INFECTION AND IMMUNITY, Nov. 1994, p. 4795–4803
0019-9567/94/$04.00+0

Vol. 62, No. 11

Phosphatidylinositol-Specific Phospholipase C from *Listeria monocytogenes* Contributes to Intracellular Survival and Growth of *Listeria innocua*

WILLIAM R. SCHWAN,* ANDREAS DEMUTH, MICHAEL KUHN, AND WERNER GOEBEL

Lehrstuhl für Mikrobiologie, Theodor-Boveri-Institut für Biowissenschaften, Universität Würzburg, 97074 Würzburg, Germany

Received 25 May 1994/Returned for modification 8 July 1994/Accepted 9 August 1994

***Listeria monocytogenes* is a facultative intracellular organism that is capable of replicating within macrophage and macrophage-like cells. The species secretes a phosphatidylinositol-specific phospholipase C (PI-PLC) encoded by the *plcA* gene. A *plcA* gene from *L. monocytogenes* was cloned downstream of a gram-positive promoter in the plasmid pWS2-2. To determine what effect *plcA* would have on intracellular survival when introduced into *Listeria innocua*, a species that does not grow intracellularly or contain *plcA*, transformation with the recombinant pWS2-2 plasmid was performed. Phospholipase C activity in *Listeria innocua*/pWS2-2 was confirmed on a brain heart infusion-phosphatidylinositol agar plate, whereas wild-type *L. innocua* did not produce PI-PLC activity. Intracellular growth of *L. innocua*/pWS2-2 was subsequently measured in the macrophage-like cell line J774 by Giemsa staining and viable count determinations at specific time points following infection. The J774 cells infected with wild-type *L. innocua* showed a falling viable count through 8 h postinfection. Although J774 cells infected with *L. innocua*/pWS2-2 also initially displayed reduced viable counts, the viable count rose after 6 h postinfection and increased further at 8 h postinfection before a subsequent decline again at 16 h postinfection. Giemsa staining revealed fewer than 6 bacteria in individual macrophage cells at 2 h postinfection, and yet approximately 15% of the J774 cells had 6 to 12 bacteria localized to one area of the macrophage cell after 6 h; moreover, electron micrographs showed that the *L. innocua*/pWS2-2 cells were replicating inside the phagosome of the host cell. Furthermore, Thoria Sol labeling demonstrated that lysosomes had fused with these phagosomes, and acridine orange staining revealed that the compartments were acidified. These results demonstrate that *L. innocua* cells transformed with the plasmid-borne *plcA* gene, and expressing functional PI-PLC, are able to grow intracellularly in what appear to be phagolysosomes, although between 3 and 6 h is needed for this to manifest itself. Intracellular growth specifically in *L. innocua* may be a secondary function associated with the *plcA* gene product. The addition of this one gene, *plcA*, to a species of *Listeria* that in the wild-type state does not replicate intracellularly apparently can now allow some of the bacteria to transiently multiply inside the phagosomes of host macrophage cells.**

Listeria monocytogenes is a gram-positive, facultative intracellular bacterial pathogen that is capable of causing disease in humans and animals (45). This species has a broad host range, and it can survive and multiply within a variety of eukaryotic cell types, including professional phagocytes (33) and epithelial cells (18). A cascade of events that are critical steps in *Listeria* intracellular survival occurs within the host cell following uptake of the bacteria. These steps consist of escape from the phagosome, intracellular replication, and actin-based motility that leads to cell-to-cell spread (51). Several genes that are part of the gene cluster associated with the steps noted above as well as the overall virulence of *L. monocytogenes* have been identified through transposon mutagenesis (39). Genes contained within this gene cluster as well as other unidentified genes are transcriptionally activated by the *prfA* (positive regulatory factor) gene product (17, 30, 35, 47). Besides PrfA encoded by the *prfA* gene, other proteins coded for in this region that are positively regulated by PrfA (39) include the following: a hemolysin, listeriolysin O, involved in lysis of the phagosome vacuole (18, 19, 26); ActA, a surface-associated protein that mediates actin assembly within the host cell (13, 27); a broad-specificity phosphatidylcholine phospholipase C (PC-PLC) (52) and a phospholipase C specific for phosphatidylinositol (PI)-containing substrates (PI-PLC) (5, 29, 34); and a metalloprotease (12, 36), which appears to be necessary for processing both PC-PLC (40) and ActA (47) into active forms of each protein.

PI-PLC is an extracellular product encoded by the gene *plcA* (5, 29, 34). This activity is expressed only in pathogenic *Listeria* species (*L. monocytogenes* and *Listeria ivanovii*) (29, 38), not in nonpathogenic species such as *Listeria innocua*. The enzyme specifically cleaves PI-containing compounds such as eukaryotic PI-glycan-ethanolamine anchors found as part of many eukaryotic membrane proteins (22, 31, 34). Mutations in *plcA* have supported a role for the PI-PLC in pathogenesis because of the reduced virulence and longer retention in phagosomes when these transposon mutants are used in infection models (5, 34, 49). However, mutations in *plcA* may cause polar effects on *prfA* (35). Previously, it was suggested that PI-PLC may impair cell-to-cell spread (49); however, a more recent study has shown that defects in cell-to-cell spread were probably the result of a polar effect on *prfA* and not attributed to *plcA* (6). By blocking readthrough transcription of *prfA* from the *plcA* promoter, many genes in that strain were presumably downregulated, which in turn supposedly led to the impairment of cell spreading. Postulated roles for PI-PLC still include escape from the phagosome and intracellular growth (6, 22). In fact, in

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Theodor-Boveri-Institut für Biowissenschaften, Universität Würzburg, Am Hubland, 97074 Würzburg, Germany. Phone: (0931) 888-4418. Fax: (0931) 888-4402.

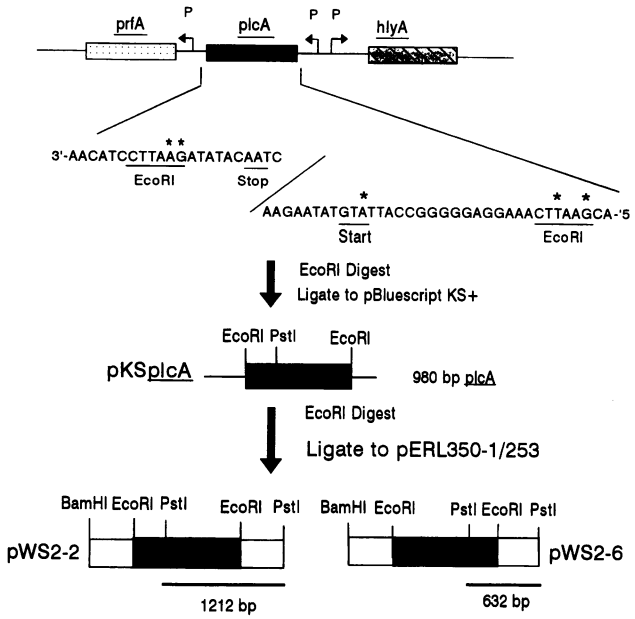

FIG. 1. Construction of recombinant vectors pKS*plcA*, pWS2-6, and pWS2-2. *L. monocytogenes* EGD DNA was amplified by using primers M328 and M329. Both primers display bases marked by asterisks that differ from the sequence taken from *L. monocytogenes* EGD, creating a new start site with an ATG and *Eco*RI sites flanking the *plcA* gene for easier cloning. The insert DNA is indicated by a dark bar.

vivo studies have shown that PI-PLC may play a part in escape from primary murine macrophage phagosomes (6).

Since mutational analyses are fraught with difficulties, we have used a different approach to delineate the putative role that *L. monocytogenes* PI-PLC has in intracellular survival. The *plcA* gene from *L. monocytogenes* was cloned into a gram-positive expression vector and used to transform a *Listeria* species that normally lacks PI-PLC. In this report, we show that the addition of plasmid-borne *plcA* to normally nonreplicating *L. innocua* cells now leads to short-term intracellular multiplication of these bacterial cells in the phagosomes of a macrophage-like cell line.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Escherichia coli* strain DH5α (*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA)U169*; Gibco/BRL, Gaithersburg, Md.) was used for transformation of plasmid DNA. Wild-type *L. monocytogenes* EGD Sv 1/2a was provided by S. H. E. Kaufmann, Ulm, Germany. *L. innocua* Sv 6a (NCTC 11288) was obtained from the *Listeria* strain collection of the Institute of Hygiene and Microbiology, Würzburg, Germany. All *Listeria* strains were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 37°C with shaking or on BHI agar plates unless otherwise noted. *E. coli* DH5α was grown in Luria-Bertani broth or agar at 37°C (37). The following antibiotics were added to the media when required: erythromycin, 5 μg/ml (*Listeria* strains) or 300 μg/ml (*E. coli*); ampicillin, 50 μg/ml (*E. coli*).

Plasmids. The plasmid pKS*plcA* was constructed as described in Fig. 1 by using *L. monocytogenes* EGD chromosomal DNA from whole cells amplified with a set of primers for the *plcA* gene described below. After PCR amplification, the 980-bp DNA fragment was eluted from a 1% agarose gel (43) and digested with *Eco*RI. This fragment was then ligated to *Eco*RI-digested pBluescriptKS+ (Stratagene) that had been treated with alkaline phosphatase. Transformants were selected on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Gibco/BRL) plates containing ampicillin. pERL3 50-1 has been described previously (30). It carries the origin of replication from the plasmid pAMβ1, an erythromycin resistance gene, the pBR322 replicon, two *Eco*RI sites, and the *prfA* gene with two of its promoters, $prfA_{P1}$ and $prfA_{P2}$ (16). A derivative of pERL3 50-1, pERL3 50-1/253, was constructed to remove the *Eco*RI site far downstream of the *prfA* promoters by performing a partial *Eco*RI digestion of pERL3 50-1, generating blunt ends by filling in with Klenow fragment, and doing a blunt-end ligation of the plasmid itself by standard procedures (43). This series of procedures left one *Eco*RI site present in the N terminus of the *prfA* gene 20 bp downstream of the translational start site of *prfA* on the vector pERL3 50-1/253. Plasmids pWS2-2 and pWS2-6, representing both orientations of the *plcA* gene in pERL3 50-1/253, were created by digesting the pKS*plcA* construct with *Eco*RI and cloning the *plcA* gene into the remaining *Eco*RI site of pERL3 50-1/253 found in the N terminus of the *prfA* open reading frame (Fig. 1). Orientation of the *plcA* gene in pWS2-2 and pWS2-6 was confirmed by *Pst*I digestion of the plasmid DNA.

Recombinant DNA techniques. Plasmid DNA from *E. coli* was isolated by the method of Clewell and Helinski (8) or by the alkaline lysis method of Birnboim and Doly (3). *E. coli* strains were transformed by the $CaCl_2$ procedure (10). *Listeria* plasmid DNA isolation and transformation of *L. innocua* cells followed the procedures of Wuenscher et al. (53). Transformants of *L. innocua* were visible on DM-3 regeneration plates (7a) after 3 to 4 days at 30°C. Restriction endonucleases (Pharmacia) and Klenow fragment, alkaline phosphatase, and T4 DNA ligase (all from Boehringer GmbH, Mannheim, Germany) were used as suggested by the manufacturers.

PCR. The PCR was performed by the method of Coen (9). Briefly, a colony of *L. monocytogenes* EGD was lysed for 10 min at 100°C in H_2O, and part of the lysate was added to a mixture of *Taq* polymerase buffer, 1.25 mM deoxynucleoside triphosphate (Pharmacia), 50 pmol of each primer, and *Taq* polymerase (Pharmacia) in a final volume of 100 μl. Amplification was done in a Thermocycler 60/2 (Bio-Med, Theres, Germany) for 30 cycles: initial denaturation for 2 min at 94°C; primary denaturation for 15 s at 94°C; primer annealing for 30 s at 55°C; primer extension for 2 min at 72°C; and a prolonged primer extension following the last cycle for 2 min at 72°C. The two primers used for PCR were prepared with an automated synthesizer (model 380B; Applied Biosystems, Foster City, Calif.). The primers were as follows: M328, a 38-mer, 5′ TATACGAATTCAAAGGAGGGGGCCATTATGTATAA GAA 3′, and M329, a 22-mer, 5′ TTGTAGGAATTCTATAT GTTAG 3′. These primers changed four bases, as depicted in Fig. 1 (two on each side of the amplified product), to generate flanking *Eco*RI sites convenient for cloning. Additionally, the GTG start site was converted to an ATG start site by using the M328 primer.

Detection of PI-PLC. PI-PLC activity of recombinant *L. innocua* strains was detected on BHI-egg yolk agar plates (BHI agar, 2.5% egg yolk, 2.5% phosphate-buffered saline [PBS]) (6) and confirmed on BHI-PI agar (BHI agar, with a 1% PI substrate [Sigma] in 1% agarose overlay) (29). Turbid zones surrounding the bacterial growth after incubation at 37°C for 48 h indicated a positive response.

Mammalian cell culture. The macrophage-like J774 cell line derived from a reticulum cell sarcoma (41) was cultured in

RPMI 1640 medium (Gibco/BRL) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml; all from Gibco/BRL) under 5% CO_2–95% air at 37°C. The RPMI 1640 medium with glutamine and fetal calf serum but without antibiotics will be referred to as RPMI 1640 complete medium.

Infection of J774 cells. The J774 cells were seeded 24 h prior to infection in 45-mm-diameter tissue culture plates (Greiner) for intracellular staining (4×10^5 cells per plate) or in 12-well tissue culture plates (Greiner) for viable count determinations (5×10^5 cells per well). Macrophage cells were washed twice with PBS, covered with RPMI 1640 complete medium, and infected with *Listeria* bacteria at a multiplicity of infection of 50 bacteria per eukaryotic cell (50:1). Bacteria used for infection were grown to the mid-log phase (120 to 160 Klett units), washed with PBS, and suspended in PBS with 15% glycerol, and aliquots were frozen at −80°C. The numbers of CFU per milliliter were determined by 10-fold serial dilutions of the bacteria in PBS and plating onto BHI agar plates that were then incubated at 37°C. For infection, frozen aliquots were thawed and applied to the J774 monolayers. After a 45-min incubation at 37°C under 5% CO_2–95% air to allow optimal phagocytosis of the bacteria, the wells or plates were washed three times with PBS. Fresh RPMI 1640 complete medium with 50 μg of gentamicin (Serva) per ml was added to kill extracellular bacteria and prevent reinfection. The initial time when gentamicin was added was designated the 0 h postinfection time point.

Intracellular growth assays. To study intracellular multiplication, two procedures were used. Giemsa staining of the cells was used initially. Briefly, J774 cells (4×10^5) in 45-mm-diameter tissue cultures plates infected at a multiplicity of infection of 50:1 as described above were washed two times with PBS and fixed for 5 to 7 min with methanol at room temperature. Plates were air dried and stained for 15 to 60 min with Giemsa stain (Sigma) prepared as described in the manufacturer's instructions. After the plates were washed three times with distilled water, they were air dried and observed under oil immersion. Time points of 0, 1, 2, 4, 6, and 8 h postinfection were examined.

Viable count determinations were also used to determine intracellular replication. J774 cells (5×10^5) in 12-well tissue culture plates infected as noted above were washed two times with PBS. Macrophages were lysed with 200 μl of cold distilled water applied for 3 to 5 min. Lysates were removed from the wells, 10-fold serially diluted in PBS, and plated onto BHI agar plates. Plates were grown at 37°C overnight, and CFU were counted.

Electron microscopy. Circular coverslips in 24-well tissue culture plates (Greiner) were seeded with 10^5 J774 cells 24 h before infection. After infection with a multiplicity of infection of 50:1 (*Listeria* cells to J774 cells), coverslips were treated at different time points as described by Karunasagar et al. (25), except for the following addition. Once the coverslips were washed with H_2O, they were submerged in 0.5% uranyl acetate and left at 4°C overnight. Cells were then dehydrated and embedded in Epon 812 (Serva, Heidelberg, Germany). Serial ultrathin sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Zeiss EM 900 electron microscope at 80 kV.

Thoria Sol labeling. J774 macrophage monolayers on coverslips (3×10^5 macrophage cells) prepared as described above were incubated for a total of 4 h at 37°C under 5% CO_2 with electron-dense colloidal Thoria Sol (Polysciences, Warrington, Pa.) diluted 1:80 in RPMI complete medium as described previously (46). After 2 h, the excess Thoria Sol was removed by washing three times with PBS, and then the coverslips were incubated for an additional 2 h for the total time of 4 h. Monolayers were infected at a multiplicity of infection of 50:1, and at 3 h postinfection with bacteria, the coverslips were prepared for electron microscopy as noted above.

Acridine orange staining. Coverslips were seeded with 3×10^5 J774 cells 16 h before use as described above. A qualitative measurement of pH was performed on the *Listeria*-infected J774 cell monolayers by treating the coverslips for 5 min with 5 μM acridine orange in PBS (1). Phagosome interiors stain green at neutral pH and red in acidified compartments under fluorescent light following acridine orange staining (50).

RESULTS

Expression of PI-PLC in *L. innocua*. *L. monocytogenes* EGD produces a very low level of PI-PLC (47). The *plcA* gene used in this study was cloned from *L. monocytogenes* EGD as depicted in Fig. 1. This gene was cloned in both orientations on the plasmid pERL3 50-1/253, resulting in the constructs pWS2-2 (correct orientation) and pWS2-6 (incorrect orientation). The *plcA* gene was cloned into the remaining *Eco*RI site found 20 nucleotides downstream of the *prfA* translational start site in the vector pERL3 50-1/253. To determine if the *plcA* gene cloned from *L. monocytogenes* EGD expressed functional PI-PLC activity, the recombinant plasmids pWS2-2 (correct orientation of *plcA*) and pWS2-6 (reverse orientation of *plcA*) were transformed into *L. innocua* cells, and some of these recombinant cells were inoculated onto a BHI-egg yolk agar plate. As shown in Fig. 2A, a large halo (7 to 8 mm) was observed around the recombinant *L. innocua*/pWS2-2 strain but not around the recombinant *L. innocua*/pWS2-6 strain grown on BHI-egg yolk agar. Previously, it has been shown that a *plcA* mutant of *L. monocytogenes* EGD formed no halo around the bacterial growth on BHI-egg yolk agar, whereas a *plcB* mutant revealed a halo similar to that of the wild-type strain (44), suggesting that the halo was the result of *plcA* activity. We also grew *plcA* and *plcB* mutants of *L. monocytogenes* EGD on BHI-egg yolk agar and found similar results (data not shown). Because both lecithinase and PI-PLC activities can be measured on egg yolk agar, the two recombinant strains as well as wild-type *L. innocua* and *L. monocytogenes* EGD were passaged on BHI agar containing a PI overlay. Once again, the recombinant *L. innocua*/pWS2-2 strain exhibited a large halo (7 to 8 mm) around the bacterial growth (Fig. 2B) that was similar to that observed on BHI-egg yolk agar (Fig. 2A), indicating functional PI-PLC activity. However, both the other recombinant *L. innocua* strain transformed with pWS2-6 as well as the wild-type *L. innocua* strain failed to show a halo around the bacterial growth. *L. monocytogenes* EGD elicited a very small halo (1 mm) on the BHI-PI agar plate (Fig. 2B). Plasmid DNA of the correct size and restriction endonuclease pattern was isolated from transformed *L. innocua* cells. Thus, the recombinant plasmid pWS2-2 has an active *plcA* gene that conveys PI-PLC expression in *L. innocua* cells.

Intracellular growth of *L. innocua* containing the *plcA* recombinant plasmid. As shown above, a functional *plcA* gene was demonstrated in *L. innocua*/pWS2-2 grown on BHI-PI agar (Fig. 2B). Previous investigations of PI-PLC have relied on mutational analyses to knock out the *plcA* gene (5, 34, 49). The transfer of the *plcA* gene to a species that does not carry the gene offered an opportunity to study the potential role of PI-PLC in intracellular survival. Because PI-PLC is suggested to assist in intracellular proliferation of the bacteria (22), *L.*

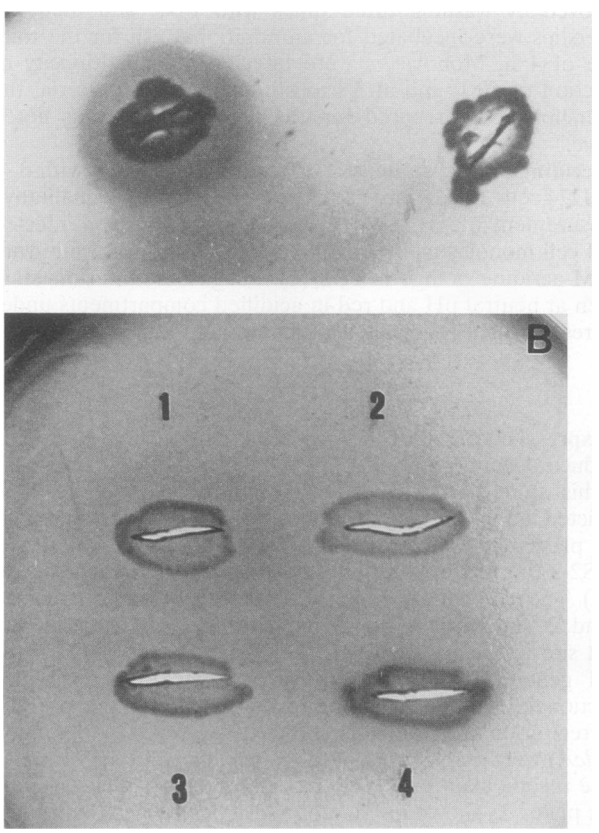

FIG. 2. Evidence of PI-PLC activity in wild-type and recombinant *Listeria* strains. All strains were stabbed and streaked on either BHI-egg yolk or BHI-PI agar and then grown for 48 h at 37°C. The presence of a halo was a positive result. (A) Comparison of both recombinant *L. innocua* strains (*L. innocua*/pWS2-2 [left] and *L. innocua*/pWS2-6 [right]) after growth on BHI-egg yolk agar; (B) PI-PLC activity determined after growth of *L. innocua*/pWS2-2 (1), *L. innocua*/pWS2-6 (2), wild-type *L. innocua* (3), or *L. monocytogenes* EGD (4) on a BHI-PI agar plate.

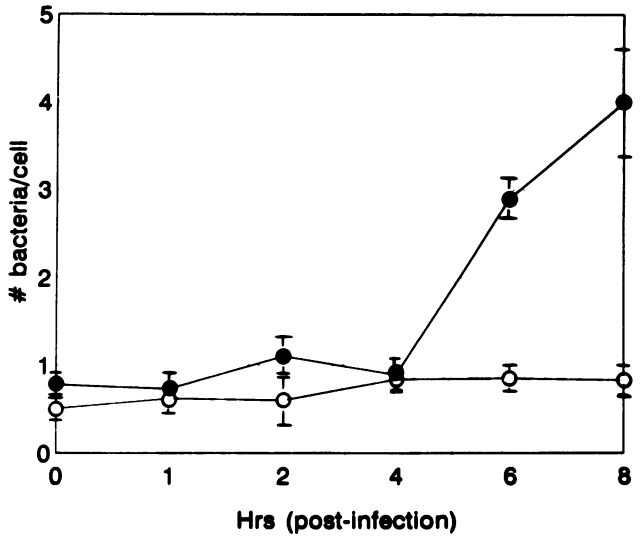

FIG. 3. Graph of counts of *Listeria* bacteria per J774 cell resulting from Giemsa staining of *L. innocua* (open circles)- and *L. innocua*/pWS2-2 (closed circles)-infected J774 macrophage cells at 0 to 8 h postinfection. The graph represents an average of three separate experiments.

innocua/pWS2-2 was used to infect the macrophage-like cell line J774 and intracellular survival and growth were assessed. Initially, Giemsa staining was used to measure the mean number of *Listeria* bacteria per J774 cell during a time course from 0 to 8 h postinfection, comparing wild-type *L. innocua* cells with *L. innocua* cells containing the recombinant plasmid pWS2-2. For wild-type *L. innocua* cells, the average cell number remained constant over the 8-h time period in three separate experiments (Fig. 3) (0.52 ± 0.15 at 2 h to 0.78 ± 0.09 at 8 h), and most J774 cells contained 0 to 2 *Listeria* cells per macrophage (Fig. 4). Although *L. innocua*/pWS2-2 also showed a pattern similar to that of *L. innocua* through early time points (mean cell number at 4 h postinfection, 0.78 ± 0.04) and no more than five bacteria in a single macrophage cell, the *Listeria* cell number showed an increase at 6 h postinfection (2.9 ± 0.14) and an additional increase at 8 h postinfection (4.0 ± 0.57) (Fig. 3). About 15% of the J774 cells had 6 to 12 *Listeria* cells localized to one area of the macrophage cell after 6 h (Fig. 4).

Giemsa staining is a qualitative measurement of bacterial growth, and one cannot discern between dead and living bacterial cells. A viable count series was then used to quantitate the number of *Listeria* cells in the J774 cells. An average of three separate trials indicated that the number of *L. innocua* CFU decreased at every time point (Fig. 5), demonstrating that the macrophage cell line was capable of killing the nonreplicating wild-type *L. innocua* cells. Furthermore, an additional control represented by the recombinant *L. innocua*/pWS2-6 strain, containing *plcA* in the wrong orientation, also displayed an overall decrease in bacterial cell number over time (35% at 2 h postinfection versus 13% at 8 h postinfection). The death curve was not as steep as that for wild-type *L. innocua*, but no increase in viable cell number was apparent. On the other hand, *L. innocua*/pWS2-2 cells expressing PI-PLC exhibited an initial decrease in cell number through 4 h postinfection (11%), but at 6 h postinfection the viable counts rose (23%) and these counts increased again at 8 h postinfection (34%). However, after 16 h postinfection, the number of *L. innocua*/pWS2-2 CFU declined again (4%), although not to the same level as wild-type *L. innocua* (0.2%) or *L. innocua* transformed with the pWS2-6 recombinant plasmid possessing *plcA* in the wrong orientation (0.6%). These cumulative increases over 8 h postinfection would correspond to approximately two doublings of the bacteria during this time. *L. innocua* transformed with pERL3 50-1 showed viable count determinations similar to those of wild-type *L. innocua* (data not shown). These results point to transient intracellular replication of the *L. innocua* cells transformed with the *plcA* gene correctly oriented on a recombinant plasmid.

Localization of *L. innocua*/pWS2-2 within the host macrophage cells. The Giemsa stain and viable count experiments suggested that intracellular growth was occurring, but they did not reveal where this multiplication happened. Electron microscopic analyses were undertaken to determine if the bacteria had escaped from the phagosome and were growing in the cytoplasm of the macrophage cells. At a time point at which replication had been suggested to result (8 h postinfection), electron micrographs were taken of J774 macrophage cells infected with *L. monocytogenes* EGD, wild-type *L. innocua*, or *L. innocua*/pWS2-2 (PI-PLC$^+$). The results showed that *L. monocytogenes*-infected J774 cells had bacteria predominately free in the cytoplasm (82 of 100 bacterial cells counted), with

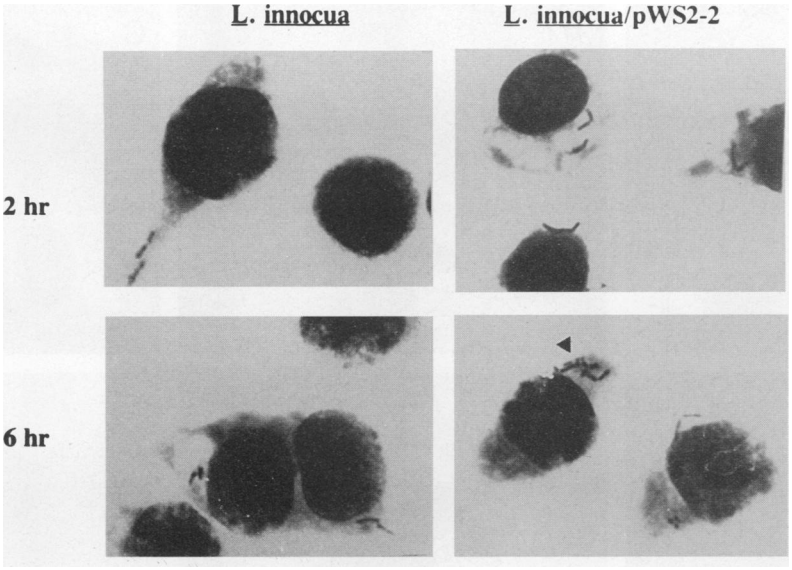

FIG. 4. Light microscope photographs of Giemsa-stained J774 macrophage cells infected with either *L. innocua* or *L. innocua*/pWS2-2. Photographs were taken at 2 or 6 h postinfection with the *Listeria* bacteria. The arrowhead points to a J774 macrophage cell containing 6 to 12 bacteria. Magnification, ×1,000.

actin polymerization surrounding these bacterial cells (Fig. 6A). Wild-type *L. innocua* cells were all contained within membrane-bound vacuoles (100 of 100 counted) as single cells (Fig. 6B) or sometimes two bacterial cells per phagosome. Examination by electron microscopy of *L. innocua*/pWS2-2-infected macrophage cells showed every bacterium within a membrane-bound vacuole. Sometimes there were 6 to 12 bacteria inside a phagosome (Fig. 6C), and frequently these bacteria appeared to be undergoing replication inside the phagosome (Fig. 6D). No apparent gross breaks in the vacuoles were visible at the magnification used. These results indicate that the bacteria are multiplying within the phagosome and not the cytoplasm of the host cell.

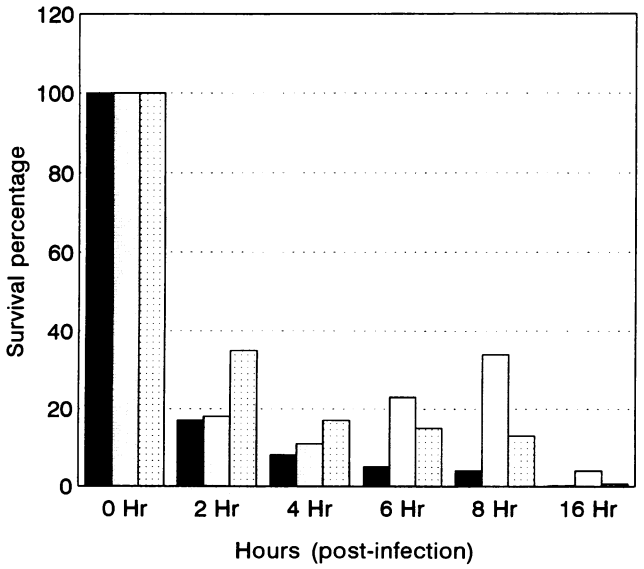

FIG. 5. Viable count determinations in CFU survival percentages of J774 macrophage cells infected with *L. innocua* (black bar), *L. innocua*/pWS2-2 (PI-PLC$^+$) (gray hatched bar), or *L. innocua*/pWS2-6 (PI-PLC$^-$) (stippled bar). Time points tested ranged from 0 to 16 h postinfection. Invasion percentages were standardized to 100% at the time point following addition of gentamicin (0 h), and subsequent survival rates were determined on the basis of comparisons to these 0-h values. The graph represents an average of three separate experiments.

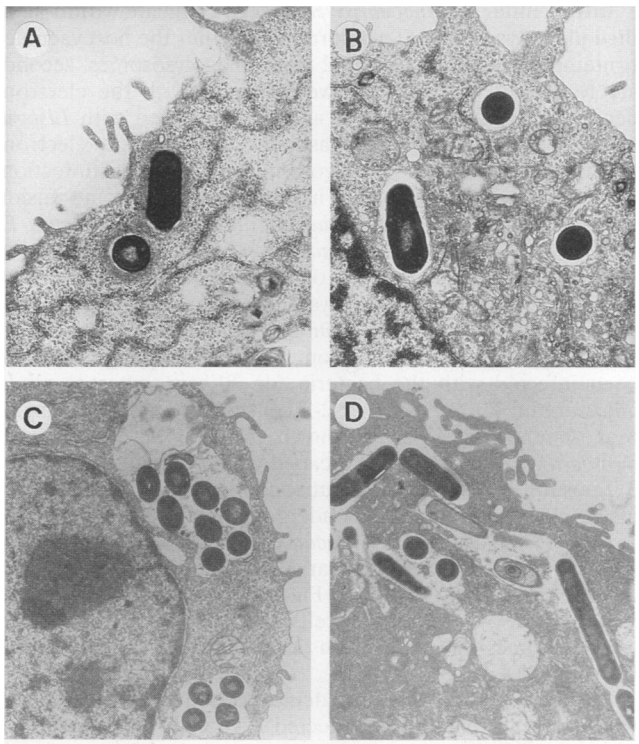

FIG. 6. Electron micrographs of 8-h-postinfection thin sections of J774 macrophage cells infected with *L. monocytogenes* (A), *L. innocua* (B), and *L. innocua*/pWS2-2 (C and D). Panel D shows *L. innocua*/pWS2-2 replicating inside the phagosome. Magnification, ca. ×12,000.

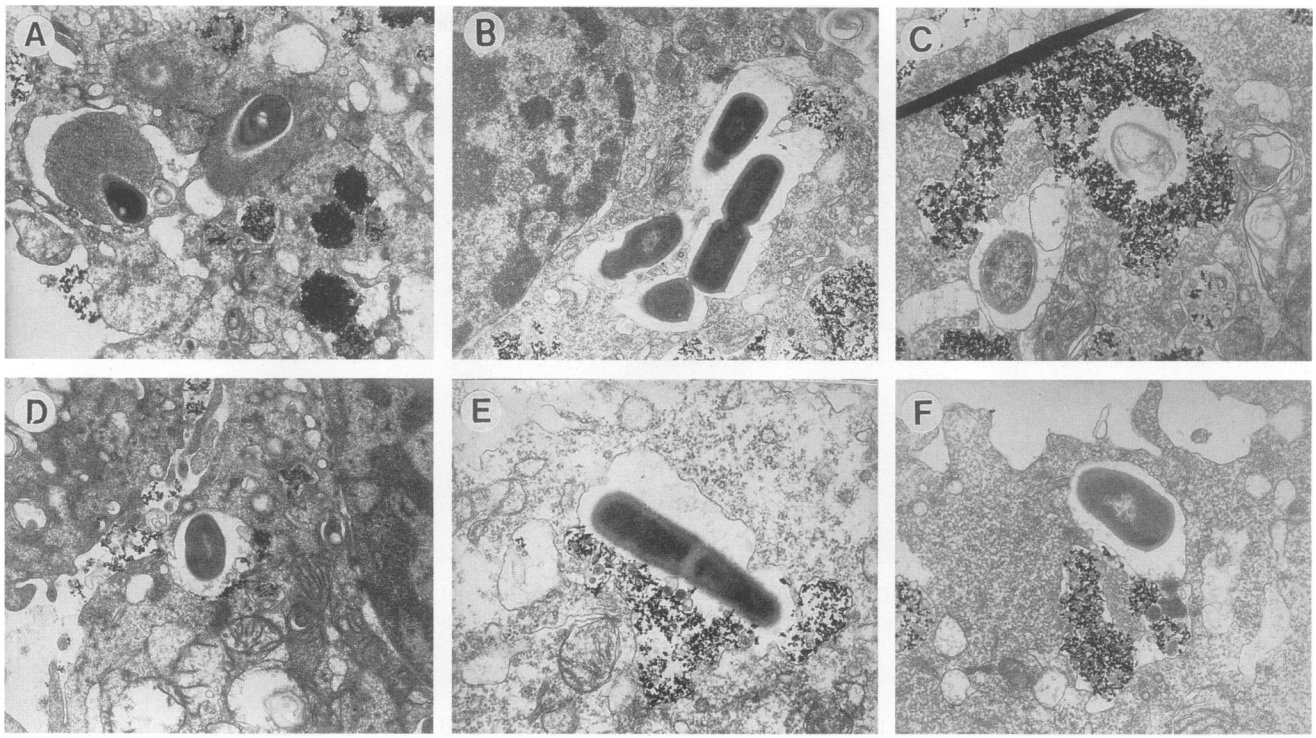

FIG. 7. Electron micrographs of 3-h-postinfection thin sections of J774 macrophage cells following Thoria Sol labeling of secondary lysosomes and infection with *Listeria* bacteria. (A) *L. monocytogenes* free in cytoplasm; (B) *L. innocua*/pWS2-2 replicating in the phagosome; (C) dead *L. innocua* cells inside phagolysosome; (D) *L. monocytogenes* in phagosome; (E) *L. innocua*/pWS2-2 in phagosome; (F) *L. innocua* cells within phagolysosomes. Lysosomes are represented by the small dark-staining granules inside the cell. Magnification, ca. ×22,000.

Intracellular *L. innocua*/pWS2-2 cells replicate within acidified phagolysosomes. To determine whether the host vacuoles containing *L. innocua*/pWS2-2 were phagolysosomes, secondary lysosomes of J774 cells were labeled with the electron-dense compound Thoria Sol and then infected with *Listeria* bacteria. Lysosome fusion was indicated by dark, electron-dense granules inside the phagosome. At 30 min postinfection, no difference was observed in phagosome-lysosome fusion frequencies among all *Listeria* populations (*L. innocua*, *L. innocua*/pWS2-2, and *L. monocytogenes* EGD). Each group had a 38 to 40% frequency of fusion between phagosomes containing bacteria and the lysosomes of the host cells. Approximately 40% of the *L. monocytogenes* EGD bacterial cells had escaped from the phagosome after macrophage cells had been infected with the bacteria for 30 min, whereas all *L. innocua* and *L. innocua*/pWS2-2 cells were confined to membrane-bound vacuoles. As shown in Fig. 7A, wild-type *L. monocytogenes* cells usually escaped from the phagosome after 3 h postinfection (Table 1), but some bacterial cells remained inside phagosomes that had undergone lysosome fusion (Table 1; Fig. 7D). Wild-type *L. innocua* cells were always present in membrane-bound vacuoles, and lysosome fusion was frequently observed (Table 1; Fig. 7C and F). Likewise, *L. innocua*/pWS2-2 (PI-PLC$^+$) cells were also found exclusively in phagosomes that were often fused with lysosomes (Table 1; Fig. 7B and E).

Acridine orange staining demonstrated that 20% of *L. monocytogenes* bacteria in infected J774 cells were contained within acidified membrane-bound vacuoles at 2 h postinfection, whereas 100% of wild-type *L. innocua* and *L. innocua*/pWS2-2 were in acidified phagosomes (Table 1). By 6 h postinfection, the percentage of *L. monocytogenes* cells still in phagosomes had dropped to 10%, and these vacuoles still appeared to be acidified (Table 1). Wild-type *L. innocua* and *L. innocua*/pWS2-2 cells were always found within acidified compartments at 6 h postinfection (Table 1), which was similar to the observations at 2 h postinfection. These data suggest that *L. innocua*/pWS2-2 is capable of multiplying inside acidified phagolysosomes of the host cell.

DISCUSSION

L. monocytogenes can survive and replicate within host macrophage and epithelial cells. Several proteins have been implicated in these virulence traits (39), including PI-PLC encoded by the *plcA* gene (5, 29, 34). Data presented here show that the *L. monocytogenes plcA* gene can confer transient

TABLE 1. Percentage of *Listeria* cells in phagosomes that have fused with lysosomes or have become acidified

Strain	% of *Listeria* cells after:			
	Phagolysosome fusion		Acridine orange staining	
	In phagosomes	Fused[a]	In vacuoles (2 h/6 h)[b]	Acidified[c] (2 h/6 h)[b]
L. monocytogenes	16 ± 3[d]	86 ± 4	20 ± 6/10 ± 3	100/100
L. innocua	100	78 ± 6	100/100	100/100
L. innocua/pWS2-2	100	74 ± 4	100/100	100/100

[a] Percentage of 100 bacteria in phagosomes that have fused with lysosomes. Lysosomes detected by Thoria Sol labeling.
[b] Postinfection time points when observations were made.
[c] Percentage of 100 bacteria in vacuoles that have undergone acidification.
[d] Values are means ± standard errors for at least two experiments.

intracellular survival and replicative ability on the nonpathogenic, intracellularly nonreplicating species *L. innocua*. Moreover, we found the *L. innocua* cells transformed with the *plcA* recombinant plasmid (pWS2-2) grow inside apparently acidified phagosomes that have undergone phagosome-lysosome fusion.

In this study, we have cloned the *plcA* gene under the control of the *prfA* promoter region located between the *plcA* gene and the *prfA* gene (7, 16, 30). PI-PLC was produced by *L. innocua*/pWS2-2; however, a larger halo was revealed around this bacterial growth on BHI-PI agar plates compared with that of wild-type *L. monocytogenes* EGD where the gene originated. The very small halo around *L. monocytogenes* EGD is compatible with the low level of PI-PLC secreted by this strain (47). We attribute the substantial increase in PI-PLC expression from *L. innocua*/pWS2-2 to multicopy effects from the plasmid and to the alteration of the translational start site from a GTG in wild-type *L. monocytogenes* EGD to an ATG in the recombinant plasmid that was created by PCR amplification with the M328 primer. No detectable PI-PLC activity was observed in the *L. innocua*/pWS2-6 recombinant strain, which had *plcA* cloned in the opposite orientation and not under the control of the gram-positive promoter. This does not preclude the possibility that some readthrough of *plcA* did not occur; there was just no measurable activity in this assay.

The recombinant strain *L. innocua*/pWS2-2 produced functional PI-PLC activity, and the question was whether this had any advantage inside a host cell. This strain showed an elevation of bacterial numbers within the macrophage-like J774 cells (by Giemsa stain and viable count determinations), but it took 6 h postinfection for this result to be observable. Recombinant *L. innocua*/pWS2-6 cells, which served as a control as a result of the *plcA* gene being cloned in the wrong orientation, displayed a viable count curve very similar to that of *L. innocua*/pWS2-2 through 4 h, but beyond this time, the *L. innocua*/pWS2-6 curve continued to fall as did the viable counts for wild-type *L. innocua*. Recently, it has been shown that only a small percentage of an *L. monocytogenes* population that enters macrophage cells undergoes intracellular growth, while the rest of the bacterial population is killed (11, 42). Additionally, *L. innocua*/pERL3 50-1 also showed a viable count curve similar to that of wild-type *L. innocua* (data not shown). This confirmed that neither the addition of the plasmid nor the *prfA* gene alone conferred intracellular replication ability on the *L. innocua* cells; only functional PI-PLC appeared to increase the viable *Listeria* population inside J774 macrophage cells. Giemsa staining also revealed that at 2 h postinfection with *L. innocua*/pWS2-2, there were always fewer than 6 *Listeria* bacteria per individual macrophage cell, but approximately 15% of the macrophage cells had 6 to 12 *L. innocua*/pWS2-2 bacteria localized in one area of the host cell after 6 h postinfection. The failure to observe so many bacteria (i.e., 6 to 12) in individual macrophage cells at 2 h postinfection suggested that the increased number of bacteria in the host cells after 6 h postinfection was a result of replication and not uptake of aggregates of bacteria.

Since one of the attributed properties of PI-PLC is assisting in the release of *L. monocytogenes* from phagosomal compartments (5, 6, 29), the intracellular location of the bacteria was examined by electron microscopy. Membrane-bound vacuoles encircled all of the *L. innocua*/pWS2-2 cells found within the macrophage cells, an observation similar to that made when wild-type *L. innocua* cells were used. However, wild-type *L. innocua* cells were usually found as single cells residing in the phagosomes, whereas phagosomes containing 6 to 12 bacteria were often identified in *L. innocua*/pWS2-2-infected host cells. Apparently, the recombinant bacteria are now able to initiate replication inside the host cell phagosomes, supported by the fact that dividing bacteria were sometimes seen (Fig. 6D) even as early as 3 h postinfection (Fig. 7B). Other intracellular pathogens are able to survive inside the phagosomal compartments of host macrophage cells (4, 15, 24, 46, 48), but this phenomenon has never been observed before in *Listeria*-infected macrophage cells, probably because *L. monocytogenes* has a hemolysin that allows it to escape from the phagosome at early time points (28). It is interesting, however, that a hemolysin-negative mutant of *L. monocytogenes* expressing PI-PLC does not grow within a host cell at 6 h postinfection (28). Possible explanations for our results may include regulation changes due to a different promoter or an increase in PI-PLC activity for the recombinant plasmid compared with that of wild-type *L. monocytogenes* resulting from multicopy effects or the change in the translational start site, or these variations may be a result of species differences between *L. innocua* and *L. monocytogenes*.

To understand the nature of these intracellular vacuoles occupied by *L. innocua*/pWS2-2, we tested whether phagosome-lysosome fusion had occurred and if the phagosomes were acidified. Both acidification (1, 14, 24) and phagosome-lysosome fusion (15, 46, 48) have been studied before with other intracellular pathogens. Electron-dense Thoria Sol, which labels secondary lysosomes (2, 48), was used to show phagosome-lysosome fusion in host cells infected with either the recombinant *L. innocua*/pWS2-2 strain or the wild-type *L. innocua*. Dark, electron-dense granules were frequently observed in the phagosomes containing the bacterial cells. In fact, *L. innocua*/pWS2-2 cells were observed to be replicating in several phagolysosomes (Fig. 7B). Furthermore, phagosomes still containing *L. monocytogenes* cells exhibited lysosome fusion. *Yersinia pestis* (48) and *Histoplasma capsulatum* (15) can survive and grow within phagolysosomes, although the precise mechanisms for their survival have yet to be elucidated. Besides the phagosome-lysosome fusion, acidification of these compartments also occurred. Wild-type *L. innocua*, *L. innocua*/pWS2-2, and the remaining *L. monocytogenes* cells displayed acidified vacuoles after acridine orange staining. These two phenomena, phagosome-lysosome fusion and acidification, support previous work by de Chastellier and Berche (11). They demonstrated that very early following phagocytosis of *L. monocytogenes* by bone marrow-derived macrophage cells, there was both phagosome-lysosome fusion and acidification of vacuoles containing *L. monocytogenes*.

Apparently, acidification of phagocytic vacuoles occurs quite quickly, resulting in survival of some of the bacteria and death for the rest of the population. Goldfine and Knob (22) have demonstrated that PI-PLC is functional at acidic pHs and listeriolysin is optimally active at an acidified pH (21), and therefore, lower pHs should not significantly impair their activities. The species *Salmonella typhimurium* lives in acidified macrophage phagosomes, although the organism can initially attentuate the pH (1). It is possible that PI-PLC in *L. innocua*/pWS2-2 may modify the pH of the phagosomes since acridine orange staining is a qualitative test, but the population reduction after 16 h of incubation inside macrophage cells suggests that the pH is not significantly altered. Moreover, wild-type *L. monocytogenes* cells can exit the phagosome under normal circumstances, a situation confirmed by fewer bacterial cells residing in phagosomal compartments. This also argues against a moderating influence on pH by *plcA*.

What is the effect of PI-PLC in terms of mediating intracellular survival and replication in the recombinant *L. innocua*/pWS2-2 strain? The enzyme does not appear to be inhibiting

phagosome-lysosome fusion or acidification of the phagosomal compartments. One possibility is that PI-PLC is hydrolyzing glycosyl-phosphadityl (GPI)-anchored proteins that constitute parts of the extracytoplasmic leaflets of cell membranes (32). Although the electron micrographs did not show any gross perturbations in the membrane-bound vacuoles, this does not mean that the vacuoles have not been partially digested. Recent work suggests that the *L. monocytogenes* PI-PLC has very limited activity towards GPI-anchored proteins compared with *Bacillus thuringiensis* PI-PLC (20). However, the GPI proteins used in that study may not represent the GPI proteins found in the interior of the macrophage-like cell line we have used. Differences in substrate activity may be responsible for the diminished enzymatic activity of *L. monocytogenes* PI-PLC such that this type of PI-PLC may have a narrower substrate specificity that could include macrophage membrane proteins. PI in these macrophage membrane GPI anchors may serve as a nutrient source for the recombinant strain to replicate. When the lysosomes fuse with the phagosome, cytoplasmic leaflets containing abundantly more PI (23) could provide an additional source of material needed for intracellular growth. This could explain why there is a delay in the elevation of bacterial numbers. The bacteria use PI to multiply, and as long as they stay in the logarithmic phase, they may survive in the harsh phagolysosomal environment. Apparently, *S. typhimurium* has adapted to tolerating the acidic pH of macrophage phagosomes (1). Once the PI in the membranes is depleted, these cells may then enter the stationary phase and be slowly killed in the same manner as wild-type *L. innocua* cells are; hence, lower viable counts occur again after 16 h.

A precise role for PI-PLC remains unclear at this time. The *L. innocua*/pWS2-2 cells were confirmed to be undergoing intracellular multiplication but, surprisingly, did not escape from the phagosome. This intracellular multiplication capability may be a secondary function of *L. monocytogenes* PI-PLC that manifests itself in another *Listeria* species.

ACKNOWLEDGMENTS

We thank Claudia Gehrig for technical assistance with the electron microscopy and Georg Krohne (Abteilung für Elektronenmikroskopie, Universität Würzburg) for stimulating discussions concerning electron microscopic methods. We also thank Roy Gross for critical reading of the manuscript.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 165-B4).

REFERENCES

1. **Alpuche Aranda, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller.** 1992. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. Proc. Natl. Acad. Sci. USA **89:**10079–10083.
2. **Armstrong, J. A., and P. D. Hart.** 1975. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. J. Exp. Med. **142:**1–16.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Buchmeier, N. A., and F. Heffron.** 1991. Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. Infect. Immun. **59:**2232–2238.
5. **Camilli, A., H. Goldfine, and D. A. Portnoy.** 1991. *Listeria monocytogenes* mutants lacking phosphatidylinositol-specific phospholipase C are avirulent. J. Exp. Med. **173:**751–754.
6. **Camilli, A., L. G. Tilney, and D. A. Portnoy.** 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. **8:**143–157.
7. **Chakraborty, T., M. Leimeister-Wächter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174:**568–574.

7a. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168:**111–115.

8. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular form. Proc. Natl. Acad. Sci. USA **62:**1159–1166.
9. **Coen, D. M.** 1990. The polymerase chain reaction, p. 15.0.1–15.1.7. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
10. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli*. Gene **6:**23–28.
11. **de Chastellier, C., and P. Berche.** 1994. Fate of *Listeria monocytogenes* in murine macrophages: evidence for simultaneous killing and survival of intracellular bacteria. Infect. Immun. **62:**543–553.
12. **Domann, E., M. Leimeister-Wächter, W. Goebel, and T. Chakraborty.** 1991. Molecular cloning, sequencing, and identification of a metalloprotease gene from *Listeria monocytogenes* that is species specific and physically linked to the listeriolysin gene. Infect. Immun. **59:**65–72.
13. **Domann, E., J. Wehland, M. Rhode, S. Pistor, W. Goebel, M. Hartl, M. Leimeister-Wächter, M. Wuenscher, and T. Chakraborty.** 1992. A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J. **11:**1981–1990.
14. **Eissenberg, L. G., W. E. Goldman, and P. H. Schlesinger.** 1993. *Histoplasma capsulatum* modulates the acidification of phagolysosomes. J. Exp. Med. **177:**1605–1611.
15. **Eissenberg, L. G., P. H. Schlesinger, and W. E. Goldman.** 1988. Phagosome-lysosome fusion in P388D_1 macrophages infected with *Histoplasma capsulatum*. J. Leukocyte Biol. **43:**483–491.
16. **Freitag, N. E., L. Rong, and D. A. Portnoy.** 1993. Regulation of the *prfA* transcriptional activator of *Listeria monocytogenes*: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. Infect. Immun. **61:**2537–2544.
17. **Freitag, N. E., P. Youngman, and D. A. Portnoy.** 1992. Transcriptional activation of the *Listeria monocytogenes* hemolysin gene in *Bacillus subtilis*. J. Bacteriol. **174:**1293–1298.
18. **Gaillard, J. L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55:**2822–2829.
19. **Gaillard, J. L., P. Berche, and P. Sansonetti.** 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **52:**50–55.
20. **Gandhi, A. J., B. Perussia, and H. Goldfine.** 1993. *Listeria monocytogenes* phosphatidylinositol (PI)-specific phospholipase C has low activity on glycosyl-PI-anchored proteins. J. Bacteriol. **75:** 8014–8017.
21. **Geoffroy, C., J.-L. Gaillard, J. E. Alouf, and P. Berche.** 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. Infect. Immun. **55:**1641–1646.
22. **Goldfine, H., and C. Knob.** 1992. Purification and characterization of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C. Infect. Immun. **60:**4059–4067.
23. **Higgins, J. A., B. W. Hitchin, and M. G. Low.** 1989. Phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis* as a probe for the distribution of phosphatidylinositol in hepatocyte membranes. Biochem. J. **259:**913–916.
24. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. J. Cell Biol. **99:**1936–1943.
25. **Karunasagar, I., G. Krohne, and W. Goebel.** 1993. *Listeria ivanovii* is capable of cell-to-cell spread involving actin polymerization. Infect. Immun. **61:**162–169.
26. **Katharíou, S., P. Metz, H. Hof, and W. Goebel.** 1987. Tn*916*-induced mutations in the hemolysin determinant affecting viru-

lence of *Listeria monocytogenes*. J. Bacteriol. **169:**1291–1297.
27. **Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart.** 1992. *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. Cell **68:**521–531.
28. **Kuhn, M., S. Kathariou, and W. Goebel.** 1988. Hemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes*. Infect. Immun. **56:**79–82.
29. **Leimeister-Wächter, M., E. Domann, and T. Chakraborty.** 1991. Detection of a gene encoding a phosphatidylinositol-specific phospholipase C that is co-ordinately expressed with listeriolysin in *Listeria monocytogenes*. Mol. Microbiol. **5:**361–366.
30. **Leimeister-Wächter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty.** 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. Proc. Natl. Acad. Sci. USA **87:**8336–8340.
31. **Low, M. G.** 1989. The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim Biophys. Acta **988:**427–454.
32. **Low, M. G., and A. R. Saltiel.** 1988. Structural and functional roles of glycosyl-phosphatidylinositol in membranes. Science **239:**268–275.
33. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116:**381–406.
34. **Mengaud, J., C. Braun-Breton, and P. Cossart.** 1991. Identification of phosphatidylinositol-specific phospholipase C activity in *Listeria monocytogenes*: a novel type of virulence factor? Mol. Microbiol. **5:**367–372.
35. **Mengaud, J., S. Dramsi, E. Gouin, J. A. Vasquez-Boland, G. Milon, and P. Cossart.** 1991. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. Mol. Microbiol. **5:**2273–2283.
36. **Mengaud, J., C. Geoffroy, and P. Cossart.** 1991. Identification of a new operon involved in *Listeria monocytogenes* virulence: its first gene encodes a protein homologous to bacterial metalloproteases. Infect. Immun. **59:**1043–1049.
37. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Notermans, S. H. W., J. Dufrenne, M. Leimeister-Wächter, E. Domann, and T. Chakraborty.** 1991. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and nonpathogenic *Listeria* species. Appl. Environ. Microbiol. **57:**2666–2670.
39. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:**1263–1267.
40. **Poyart, C., E. Abachin, I. Razafimanantsoa, and P. Berche.** 1993. The zinc metalloprotease of *Listeria monocytogenes* is required for maturation of phosphatidylcholine phospholipase C: direct evidence obtained by gene complementation. Infect. Immun. **61:**1576–1580.
41. **Ralph, P., J. Prichard, and M. Cohn.** 1975. Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. J. Immunol. **114:**898–905.
42. **Raybourne, R. B., and V. K. Bunning.** 1994. Bacterium-host cell interactions at the cellular level: fluorescent labeling of bacteria and analysis of short-term bacterium-phagocyte interaction by flow cytometry. Infect. Immun. **62:**665–672.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Schüller, S.** 1993. M.S. thesis. University of Würzburg, Würzburg, Germany.
45. **Seeliger, H. P. R.** 1988. Listeriosis—history and actual developments. Infection **16:**81–85.
46. **Sibley, L. D., S. G. Franzblau, and J. L. Krahenbuhl.** 1987. Intracellular fate of *Mycobacterium leprae* in normal and activated mouse macrophages. Infect. Immun. **55:**680–685.
47. **Sokolovic, Z., J. Riedel, M. Wuenscher, and W. Goebel.** 1993. Surface-associated, PrfA-regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. Mol. Microbiol. **8:**219–227.
48. **Straley, S. C., and P. A. Harmon.** 1984. *Yersinia pestis* grows within phagolysosomes in mouse peritoneal macrophages. Infect. Immun. **45:**655–659.
49. **Sun, A. N., A. Camilli, and D. A. Portnoy.** 1990. Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. Infect. Immun. **58:**3770–3778.
50. **Swanson, J.** 1989. Fluorescent labeling of endocytic compartments. Methods Cell Biol. **29:**137–151.
51. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell Biol. **109:**1597–1608.
52. **Vasquez-Boland, J., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart.** 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60:**219–230.
53. **Wuenscher, M. D., S. Köhler, W. Goebel, and T. Chakraborty.** 1991. Gene disruption by plasmid integration in *Listeria monocytogenes*: insertional inactivation of the listeriolysin determinant *lisA*. Mol. Gen. Genet. **228:**177–182.