in labelled diacylglycerol in in-

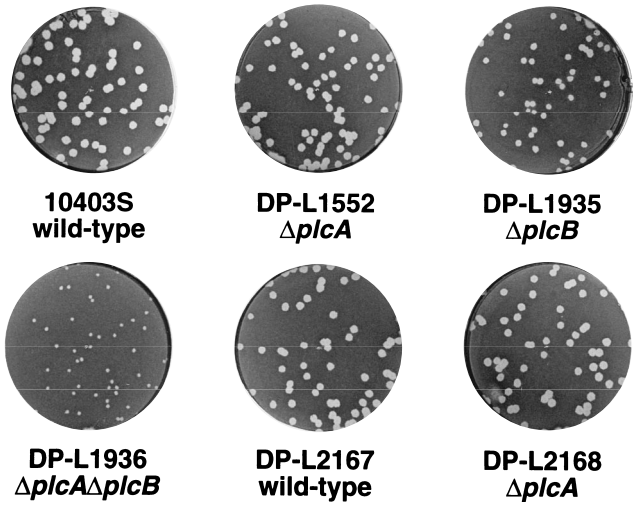

FIG. 1. Plaque formation by *L. monocytogenes* in L2 cell monolayers. Strains are indicated below each well.

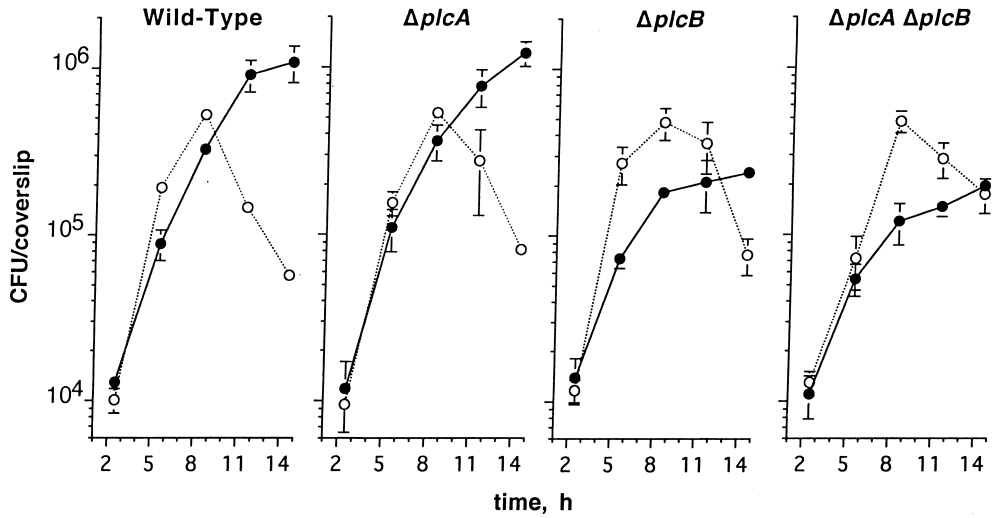

FIG. 2. Intracellular growth of *L. monocytogenes* strains in the Henle 407 human epithelial cell line. Symbols: ●, growth in the absence of cytochalasin D; ○, growth in the presence of 0.25 μg of cytochalasin D per ml. The data represent the means ± standard deviations of three coverslips from a representative experiment.

fected cells compared with that in control cells, beginning 3 to 4 h after infection with 2 to 4 bacteria per cell. The results of an experiment with the wild type, the Δ*plcB* mutant, and the Δ*plcA* Δ*plcB* double mutant are shown in Fig. 4. Significant differences in diacylglycerol radioactivity between cells infected with wild-type or mutant strains and uninfected control cells were observed 4 and 5 h after infection. Labelled diacylglycerol was highest in cells infected with the wild type. Mutation of one or both phospholipases resulted in progressively smaller amounts of labelled diacylglycerol.

Diacylglycerol and ceramide levels in infected cells. The preceding experiments showed that infected cells experienced increased lipid turnover resulting in elevated diacylglycerol labelling but did not permit quantitative measurement of the content of diacylglycerol. They also provided no information on the levels of ceramide, a potential product of sphingomyelin hydrolysis by PC-PLC, in infected cells.

Diacylglycerol and ceramide were measured in cells infected with the wild type, the Δ*plcA* Δ*plcB* double mutant, and the Δ*actA* mutant. Cells were infected as described above and harvested at $t = 4$ and 5 h. Diacylglycerol and ceramide were quantified by the diacylglycerol kinase assay since the enzyme from *E. coli* is also capable of phosphorylating ceramide (46). These experiments revealed significant elevations of diacylglycerol and ceramide at $t = 4$ and 5 h in wild-type infections compared with those in uninfected cells (Table 2). A smaller but significant elevation in diacylglycerol was observed at $t = 4$ h in cells infected with the Δ*plcA* Δ*plcB* double mutant; however, at $t = 5$ h, there was no significant difference between cells infected with the double mutant and uninfected cells. No increased ceramide was observed at either time in cells infected with the double mutant. Cells infected with a Δ*actA* mutant, like those infected with wild-type bacteria, had significantly elevated diacylglycerol levels at both times, but the increase in ceramide was only significant at $t = 4$ h. These data were corrected for the number of bacteria per monolayer and for the diacylglycerol content of *L. monocytogenes* cells (Table 2). The relationships described above were similar even when the differences in diacylglycerol and ceramide were corrected for the bacterial load. Correction for the bacterial diacylglycerol content shows that all of the increase seen in cells infected with the double mutant at 5 h postinfection could be accounted for by bacterial diacylglycerol and probably did not arise from the host cell. *L. monocytogenes* does not contain ceramide (22).

DISCUSSION

The results of this study show that an in-frame deletion in either of the *L. monocytogenes* phospholipases has a relatively minor effect on virulence in mice or on plaque size in vitro. In contrast, a strain lacking both PI-PLC and PC-PLC is considerably less virulent, suggesting that the phospholipases have an overlap in function. PI-PLC and PC-PLC have been implicated in escape from the primary vacuole (6) and in cell-to-cell spread (47), respectively. We have therefore examined the single and double mutants in these processes and have looked for evidence of the phospholipase enzymatic activities during the course of intracellular infection.

By electron microscopy, a Δ*plcA* mutant strain of *L. mono-*

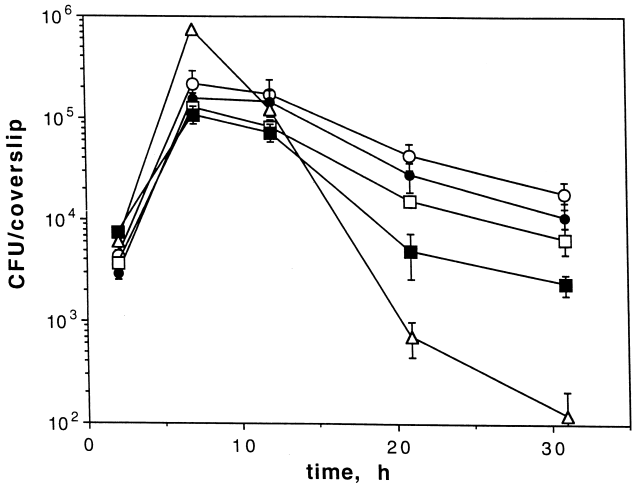

FIG. 3. Intracellular growth of *L. monocytogenes* strains in the J774 murine macrophage-like cell line. Symbols: ○, wild-type strain 10403S; ●, DP-L1552 (Δ*plcA*); □, DP-L1935 (Δ*plcB*); ■, DP-L1936 (Δ*plcA* Δ*plcB*); △, DP-L1942 (Δ*actA*). The data represent the means ± standard deviations of three coverslips from one of three experiments.

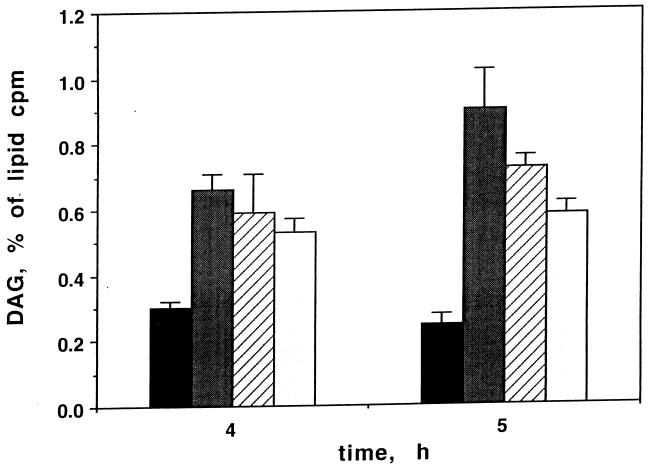

FIG. 4. Formation of [^{14}C]diacylglycerol (DAG) in J774 cells prelabelled with [U-^{14}C]glycerol and infected with *L. monocytogenes* strains. Bars: solid, uninfected; dark stippling, wild type; cross hatched, Δ*plcB*; open, Δ*plcA* Δ*plcB*. The data represent the means ± standard deviations of three dishes. At both times, the differences between all infected cells and the uninfected control were significant ($P \leq 0.05$) by the Student *t* test. The differences between the cells infected with the Δ*plcA* Δ*plcB* strain and the cells infected with the wild-type strain were significant at both times ($P = 0.02$ at 4 h; $P = 0.04$ at 5 h).

cytogenes was previously found to be partially defective in escape from the primary vacuole in bone marrow macrophages (6). We have confirmed this observation by an actin nucleation assay. In contrast to the Δ*plcA* mutant strain, a Δ*plcB* mutant had no defect in escape from the vacuole. We have recently demonstrated that PI-PLC but not PC-PLC synergizes with LLO to mediate disruption of membranes in vitro and that this synergism is independent of PI-PLC catalytic activity (17). Thus, the escape defect present in a Δ*plcA* mutant but absent in a Δ*plcB* mutant may be explained if this synergism functions in vivo to enhance escape from the primary phagosome.

The Δ*plcA* Δ*plcB* double mutant displayed a defect greater than that of Δ*plcA* alone, which was also observed as an early decrease in growth rate in J774 cells. These observations indicate that the two phospholipases share an overlapping function in mediating escape from the primary phagosome. The nature of this overlap is unknown but may stem from the similar enzymatic activities of the two proteins or from independent lytic mechanisms inherent to both PC-PLC activity and the observed PI-PLC synergism with LLO (17). The PC-PLC has recently been shown to actuate escape, and the PI-PLC has been shown to enhance escape, from the primary vacuole of Henle 407 cells in the absence of LLO, supporting a role for phospholipase activity in this process (30).

The phospholipases also appeared to have an overlapping function in promoting cell-to-cell spread. Quantitative electron microscopy has been employed previously to detect a spreading defect in a *plcB* insertional mutant, with bacteria in double-membrane vesicles used as a marker of cell-to-cell spread. The insertion mutant was also reported to show a decrease in plaque size (47). The plaque sizes of the Δ*plcA* and Δ*plcB* single mutants reported here are in agreement with those described previously (6, 47). The Δ*plcA* Δ*plcB* double mutant demonstrated a decrease in plaque size that was greater than the sum of the decreases of the single mutants. Because the single and double mutants have maximal growth rates in J774 cells equivalent to that of the wild type (data not shown), the plaquing defect of the mutant strains was presumably due to a cell-to-cell spreading defect. This is corroborated by data collected with Henle 407 cells. During infection of these cells, the growth rates of the Δ*plcB* mutant and the double Δ*plcA* Δ*plcB* mutant strains declined 6 h postinfection. The growth defects were complemented by treatment with low levels of cytochalasin D, presumably by inhibiting bacterial actin-dependent intracellular movement and subsequent cell-to-cell spread. The ability of this inhibitor to restore the growth rates of the Δ*plcB* and Δ*plcA* Δ*plcB* mutants suggested that these strains enter a nutrient-poor environment, which we hypothesize to be the double-membrane vacuole. Since the wild-type and the Δ*plcA* strains did not have this actin polymerization-dependent growth defect, it was likely that the presence of PC-PLC bypasses or promotes escape from the nutrient-poor environment.

The loss of viability of the Δ*plcB* mutant and the Δ*plcA* Δ*plcB* mutant in J774 cells after 7 h of infection is also consistent with a defect in spread from cell to cell, resulting in eventual loss of host cell viability, its permeabilization to gentamicin, and consequent bacterial killing. Furthermore, the rate at which bacterial counts decreased was dependent on the ability of each strain to spread cell to cell and thus escape the dying host cell. This was demonstrated with the Δ*actA* strain, which, completely lacking the ability to spread cell to cell, lost viable bacterial numbers at the fastest rate. The phospholipase mutants exhibited decreases in viable counts intermediate to those of the wild type and the Δ*actA* strains, which correlated with plaque size. Thus, all three of these assays support the conclusion that the two phospholipases share an overlapping function in mediating cell-to-cell spread.

TABLE 2. Diacylglycerol (DAG) and ceramide levels in *L. monocytogenes*-infected J774 cells

Strain (genotype)	Time (h)	*n*[a]	Level (nmol/dish)						Bacteria/ dish (10^8)	ΔDAG/10^8 bacteria	ΔCeramide/ 10^8 bacteria
			Infected DAG	Control DAG	ΔDAG[b]	Infected ceramide	Control ceramide	ΔCeramide[b]			
10403S	4	6	1.06 ± 0.46	0.797 ± 0.36	0.267***	0.340 ± 0.16	0.246 ± 0.15	0.094*	0.60[c]	0.45 (0.32)[d]	0.16
	5	4	0.952 ± 0.38	0.673 ± 0.32	0.279***	0.534 ± 0.20	0.298 ± 0.11	0.237***	1.13	0.25 (0.12)	0.21
DP-L1936 (Δ*plcA* Δ*plcB*)	4	5	0.975 ± 0.40	0.845 ± 0.35	0.130**	0.287 ± 0.11	0.274 ± 0.14	0.012	0.42	0.31 (0.18)	0.03
	5	3	0.781 ± 0.41	0.699 ± 0.33	0.082	0.344 ± 0.14	0.348 ± 0.07	0	0.77	0.11 (0)	0
DP-L1942 (Δ*actA*)	4	2	0.668 ± 0.15	0.438 ± 0.10	0.230**	0.277 ± 0.03	0.190 ± 0.036	0.087**	0.635	0.36 (0.23)	0.14
	5	2	0.734 ± 0.20	0.425 ± 0.18	0.311**	0.247 ± 0.02	0.189 ± 0.058	0.058	1.25	0.25 (0.12)	0.046

[a] *n*, number of separate experiments each done in triplicate.
[b] ΔDAG and Δceramide, increase in DAG and ceramide, respectively, over that in control, uninfected cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by paired *t* test).
[c] The values for bacterial cell number represent the average of all the bacterial counts obtained from plating of three coverslips at each harvest for each experiment.
[d] The values in parentheses are corrected for 0.13 nmol of DAG per 10^8 bacteria.

Because a likely source of functional redundancy in the phospholipases was enzymatic activity, we looked for evidence of bacterial phospholipase activity during the course of intracellular infection. J774 cells prelabelled with [^{14}C]glycerol and infected with wild-type *L. monocytogenes* demonstrated increased phospholipid turnover as measured by increased levels of radioactive diacylglycerol in cells 3 to 4 h after infection. Time course experiments indicated that the increased labelling of diacylglycerol continued for at least 3 h more (data not shown). Measurements of diacylglycerol and ceramide mass confirmed the presence of higher than basal levels of PLC products which were completely (ceramide) or partially (diacylglycerol) dependent on the activities of the bacterial phospholipases. Since these differences were small, we have not attempted to study the effects of deletion of each phospholipase independently. The finding that ceramide, which was presumably produced by hydrolysis of sphingomyelin by bacterial PC-PLC, returned to near basal levels in J774 cells infected with an *actA* mutant at 5 h postinfection but continued to increase in wild-type infections is also consistent with a role for PC-PLC in cell-to-cell spread.

The finding of elevated diacylglycerol in cells infected with a strain lacking both phospholipases indicates that infection with *L. monocytogenes* activates endogenous host phospholipases by as-yet-unknown pathways. It is known that cytokines, including tumor necrosis factor alpha, interleukin-1α, interleukin-1β, and interleukin-6, are expressed by host cells as a result of *L. monocytogenes* infection (18, 26, 38, 48). By autocrine effects, these factors could trigger host signalling pathways involving endogenous PLCs (42).

The most striking defect of the double phospholipase mutant was seen in the mouse LD_{50}. The Δ*plcA* Δ*plcB* mutant strain was 500-fold less virulent than the wild type. In contrast, the Δ*plcA* and Δ*plcB* single mutants were 3-fold and 20-fold less virulent, respectively. This dramatic difference in phenotype may be due to the differences in escape from the vacuole and cell-to-cell spread as described here. However, the phospholipases may have other important functions not readily detectable in tissue culture models of infection. In this context, one interesting possible role for the phospholipases could be in host cell growth activation. Diacylglycerol and ceramide are known to be potent biological second messengers which have been implicated in a wide variety of physiological processes (10, 34, 42). It is of interest to note that stable transfection of NIH 3T3 cells with the gene for PC-PLC from *Bacillus cereus* led to their transformation in tissue culture. These cells exhibited increased DNA synthesis and could proceed through the cell cycle without added mitogens (21). Although signals that stimulate progression through the cell cycle may not in themselves be important in cells heavily infected with *L. monocytogenes*, the induction of host cell growth may promote growth of the bacteria by providing nutrients. Many cellular responses are associated with the reinitiation of cell proliferation, including increased nutrient uptake, the induction of transport systems, and increased energy metabolism (40, 41). The rapid intracellular growth of *L. monocytogenes* could compete with the host for glucose and other nutrients. Although there is no evidence of this in the transformed tissue culture cells used in these studies, differentiated tissues of the animal host may limit bacterial growth when the PLCs are absent.

It is now clear that the *L. monocytogenes* phospholipases are essential determinants of pathogenicity. The enzymes function together throughout the course of cellular infection to promote *L. monocytogenes* access to the host cytoplasm both during the initial invasion of host cells and upon subsequent cell-to-cell spread. In addition, the severe defect of the double phospholipase mutant demonstrated by the mouse LD_{50} suggests that the phospholipases may have additional roles during animal infection.

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