INFECTION AND IMMUNITY, Apr. 1999, p. 1844–1852 Vol. 67, No. 4
0019-9567/99/$04.00+0

Examination of *Listeria monocytogenes* Intracellular Gene Expression by Using the Green Fluorescent Protein of *Aequorea victoria*

NANCY E. FREITAG* AND KATHLEEN E. JACOBS

Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan

Received 7 October 1998/Returned for modification 25 November 1998/Accepted 19 January 1999

The ActA protein of *Listeria monocytogenes* is an essential virulence factor and is required for intracellular bacterial motility and cell-to-cell spread. *plcB*, cotranscribed with *actA*, encodes a broad-specificity phospholipase C that contributes to lysis of host cell vacuoles and cell-to-cell spread. Construction of a transcriptional fusion between *actA-plcB* and the green fluorescent protein gene of *Aequorea victoria* has facilitated the detailed examination of patterns of *actA*/*plcB* expression within infected tissue culture cells. *actA*/*plcB* expression began approximately 30 min postinfection and was dependent upon entry of *L. monocytogenes* into the host cytosol. *L. monocytogenes* Δ*hly* mutants, which are unable to escape from host cell vacuoles, did not express *actA*/*plcB* at detectable levels within infected tissue culture cells; however, complementation of the *hly* defect allowed entry of the bacteria into the host cytoplasm and subsequent *actA*/*plcB* expression. These results emphasize the ability of *L. monocytogenes* to sense the different host cell compartment environments encountered during the course of infection and to regulate virulence gene expression in response.

Listeria monocytogenes is a facultative intracellular bacterial pathogen responsible for serious disease in immunocompromised patients and pregnant women (19, 42). *L. monocytogenes* enters host cells and escapes from membrane-bound vacuoles into the cytoplasm, where it begins to replicate (34, 53). Shortly after entry into the cytosol, the bacteria begin to move and spread to adjacent cells by using a host actin polymerization-based motility (11, 34, 41, 51, 53). Several gene products that are important for intracellular bacterial growth and cell-to-cell spread have been identified and described (reviewed in reference 22, 36, 43, and 46). These gene products include the hemolysin listeriolysin O, encoded by *hly*, which is required for escape of *L. monocytogenes* from host cell vacuoles; ActA, which is essential for actin polymerization-based bacterial motility and cell-to-cell spread; and a broad-range phospholipase C (PC-PLC) encoded by *plcB* that enhances escape from primary and secondary vacuoles (30, 45).

L. monocytogenes is similar to several other intracellular bacterial pathogens, such as *Salmonella typhimurium* (1, 5, 20, 29) and *Legionella pneumophila* (2, 3, 4, 49), in that it possesses gene products that are preferentially expressed within host cells or tissues. Gene products that facilitate escape of *L. monocytogenes* from primary vacuoles (listeriolysin O and *plcA*-encoded phosphatidylinositol-specific phospholipase C) are expressed when bacteria are grown in standard broth culture. However, gene products that contribute to intracellular spread of *L. monocytogenes*, such as those encoded by *mpl*, *actA*, and *plcB*, are expressed at low-to-undetectable levels by bacteria grown in standard broth culture, and expression increases following entry of *L. monocytogenes* into the host cell cytoplasm. As an example, immunoprecipitation experiments using antibodies directed against ActA have indicated that the protein becomes one of the most abundant bacterial surface proteins expressed by intracytoplasmic *L. monocytogenes* (7). *L. monocytogenes* strains that express high levels of *actA* (as well as *hly* and *plcB*) after growth in rich broth at 37°C have been described (for a review, see reference 46), but a study of a wide panel of *L. monocytogenes* isolates has suggested that these hypersecreting strains are variants or mutants (39). Indeed, Ripio et al. (38) have recently demonstrated that several *L. monocytogenes* strains that express elevated levels of virulence factors all carry a point mutation within *prfA* that converts the gene product to an "activated" state. Additional *L. monocytogenes* gene products preferentially expressed within infected mammalian cells have been described (25). These experiments and others suggest that *L. monocytogenes* is capable of sensing the different host cell environments it encounters during the course of infection and responding with the regulated expression of specific virulence gene products.

We are interested in understanding how *L. monocytogenes* regulates gene expression within infected host cells. As an initial step, it seemed important to better define the patterns of *L. monocytogenes* gene expression within infected host cells. This study describes the use of the green fluorescent protein (GFP) reporter gene system from *Aequorea victoria* to monitor *L. monocytogenes* intracellular gene expression. The use of GFP as a reporter has several advantages over other reporter gene systems in that it requires no cofactors and can be used in examination of fixed samples (10). GFP has been used to successfully monitor the expression of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG promoters within macrophages (14), as well as in the isolation of *S. typhimurium* genes preferentially expressed following bacterium-host association (54). We report here the successful use of GFP to monitor the timing and patterns of *L. monocytogenes actA* and *plcB* expression within different host cell compartments of infected tissue culture cells. *actA*/*plcB* expression was evident within 30 min to 1 h postinfection and was dependent upon the ability of *L. monocytogenes* to reach the host cytosol. *actA*/*plcB* did not appear to be expressed by bacteria located within host cell vacuoles. Our results indicate that GFP functions as a useful reporter system for the monitoring of the timing of *L. monocytogenes* intracellular gene expression and for determination of cell compartment expression patterns within infected host cells.

* Corresponding author. Mailing address: Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48201. Phone: (313) 577-1314. Fax: (313) 577-1155. E-mail: nfreitag@med.wayne.edu.

TABLE 1. Bacterial strains used in this study

Strain[a]	Relevant characteristics(s)	Source or reference
10403S	Wild type	18
NF-L357	*actA-gfp-plcB* fusion	This work
DP-L2161	Δ*hly*	24
NF-L404	Δ*hly actA-gfp-plcB* fusion	This work
NF-L411	NF-L404 + *hly*[b]	This work
NF-L497	Δ*hly* + P_{spac}-*gfp*[c]	This work

[a] All strains were derived from 10403S.
[b] *hly* provided by plasmid pDP906 (24).
[c] IPTG-inducible *gfp* plasmid pNF496 (this work).

MATERIALS AND METHODS

Bacterial strains, growth media, and plasmids. The bacterial strains used in this study are listed in Table 1. *L. monocytogenes* 10403S (serotype 1/2b) is resistant to streptomycin, and its 50% lethal dose for mice is 2×10^4 CFU (18). *L. monocytogenes* was stored at −70°C in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) containing 20% glycerol. *Escherichia coli* HB101 or DH5α was used as the host strain for recombinant plasmids. All *E. coli* strains were grown in Luria-Bertani broth (12). Antibiotics were used at the following concentrations: carbenicillin, 50 μg/ml; chloramphenicol, 10 μg/ml.

The thermosensitive shuttle vector plasmid pKSV7 has been described previously (47). Plasmid pSPAC, a shuttle vector containing the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{spac} promoter (56), and plasmid pRAY1, containing the *A. victoria* GFP gene (32), were gifts from David Dubnau. Plasmid pDP906, containing the *hly* gene, has already been described (24).

Construction of *actA-gfp-plcB* transcriptional gene fusion mutants. Primers GFP-1 (5′-GCTCTAGAAGGAGGAAAAATATGAGTAAAGGAGAAGAAC-3′) and GFP-2A (5′-AACTGCAGCTATTTGTATAGTTCATCC-3′) were designed to amplify *gfp* coding sequences from plasmid pRAY1 by PCR (21) and to introduce a gram-positive ribosome binding site derived from SD1 of *ermC* (13) (underlined sequence of GFP-1) upstream of *gfp*. The PCR-amplified product was digested with *Xba*I and *Pst*I and subcloned into pKSV7 (47) to yield pNF320. Primers ActA-1 (5′-GCGAATTCGAGTTGAACGGGAGAGGC-3′) and ActA-2 (5′-GCTCTAGAGTGTTTTTAATTATTTTTTC-3′) were used in conjunction with *L. monocytogenes* genomic DNA to PCR amplify an approximately 760-bp product containing C-terminal sequences of *actA*. The *actA* PCR product was digested with *Eco*RI and *Xba*I and subcloned upstream of *gfp* in plasmid pNF320 to yield plasmid pNF326. Primers PlcB-1 (5′ GCCTGCAGCG AAAGAAAAAGTGAGGT 3′) and PlcB-2 (5′ GCAAGCTTCGGGTAGTCC GCTTTCGC 3′) were used in conjunction with *L. monocytogenes* genomic DNA to PCR amplify an approximately 740-bp fragment containing *plcB* upstream and N-terminal coding sequences. This PCR product was digested with *Pst*I and *Hin*dIII and subcloned downstream of *gfp* in plasmid pNF326 to yield plasmid pNF333. The pNF333 plasmid thus contains a transcriptional fusion of *gfp* to the *actA* gene of *L. monocytogenes*, as well as flanking *L. monocytogenes* chromosomal regions for introduction of the *actA-gfp-plcB* fusion into the *L. monocytogenes* chromosome via homologous recombination.

Construction of plasmid pSPAC-GFP. Primers GFP-1 and GFP-2A (described above) were used to PCR amplify *gfp* from plasmid pRAY1. The purified *gfp* PCR product was digested with *Xba*I and *Pst*I and subcloned into plasmid pSPAC to yield plasmid pSPAC-GFP.

Transfer of *actA-gfp-plcB* transcriptional fusions to the *L. monocytogenes* chromosome. Plasmid pNF333 was introduced into *L. monocytogenes* 10403S and DP-L2161 (24) by electroporation (35), and transformants were isolated by growth at 30°C on BHI agar containing chloramphenicol. *L. monocytogenes* colonies containing the *actA-gfp-plcB* transcriptional fusion in single copy on the bacterial chromosome were isolated by a procedure previously described (9, 17) and designated NF-L357 (*actA-gfp-plcB* in strain 10403S) and NF-L404 (*actA-gfp-plcB* in strain DP-L2161). Southern analysis (40) was used to confirm the existence of the *actA-gfp-plcB* fusion in the correct location and in single copy within the *L. monocytogenes* chromosome.

Transformation of *L. monocytogenes* with pSPAC-GFP and pDP906 plasmids. Plasmid pSPAC-GFP was introduced into *L. monocytogenes* 10403S and DP-L2161, and plasmid pDP906 was introduced into *L. monocytogenes* NF-L404 by electroporation (35), and transformants were isolated by growth at 37°C on BHI agar containing chloramphenicol.

Plaque formation in L2 cells. Plaque assays were carried out as previously described by Sun et al. (48). Plaque size was measured as described by Camilli et al. (9).

Intracellular growth assays. The cell lines used in these studies were the J774 mouse macrophage-like cell line and the potoroo kidney epithelial cell line PtK2; both were maintained as previously described (7, 51). Intracellular growth in J774 cells was monitored as described by Portnoy et al. (37). PtK2 cells were grown as previously described (51) on acid-washed glass coverslips and infected with approximately 2×10^7 CFU of *L. monocytogenes* 10403S or mutant strains for 60 min (multiplicity of infection, ~10 CFU/cell). In experiments designed to examine the intracellular growth and GFP fluorescence of Δ*hly L. monocytogenes* strains, infections of PtK2 cells were carried out with approximately 2×10^9 CFU of bacteria. This high concentration of bacteria resulted in the infection of nearly every PtK2 cell with one to two bacteria and increased the number of intracellular bacteria that could be visualized in each microscopic field. Following infection, cell monolayers were washed three times with 37°C phosphate-buffered saline (PBS), and then 5 ml of prewarmed medium containing 50-μg/ml gentamycin was added. At the indicated time points, coverslips were removed and processed for either bright-field or fluorescence microscopy. For bright-field microscopy, coverslips were stained with Diff-Quik (VWR Scientific, Chicago, Ill.) and mounted in Permount mounting medium (Fisher Scientific, Philadelphia, Pa.). For fluorescence microscopy, the cells were fixed by placing a drop of 3.7% formaldehyde in PBS on each coverslip and incubating it at room temperature for 5 min. The coverslips were then rinsed in PBS and mounted in Permafluor mounting medium (Immunon, Pittsburgh, Pa.) and allowed to set overnight. For experiments using rhodamine phalloidin, following formaldehyde fixation of cells, 1 drop of 0.1% Triton was placed on each of the coverslips, which were then incubated for 3 to 5 min at room temperature. Coverslips were washed by being dipped in PBS, and then 100 μl of 300 mM rhodamine phalloidin in 1-mg/ml bovine serum albumin in PBS was added to each coverslip. After 20 min at room temperature, the coverslips were washed in PBS, drained, and mounted in Permafluor. *L. monocytogenes* containing the *actA-gfp-plcB* reporter gene fusion were visualized in at least 20 separate microscopic fields for each data group. Bacteria were first visualized in a single field by using the fluorescein isothiocyanate (FITC) filter set (see below) and then scored for the presence of F-actin by switching to the rhodamine filter set. The data shown represents at least three independent experiments.

To quantitate percentages of fluorescent bacteria versus the total numbers of intracellular bacteria, PtK2 cells were infected with *L. monocytogenes actA-gfp-plcB* as described above and coverslips were removed at 30 min, 1 h, 1.5 h, and 2 h postinfection and fixed in PBS–3.2% formaldehyde. Coverslips were washed with PBS, and then 1 drop of PTB (0.1% Triton, 1-mg/ml bovine serum albumin in PBS) with a 1:320 dilution of *Listeria* O rabbit antiserum (Difco, Detroit, Mich.) was added and the coverslips were incubated at room temperature for 30 min. After washing, 1 drop of tetramethylrhodamine-conjugated goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, Oreg.) at 5 μg/ml in PTB was added and the coverslips were incubated at room temperature for an additional 30 min. Coverslips were then washed in PTB and mounted in Permafluor. The relative percentage of intracellular versus total bacteria for each time point was determined by removing additional coverslips from each dish of infected cells at each time point and following the procedure described above with the omission of 0.1% Triton. Bacteria were counted in at least 50 different microscopic fields for each time point, and the number of intracellular *L. monocytogenes* bacteria (total number of bacteria times the percentage of intracellular bacteria) that were positive for GFP fluorescence was scored.

IPTG-mediated induction of *gfp* expression in PtK2 cells infected with NF-L497. PtK2 cells were grown as previously described (51) on acid-washed glass coverslips and infected with approximately 10^9 CFU of *L. monocytogenes* NF-L497 for 60 min. Monolayers were washed three times with 37°C PBS, and then 5 ml of prewarmed medium containing 50-μg/ml gentamicin was added. After an additional 60 min, IPTG was added to a final concentration of 2 mM. At the indicated time points, coverslips were removed and processed for either bright-field or fluorescence microscopy.

Fluorescence microscopy and imaging. Cells were observed by using a Zeiss Axiophot fluorescence microscope coupled to either a Diagnostic Instruments SPOT Digital Camera or a DAGE/MTI SIT camera. The filter sets used were Zeiss filter set no. 487915 for monitoring of rhodamine fluorescence and Zeiss FITC filter set no. 487910 with the substitution of an Omega short-pass emission filter (530 nm ± 20 nm, used to reduce rhodamine bleedthrough) for monitoring of GFP fluorescence.

RESULTS

Construction of *L. monocytogenes* chromosomal *actA-gfp-plcB* transcriptional fusion mutants. The PCR was used to amplify the *A. victoria gfp* gene (10) with the addition of a gram-positive ribosome binding site. A transcriptional fusion between *actA* and *gfp* was constructed in plasmid pKSV7 (47) and introduced into the *L. monocytogenes* chromosome via homologous recombination as described in Materials and Methods (Fig. 1). Two *L. monocytogenes* strains were used as recipients of the *actA-gfp* fusion: 10403S, the wild type strain, to generate NF-L357, and DP-L2161, a strain derived from 10403S that contains an *hly* deletion (24), to generate NF-L404. NF-L357 and NF-L404 each contain a single copy of a promoterless *gfp* gene located between *actA* and *plcB* in the *L. monocytogenes* chromosome.

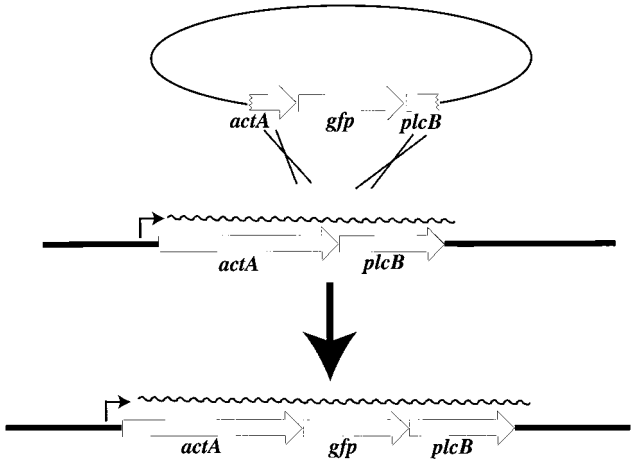

FIG. 1. Construction of an *actA-gfp-plcB* transcriptional gene fusion in *L. monocytogenes*. Expression of *actA* and *plcB* is transcriptionally coupled. A promoterless copy of *gfp* was inserted between the *actA* and *plcB* coding regions and then introduced into the *L. monocytogenes* chromosome via homologous recombination.

The *actA-gfp-plcB* transcriptional fusion does not affect intracellular bacterial growth or cell-to-cell spread. The capacity of *L. monocytogenes* to escape from a vacuole, replicate intracellularly, and spread to adjacent cells can be measured by the ability of the bacteria to form plaques in monolayers of mouse L cells. *L. monocytogenes* mutants lacking functional ActA or PC-PLC can be identified by their inability to form wild-type size plaques (26, 45, 55). We examined the plaque-forming ability of each of the *L. monocytogenes actA-gfp-plcB* fusion strains (Fig. 2). NF-L357 (Fig. 2a), which contains the *actA-gfp-plcB* chromosomal fusion in a wild-type background, formed plaques of the same approximate size and frequency as the parent strain (Fig. 2b). NF-L404, which contains a deletion within *hly* and does not escape from host cell vacuoles, did not form visible plaques in L2 cell monolayers (Fig. 2d). Complementation of NF-L404 by the introduction of plasmid-encoded *hly* (NF-L411) restored the ability of this strain to form plaques; however, the plaques were smaller than those formed by wild-type *L. monocytogenes* (Fig. 2c). The inability of *hly* on a plasmid to completely restore plaque size may result from the fact that the *hly* promoter is present in multiple copies and results in titration of PrfA, the transcriptional activator required for expression of several *L. monocytogenes* virulence genes (9, 24).

Intracellular growth of the *actA-gfp-plcB* fusion strains was also examined in the mouse macrophage-like cell line J774 and in the potoroo kidney epithelial cell line PtK2, as well as in mouse bone marrow-derived macrophages (23). Normal growth and spread were observed for NF-L357, whereas the NF-404 Δ*hly actA-gfp-plcB* strain failed to replicate intracellularly. The results of the plaque assay and tissue culture infection experiments indicate that the introduction of *gfp* into the *L. monocytogenes* chromosome between *actA* and *plcB* produced no discernible effect on intracellular growth or cell-to-cell spread of the bacteria.

Characterization of intracellular *L. monocytogenes actA-gfp-plcB* expression by fluorescence in tissue culture cell lines. *L. monocytogenes* mutants containing the *actA-gfp-plcB* chromosomal fusion were examined by fluorescence microscopy following infection of mouse macrophage-like cell line J774 (Fig. 3). Fluorescent bacteria of strain NF-L357 were detectable between 30 min and 1 h postinfection. The numbers of fluorescent bacteria and the levels of fluorescence observed increased as the bacteria multiplied and spread to adjacent cells (Fig. 3, middle panels). No bacterial fluorescence was detected in cells infected with the 10403S parent strain at any time point postinfection (Fig. 3, bottom panel).

We also investigated infections of PtK2 cells, a potoroo kidney epithelial cell line that is relatively flat. Fluorescent bacteria were more easily visualized in this cell line (in comparison to J774 cells) with less background contributed from surrounding planes of focus. Fluorescent bacteria were initially detected beginning at approximately 30 min to 1 h postinfection. At the earliest time point examined, 30 min postinfection, approximately 80% of the intracellular NF-L357 bacteria were expressing *actA-gfp*, and by 2 h, at least 95% of the intracellular NF-L357 bacteria were scored as GFP positive (23). To obtain a functional correlation of *actA* expression with the expression patterns detected via *actA*-dependent GFP fluorescence, infected cells were permeabilized and stained with rhodamine phalloidin to allow detection of F-actin filaments accumulating around intracytoplasmic bacteria (Fig. 4 and Table 2). With a multiplicity of infection of approximately 10 CFU per cell, at 1.5 h postinfection *L. monocytogenes* bacteria expressing GFP are visible but detection of bacteria requires examination of large numbers of infected cells. Approximately 80% of the bacteria detected are expressing GFP in the absence of F-actin staining. The number of bacteria that express GFP and colocalize with F-actin increases steadily, such that at 2.5 h postinfection, over 70% colocalize, and at 4 h postinfection, greater than 90% of the bacteria colocalize with F-actin as detected by fluorescence staining. At 6 h postinfection, bacteria could be clearly seen either surrounded by actin filaments or followed by actin filament comet tails (Fig. 4C). These results indicated that *actA* expression, as detected by GFP fluorescence, preceded the accumulation of F-actin around intracytoplasmic

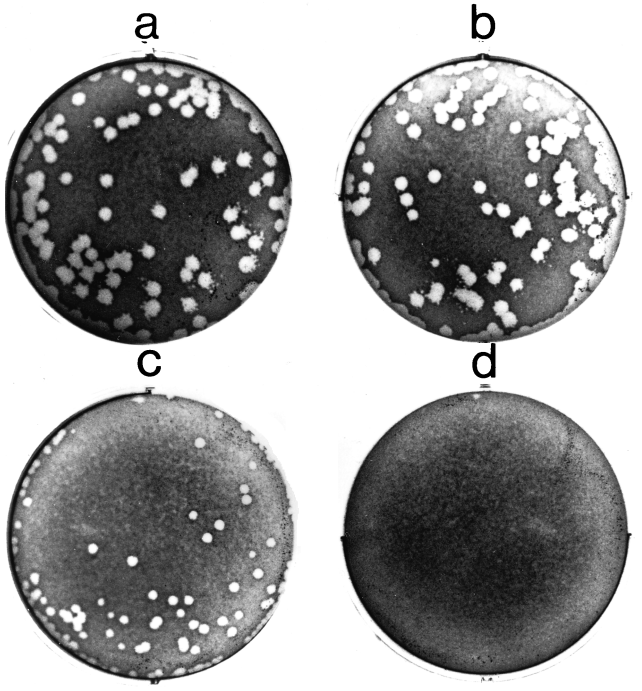

FIG. 2. Plaque assay of intracellular growth and cell-to-cell spread of *L. monocytogenes* strains containing *actA-gfp-plcB* fusions. Confluent monolayers of mouse L2 fibroblast cells were infected with the indicated *L. monocytogenes* strain and stained after 3 days with neutral red. Panels: a, wild-type 10403S; b, NF-L357; c, NF-L411; d, NF-L404.

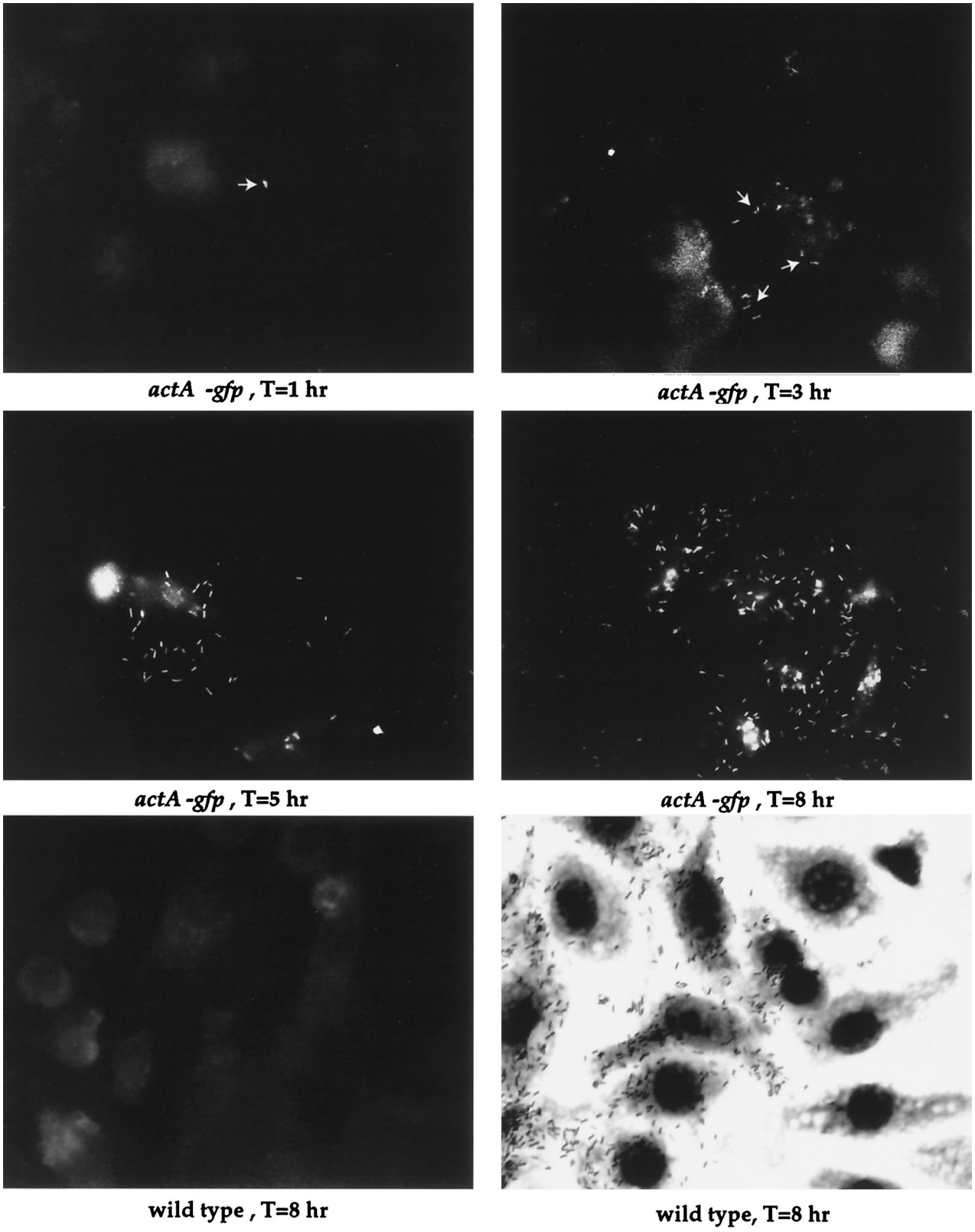

FIG. 3. Intracellular fluorescence of NF-L357 in mouse macrophage-like cell line J774. Monolayers of J774 cells were grown on glass coverslips and infected with either NF-L357 or the wild-type 10403S strain as indicated. Coverslips were removed at the indicated time points and fixed prior to fluorescence microscopy. T, time postinfection.

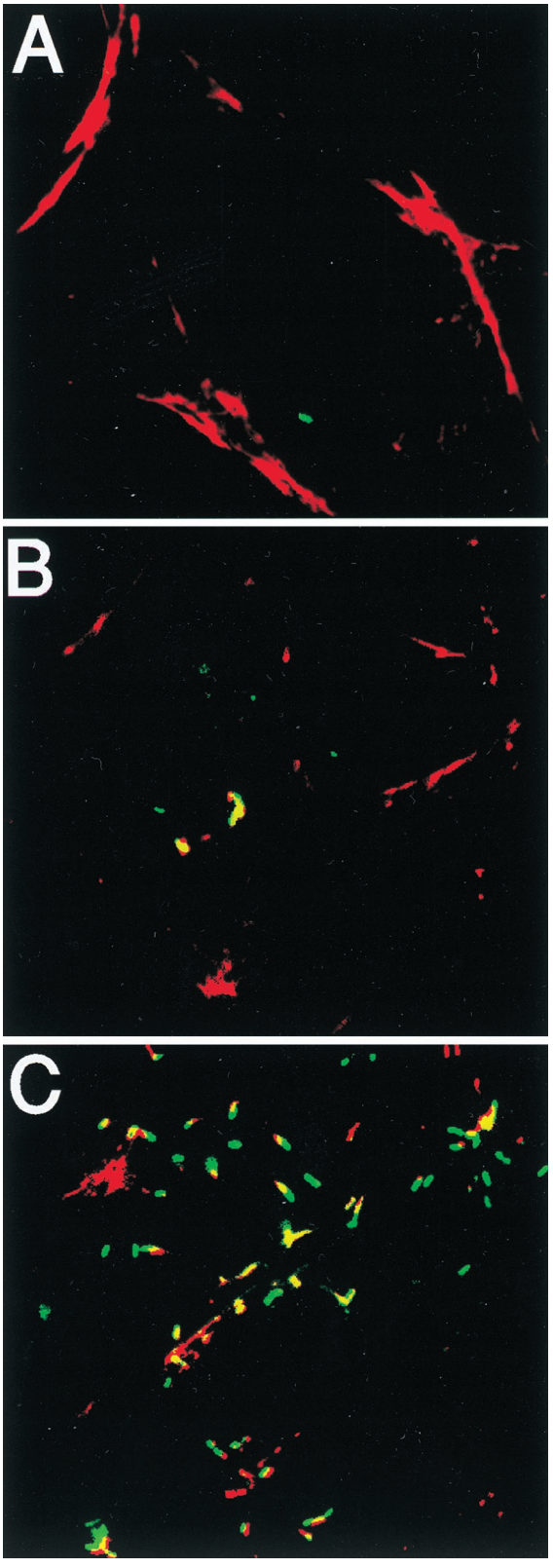

FIG. 4. Intracellular fluorescence and F-actin staining of *L. monocytogenes*-infected PtK2 cells. Monolayers of PtK2 cells were grown on coverslips and infected with the NF-L357 *actA-gfp-plcB* fusion strain. Coverslips were removed at 3 h postinfection (A), 3 h postinfection (B) (an independent field adjacent to that shown in panel A), and 6 h postinfection (C) and treated with rhodamine phalloidin to identify F-actin. The yellow areas indicate overlapping fluorescence of GFP (green fluorescence) and F-actin (red fluorescence).

TABLE 2. Quantitation of GFP expression versus F-actin staining of *L. monocytogenes actA-gfp-plcB* mutants following infection of PtK2 cells

Expt no. and time (h) post-infection	No. of GFP-positive bacteria[a]	No. of GFP- and F-actin- positive bacteria[b]	% GFP$^+$ and:	
			F-actin$^-$	F-actin$^+$
1				
1.5	26	2	92	8
2.0	34	8	76	24
2.5	61	50	18	82
4.0	65	63	3	97
2				
1.5	15	3	80	20
2.0	50	17	66	34
2.5	59	42	29	71
4.0	60	53	12	88
3				
1.5	63	17	73	27
2.0	62	27	56	44
2.5	57	38	34	66
4.0	55	51	7	93
Mean ± SD[c]				
1.5			82 ± 7	18 ± 7
2.0			66 ± 7	34 ± 7
2.5			27 ± 6	73 ± 6
4.0			7 ± 3	93 ± 3

[a] Number of intracellular *L. monocytogenes* bacteria that scored positive for GFP expression following examination of infected cells by fluorescence microscopy. Numbers are taken from at least 20 different microscopic fields equivalent in size.

[b] Number of intracellular *L. monocytogenes* bacteria that scored positive for GFP expression and F-actin accumulation following examination of cells by fluorescence microscopy. Numbers are taken from at least 20 different microscopic fields equivalent in size.

[c] Mean values derived from three individual experiments are shown. Similar results were obtained from two additional independent experiments (data not shown).

bacteria. *actA/gfp/plcB* expression patterns therefore closely correlated with the functional appearance of ActA protein.

***L. monocytogenes actA/plcB* expression is influenced by intracellular compartment location.** *L. monocytogenes* encounters several different host cell compartment environments during the course of infection, and these environments are thought to influence bacterial gene expression. As mentioned previously, immunoprecipitation experiments have demonstrated that ActA becomes one of the dominant surface proteins expressed by *L. monocytogenes* in the cell cytoplasm (7). Dietrich et al. (15) have recently reported that the *actA* promoter is preferentially activated in the cytosol of the infected host cell; however, it has not been definitively shown whether *actA* transcription occurs while *L. monocytogenes* resides within host cell phagosomes or if expression is absolutely dependent upon entry of the bacteria into the cytoplasm. *L. monocytogenes* NF-L404, which contains the *actA-gfp-plcB* transcriptional fusion in a Δ*hly* background (24), was used to examine *actA/plcB* expression by bacteria trapped within host primary vacuoles. When PtK2 cells were infected with the *L. monocytogenes* Δ*hly actA-gfp-plcB* fusion mutant, no fluorescence was detected at any time point postinfection (Fig. 5B). However, when the *hly* defect of this mutant was complemented by the introduction of plasmid-encoded *hly*, bacteria were able to escape from host vacuoles and multiply within the cytoplasm, where *actA/gfp/plcB* expression was readily detectable (Fig. 5D). These results indicate that *actA/plcB* expression does not occur while bacte-

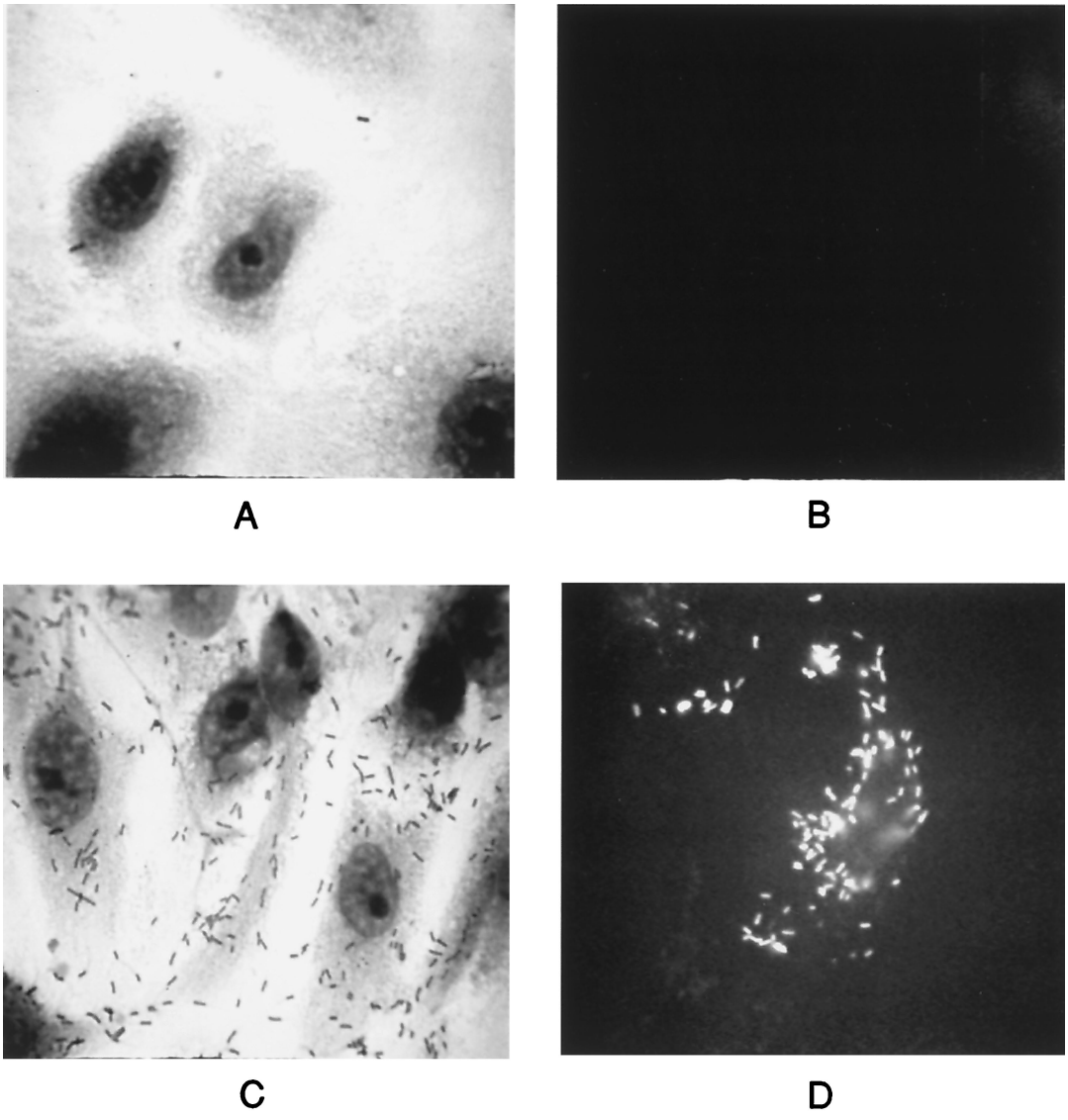

FIG. 5. *L. monocytogenes actA/plcB* is not expressed in primary host cell vacuoles. PtK2 cells were infected with NF-L404 (Δ*hly actA-gfp*; A and B) or NF-L411 (NF-L404 plus plasmid-encoded *hly*; C and D). Infected cells were fixed and stained (A and C) or examined for fluorescence (B and D) at 8 h postinfection. At least 50 microscopic fields were examined per experiment; one of three experiments with similar results is shown.

ria are located within host cell primary vacuoles (or occurs at low levels not detectable by this assay) and suggest that expression is triggered when the bacteria encounter a specific target host cell environment such as the cytosol.

To verify that gene expression occurring in bacteria located within primary vacuoles would be detectable in our system using GFP as a reporter, *L. monocytogenes* Δ*hly* mutants were transformed with plasmid pNF496, which contains *gfp* under the control of the IPTG-inducible P_{spac} promoter (56), to generate NF-L497. PtK2 cells were infected for 1 h with NF-L497 in the absence of IPTG induction. Infected cells were then washed, and gentamicin was added to kill any remaining extracellular bacteria. Following 1 h of gentamicin treatment, IPTG was added at a final concentration of 2 mM. Under these conditions, *gfp* expression should be induced only in the viable *L. monocytogenes* bacteria that were located within host cell vacuoles and that were thereby protected from gentamicin exposure. After 4 h of IPTG induction, the PtK2 cells were fixed and examined for fluorescent bacteria. Fluorescent bacteria located within host cell vacuoles were easily detectable (Fig. 6A and B), although it should be noted that these bacteria represented a relatively small percentage (approximately 10%) of the intracellular *L. monocytogenes*. The small numbers of fluorescent bacteria detected may be due in part to the requirement for IPTG to diffuse into *L. monocytogenes*-containing vacuoles at sufficient concentrations for induction of gene expression. These experiments demonstrate that it was therefore possible to observe GFP-mediated fluorescence from individual bacteria located within host vacuoles provided that *gfp* expression was occurring.

DISCUSSION

It has been possible to define in detail the patterns of *L. monocytogenes actA-plcB* expression within infected host cells through the construction of transcriptional gene fusions with the *gfp* gene of *A. victoria*. Observation of infected tissue culture cells by fluorescence microscopy allowed determination of both the

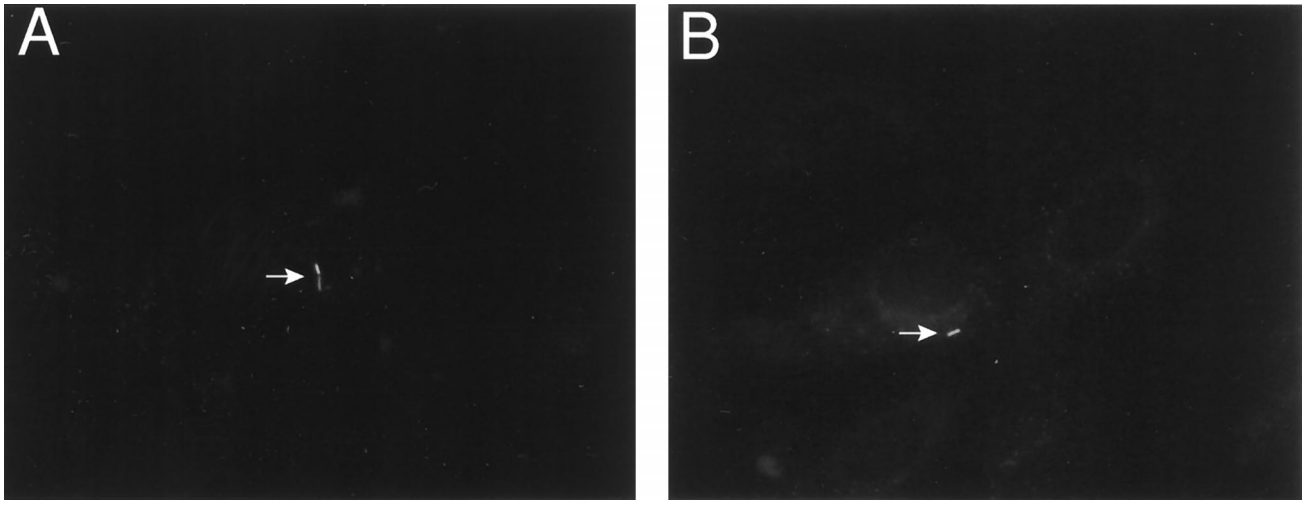

FIG. 6. Induced expression of *gfp* in *L. monocytogenes* located within primary host cell vacuoles. NF-L497, a Δ*hly* strain that contains a plasmid encoding an IPTG-inducible copy of *gfp*, was used in infection assays of PtK2 cells. At 1 h postinfection, monolayers were washed and treated with gentamicin to kill extracellular bacteria. At 2 h postinfection, 2 mM IPTG was added to induce *gfp* expression. Cells were fixed and examined for fluorescent bacteria at 4 h postinfection. White arrows identify fluorescent bacteria located within host cell vacuoles. At least 50 microscopic fields were examined per experiment; one of two experiments with similar results is shown.

timing of *actA-plcB* expression and the intracellular location at which expression occurred. Such information should prove useful in gaining a better understanding of how *L. monocytogenes* responds to the different host cell compartment environments it encounters during the course of infection.

ActA and PC-PLC are critical components of *L. monocytogenes* pathogenesis. *L. monocytogenes* mutants that contain insertions or in-frame deletions within *actA* invade host cells and multiply within the cytoplasm but do not spread to adjacent cells and are 1,000-fold less virulent than wild-type bacteria in a mouse model of infection (7, 16, 26). ActA-dependent actin polymerization by *L. monocytogenes* has been examined in many cell types (7, 11, 16, 26–28, 51–53), as well as in cell extracts (50). Examination of the precise function and sites of action of PC-PLC has been more difficult. *plcB* deletion mutants are approximately 10-fold less virulent in mouse models of infection (45). PC-PLC is secreted as an inactive proenzyme; the activated form has recently been shown to localize within Lamp1-positive vacuoles thought to be formed during bacterial cell-to-cell spread (31). Our results suggest that activation of *actA*/*plcB* expression occurs shortly after *L. monocytogenes* gains access to the host cell cytosol. Timing of *actA-plcB* expression as monitored by GFP fluorescence correlated well with the detection of actin filaments surrounding the bacteria in our studies and agreed with previous actin filament data obtained in other laboratories (11, 26–28, 53). Preliminary data obtained at early time points postinfection suggests that *actA*/*plcB* expression may occur more rapidly in phagocytic cells, such as J774 cells, than in nonprofessional phagocytic cells such as PtK2 cells (23). The delay in *actA*/*plcB* expression observed in PtK2 cells may reflect the time needed for the bacteria to actively invade the host cell; alternatively, the timing of bacterial escape from the host cell vacuoles may vary between cell types.

By utilizing previously constructed and characterized *L. monocytogenes* Δ*hly* mutants (24), it was possible to demonstrate that *actA*/*plcB* does not appear to be expressed by bacteria located within host cell vacuoles. Fluorescence was observed for Δ*hly* mutants containing plasmid-encoded *gfp* under the control of an inducible promoter, indicating that fluorescence occurring within a single bacterium located in a vacuole could be detected. The IPTG-inducible P_{spac} promoter (56) is not a strong promoter in *L. monocytogenes*, thus, we believe that GFP fluorescence provides a relatively sensitive indicator of gene expression. However, previous experiments have demonstrated that PC-PLC is capable of mediating lysis of the primary vacuole in Henle 407 human epithelial cells (30), indicating that *plcB* expression does, in fact, occur in this environment. *L. monocytogenes* infections of Henle 407 cells are unusual in that bacterial lysis of the primary vacuole does not require the action of *hly*-encoded listeriolysin O (30, 37). It is unclear whether the primary vacuole of Henle 407 cells presents a unique environment in which *actA*/*plcB* expression occurs or whether Henle 407 primary vacuolar membranes are more sensitive to the action of the low levels of PC-PLC that are produced. No fluorescence of the Δ*hly actA-gfp-plcB* strain was detected in PtK2 cells up to 5 h postinfection, suggesting that if low levels of expression were occurring, there was no accumulation of GFP. Complementation of the *hly* defect in the *actA-gfp-plcB* background resulted in brightly fluorescent bacteria following *L. monocytogenes* entry into the cytosol. These data strongly suggest that *L. monocytogenes* intracellular gene expression is dependent upon the bacteria encountering and sensing the appropriate host cell compartment environment and serve to illustrate the level and degree of intracellular gene regulation used by the bacteria during the course of infection.

ActA is reportedly one of the most abundant bacterial surface proteins expressed by *L. monocytogenes* in the host cell cytoplasm (6, 7). Several conditions have been reported to induce *actA-plcB* expression in extracellularly grown cultures of *L. monocytogenes* (6, 39, 44); however, the levels of NF-L357 fluorescence observed under any of these conditions appeared to be much lower than those of intracellular bacteria (23). Moors et al. (33) have recently described a *lacZ*/*cat* reporter gene system that can be used to monitor extracellular and intracellular *L. monocytogenes* gene expression, and their results indicate that *actA* is highly (226-fold) induced in infected host cells. We are in the process of devising similar ways to accurately quantitate differences observed in levels of *actA*/*plcB* expression, and our results thus far indicate that extracellular

conditions have not yet been defined that result in levels of *actA*/*plcB* expression equivalent to those observed for intracellular bacteria (23).

The use of GFP to monitor *L. monocytogenes* intracellular gene expression presents several advantages over previous approaches. It provides a sensitive system that allows the monitoring of expression patterns of individual bacteria within an infected cell. No addition of cofactors is required; thus, gene expression that occurs within membrane-bound host cell compartments can be monitored without the complications of substrate diffusion. *gfp* fusions present in single copies on the bacterial chromosome eliminate potential artifacts resulting from multicopy plasmid-based experiments. Finally, no discernible effect on bacterial growth or viability was observed for *L. monocytogenes gfp* fusion mutants; thus, the patterns of gene expression observed are probably more reflective of the natural environment than are expression patterns obtained by using mutants with decreased viability (8).

Our results indicate that GFP provides a useful system for monitoring of the patterns of *L. monocytogenes* intracellular gene expression. We are in the process of constructing additional fusions of *gfp* to other *L. monocytogenes* genes important for intracellular growth and/or cell-to-cell spread, and it is hoped that examination of these fusion mutants will provide a more detailed understanding of host cell compartment influences on *L. monocytogenes* virulence gene regulation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI41816 from the National Institutes of Health. N.E.F. thanks the Public Health Research Institute (New York, N.Y.) for initial support and encouragement.

We thank David Dubnau and Daniel Portnoy for gifts of plasmids and bacterial strains and for many helpful discussions. We thank Vojo Deretic for helpful suggestions regarding the preparation of GFP samples for fluorescence microscopy.

REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. J. Bacteriol. **175:**3734–3743.
2. **Abu Kwaik, Y.** 1998. Induced expression of the *Legionella pneumophila* gene encoding a 20-kilodalton protein during intracellular infection. Infect. Immun. **66:**203–212.
3. **Abu Kwaik, Y., B. I. Eisenstein, and N. C. Engleberg.** 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. Infect. Immun. **61:**1320–1329.
4. **Abu Kwaik, Y., and L. L. Pederson.** 1996. The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. Mol. Microbiol. **21:**543–556.
5. **Baumler, A. J., J. G. Kusters, I. Stojiljkovic, and F. Heffron.** 1994. *Salmonella typhimurium* loci involved in survival within macrophages. Infect. Immun. **62:**1623–1630.
6. **Bohne, J., Z. Sokolovic, and W. Goebel.** 1994. Transcriptional regulation of *prfA* and PrfA-regulated virulence genes in *Listeria monocytogenes*. Mol. Microbiol. **11:**1141–1150.
7. **Brundage, R. A., G. A. Smith, A. Camilli, J. A. Theriot, and D. A. Portnoy.** 1993. Expression and phosphorylation of the *Listeria monocytogenes* ActA protein in mammalian cells. Proc. Natl. Acad. Sci. USA **90:**11890–11894.
8. **Bubert, A., H. Kestler, M. Gotz, R. Bockmann, and W. Goebel.** 1997. The *Listeria monocytogenes iap* gene as an indicator gene for the study of PrfA-dependent regulation. Mol. Gen. Genet. **256:**54–62.
9. **Camilli, A., L. G. Tilney, and D. A. Portnoy.** 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. **8:**143–157.
10. **Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher.** 1994. Green fluorescent protein as a marker for gene expression. Science **263:**802–805.
11. **Dabiri, G. A., J. M. Sanger, D. A. Portnoy, and F. S. Southwick.** 1990. *Listeria monocytogenes* moves rapidly through the host cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA **87:**6068–6072.
12. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Denoya, C. D., D. H. Bechhofer, and D. Dubnau.** 1986. Translational autoregulation of *ermC* 23S rRNA methyltransferase expression in *Bacillus subtilis*. J. Bacteriol. **168:**1133–1141.
14. **Dhandayuthapani, S., L. E. Via, C. A. Thomas, P. M. Horowitz, D. Deretic, and V. Deretic.** 1995. Green fluorescent protein as a marker for gene expression and cell biology of mycobacterial interactions with macrophages. Mol. Microbiol. **17:**901–912.
15. **Dietrich, G., A. Bubert, I. Gentschev, Z. Sokolovic, A. Simm, A. Catic, S. H. E. Kaufmann, J. Hess, A. A. Szalay, and W. Goebel.** 1998. Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide *Listeria monocytogenes*. Nat. Biotechnol. **16:**181–185.
16. **Domann, E., J. Wehland, M. Rohde, S. Pistor, M. Hartl, W. Goebel, M. Leimeister-Wachter, M. Wuenscher, and T. Chakraborty.** 1992. A novel bacterial gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J. **11:**1981–1990.
17. **Freitag, N. E., and D. A. Portnoy.** 1994. Dual promoters of the *Listeria monocytogenes prfA* transcriptional activator appear essential in vitro but are redundant in vivo. Mol. Microbiol. **12:**845–853.
18. **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.
19. **Gray, M. L., and A. H. Killinger.** 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. **30:**309–382.
20. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400–403.
21. **Innis, M. A., and D. H. Gelfand.** 1990. Optimization of PCRs, p. 3–12. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. A guide to methods and applications. Academic Press, Inc., San Diego, Calif.
22. **Ireton, K., and P. Cossart.** 1997. Host-pathogen interactions during entry and actin-based movement of *Listeria monocytogenes*. Annu. Rev. Genet. **31:**113–138.
23. **Jacobs, K. E., and N. E. Freitag.** Unpublished data.
24. **Jones, S., and D. A. Portnoy.** 1994. Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. Infect. Immun. **62:**5608–5613.
25. **Klarsfeld, A., P. L. Goossens, and P. Cossart.** 1994. Five *Listeria monocytogenes* genes preferentially expressed in infected mammalian cells: *plcA*, *purH*, *purD*, *pyrE* and an arginine ABC transporter gene, *arpJ*. Mol. Microbiol. **13:**585–597.
26. **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.
27. **Kocks, C., R. Hellio, P. Gounon, H. Ohayon, and P. Cossart.** 1993. Polarized distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. J. Cell Sci. **105:**699–710.
28. **Kuhn, M., M. C. Prevost, J. Mounier, and P. J. Sansonetti.** 1990. A nonvirulent mutant of *Listeria monocytogenes* does not move intracellularly but still induces polymerization of actin. Infect. Immun. **58:**3477–3486.
29. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.
30. **Marquis, H., V. Doshi, and D. A. Portnoy.** 1995. The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells. Infect. Immun. **63:**4531–4534.
31. **Marquis, H., H. Goldfine, and D. A. Portnoy.** 1997. Proteolytic pathways of activation and degradation of a bacterial phospholipase C during intracellular infection by *Listeria monocytogenes*. J. Cell Biol. **137:**1381–1392.
32. **Marshall, J., R. Molloy, G. W. Moss, J. R. Howe, and T. W. Hughes.** 1995. The jellyfish green fluorescent protein: a new tool for studying ion channel expression and function. Neuron **14:**211–215.
33. **Moors, M. A., B. Levitt, P. Youngman, and D. A. Portnoy.** 1999. Expression of listeriolysin O and ActA by intracellular and extracellular *Listeria monocytogenes*. Infect. Immun. **67:**131–139.
34. **Mounier, J., A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. Infect. Immun. **58:**1048–1058.
35. **Park, S. F., and G. S. A. B. Stewart.** 1990. High-efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. Gene **94:**129–132.
36. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:**1263–1267.
37. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167:**1459–1471.
38. **Ripio, M.-T., G. Dominguez-Bernal, M. Lara, M. Suarez, and J.-A. Vazquez-Boland.** 1997. A Gly145Ser substitution in the transcriptional activator PrfA

causes constitutive overexpression of virulence factors in *Listeria monocytogenes*. J. Bacteriol. **179:**1533–1540.
39. **Ripio, M. T., G. Dominguez-Bernal, M. Suarez, K. Brehm, P. Berche, and J. A. Vazquez-Boland.** 1996. Transcriptional activation of virulence genes in wild-type strains of *Listeria monocytogenes* in response to a change in the extracellular medium composition. Res. Microbiol. **147:**371–384.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Sanger, J. M., B. Mittal, F. S. Southwick, and J. W. Sanger.** 1990. Analysis of intracellular motility and actin polymerization induced by *Listeria monocytogenes* in PtK2 cells. J. Cell Biol. **111:**415A.
42. **Seeliger, H. P. R.** 1988. Listeriosis—history and actual developments. Infection **16:**81–85.
43. **Sheehan, B., C. Kocks, S. Dramsi, E. Gouin, A. D. Klarsfeld, J. Mengaud, and P. Cossart.** 1994. Molecular and genetic determinants of the Listeria monocytogenes infectious process. Curr. Top. Microbiol. Immunol. **192:**187–216.
44. **Shen, H., M. K. Slifka, M. Matloubian, E. R. Jensen, R. Ahmed, and J. F. Miller.** 1995. Recombinant *Listeria monocytogenes* as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity. Proc. Natl. Acad. Sci. USA **92:**3987–3991.
45. **Smith, G. A., H. Marquis, S. Jones, N. C. Johnston, D. A. Portnoy, and H. Goldfine.** 1995. The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. Infect. Immun. **63:**4231–4237.
46. **Smith, G. A., and D. A. Portnoy.** 1997. How the *Listeria monocytogenes* ActA protein converts actin polymerization into a motile force. Trends Microbiol. **5:**272–276.
47. **Smith, K., and P. Youngman.** 1992. Use of a new integrational vector to investigate compartment-specific expression of the *Bacillus subtilis spoIIM* gene. Biochimie **74:**705–711.
48. **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.
49. **Susa, M., J. Hacker, and R. Marre.** 1996. De novo synthesis of *Legionella pneumophila* antigens during intracellular growth in phagocytic cells. Infect. Immun. **64:**1679–1684.
50. **Theriot, J., J. Rosenblatt, D. A. Portnoy, P. J. Goldschmidt-Clermont, and T. Mitchison.** 1994. Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and cell-free extracts. Cell **76:**505–517.
51. **Theriot, J. A., T. J. Mitchison, L. G. Tilney, and D. A. Portnoy.** 1992. The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. Nature **357:**257–260.
52. **Tilney, L. G., P. S. Connelly, and D. A. Portnoy.** 1990. The nucleation of actin filaments by the bacterial intracellular pathogen, *Listeria monocytogenes*. J. Cell Biol. **111:**2979–2988.
53. **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.
54. **Valdivia, R. H., and S. Falkow.** 1997. Fluorescence-based isolation of bacterial genes expressed within host cells. Science **277:**2007–2011.
55. **Vazquez-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.
56. **Yansura, D. G., and D. J. Henner.** 1984. Use of the *Escherichia coli* lac repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **81:**439–443.

Editor: D. L. Burns