INFECTION AND IMMUNITY, June 1993, p. 2537–2544
0019-9567/93/062537-08$02.00/0

Vol. 61, No. 6

Regulation of the *prfA* Transcriptional Activator of *Listeria monocytogenes*: Multiple Promoter Elements Contribute to Intracellular Growth and Cell-to-Cell Spread

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Received 23 December 1992/Accepted 15 March 1993

The *prfA* gene product is a transcriptional activator of *Listeria monocytogenes* determinants of pathogenicity. In this study, we provide direct evidence that the PrfA protein is a site-specific DNA-binding protein. Additionally, we describe the characterization of two classes of *L. monocytogenes* mutants which contain transposon insertions either in the *prfA* structural gene (exemplified by strain DP-L1075) or within the *prfA* promoter region (exemplified by strain DP-L973). Both mutants are completely avirulent and secrete greatly reduced levels of listeriolysin O and phosphatidylinositol-specific phospholipase C, and both are fully complemented by the introduction of *prfA* on a multicopy plasmid. The behaviors of the two mutants differ markedly within cultured macrophages. Following infection, no cytoplasmic growth was observed for DP-L1075 whereas DP-L973 escaped from the phagosome and grew in the cell cytoplasm. However, DP-L973 was defective in nucleation of actin filaments and spread to adjacent cells. Transcription of *prfA* in DP-L973 was directed from a single, previously unidentified promoter (*prfAp*$_2$) located close to the *prfA* initiation codon. This promoter is therefore capable of providing sufficient *prfA* expression for escape from the host cell vacuole but is insufficient for wild-type levels of bacterially induced actin polymerization and cell-to-cell spread. Transcription directed from both *prfAp*$_1$ and *prfAp*$_2$ promoters was increased in the absence of a functional *prfA* gene product, suggesting that PrfA protein contributes to down-regulating its own expression.

The facultative intracellular bacterium *Listeria monocytogenes*, a gram-positive food-borne human pathogen, successfully inhabits a variety of environments including contaminated foods, the gut, and the intracellular environment of the mammalian cell (13, 17). Once it has entered the cell, *L. monocytogenes* is found initially within a host vacuole and subsequently directly in the cytoplasm (16, 23, 34). How *L. monocytogenes* recognizes and responds to these diverse environmental conditions remains unknown. A number of *L. monocytogenes* gene products which contribute to the infectious process have recently been identified (reviewed in reference 25); one of these is a transcriptional activator encoded by *prfA* (9, 15, 20, 21). *L. monocytogenes* mutants lacking a functional PrfA protein are avirulent and have greatly reduced transcript levels of at least five genes which contribute to *L. monocytogenes* pathogenesis (9, 20, 21). The PrfA protein has been demonstrated to increase the transcription of a target *hly-lacZ* promoter fusion more than 100-fold in a heterologous *Bacillus subtilis*-based expression system (15). Although sequence comparisons have identified no significant homologies (20, 21), the PrfA protein does contain a region which resembles the helix-turn-helix motif found in many DNA-binding proteins (15).

Two transcripts that direct the expression of *prfA* have been identified by Northern (RNA) analysis; one originates from a promoter located within the 5′ upstream region immediately adjacent to *prfA* coding sequences, and the second originates from the upstream *plcA* promoter (19, 21). In the latter case, transcription continues through a putative Rho-independent terminator-like structure at the 3′ end of the *plcA* gene and generates a transcript that contains both *plcA* and *prfA*. There is strong evidence that this readthrough transcript is required for wild-type levels of cell-to-cell spread (8). Transcription of *prfA* is regulated during the growth phase and is thermoregulated, and *prfA* positively regulates its own synthesis via activation of *plcA* transcription (19, 21). In the present study, we extend this work by identifying an additional promoter which contributes to *prfA* expression and provide evidence that *prfA* also contributes to down-regulating its own expression. We further characterize two classes of *prfA* transposon-insertion mutants which differ markedly in their abilities to escape from the host cell vacuole and grow within the host cell cytoplasm. In addition, we provide the first demonstration that PrfA protein is indeed a site-specific DNA-binding protein.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* 10403S was the parental strain used in these studies (3). The isolation of *L. monocytogenes* Tn*917*-LTV3 insertion mutants DP-L973 and DP-L1075 has been described previously (31). DP-L1303 was isolated after screening a library of 10403S::Tn*917*-LTV3 for mutants which showed a negative reaction on egg yolk agar (30). The mutant was shown to have a transposon insertion in the *plcA-prfA* intragenic region (see Fig. 2). Bacteria were grown on either brain heart infusion (Difco Laboratories, Detroit, Mich.) agar and broth or Luria-Bertani (LB) medium (12). LB broth was buffered to pH 7.5 with 77 mM K_2HPO_4, 20 mM KH_2PO_4, 7.6 mM ammonium sulfate, and 1.7 mM sodium citrate. All stock cultures were stored as suspensions of cells at −70°C in 50%

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glycerol. For routine use, bacteria were kept on brain heart infusion agar at room temperature.

Purification of PrfA protein. The *prfA* coding sequence was amplified by polymerase chain reaction (PCR) with primers LR-4 (5′-GGTCTAGAGATGAACGCTCAAGCA GAA-3′) and prf-2 (5′-GGGTCGACCAGCTCTTCTTGGT GAAG-3′), which create *Xba*I and *Sal*I sites, respectively, at the 5′ and 3′ ends of the *prfA* gene. The *prfA* PCR product was purified and cloned into *Xba*I-*Sal*I-digested plasmid pGEX-KG (18) to create an in-frame translational fusion between the glutathione *S*-transferase (GST) gene and *prfA*. The GST-PrfA gene fusion in pGEX-KG was sequenced with primer LR-5 (5′-CCAAAATCGGATCTGGTTCCG-3′) to confirm the fusion junction. The plasmid was transformed into *Escherichia coli* LE392 to produce strain DP-E1754.

The GST-PrfA fusion protein was purified by a modification of a previously described procedure (18). *E. coli* DP-E1754 was grown in 300 ml of brain heart infusion broth plus 50 μg of ampicillin per ml at 37°C with shaking to an optical density at 600 nm of 1.0. Isopropyl-β-D-thiogalactoside (IPTG) was added at a final concentration of 1 mM, and the culture was incubated for an additional 3 h and then harvested by centrifugation. The bacterial pellet was resuspended in 4 ml of PBST buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 [pH 7.3], 1% Triton X-100), sonicated, and microcentrifuged. Then 1 ml of the resulting supernatant was added to 2 ml of 50% glutathione-agarose beads (Sigma) and incubated at 4°C for 30 min. The agarose beads were collected by centrifugation and washed four times with PBST buffer and once with thrombin cleavage buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% β-mercaptoethanol). The beads were resuspended in 1.5 ml of thrombin cleavage buffer containing 5 μg of thrombin and incubated at room temperature for 30 min. Following centrifugation, the supernatant containing PrfA protein was recovered and stored at 4°C for immediate use or at −70°C. The PrfA protein concentration was determined by the Bradford method (4). The purified PrfA protein had a molecular mass of approximately 27 kDa on the basis of its mobility in sodium dodecyl sulfate-polyacrylamide gels and was greater than 99% pure as estimated by Coomassie blue staining. The purified protein contains an additional 12 amino acids (Pro-Gly-Ile-Ser-Gly-Gly-Gly-Gly-Gly-Ile-Leu-Glu) at its amino terminal as a result of the fusion construction.

Gel mobility shift assays. Two oligonucleotide primers, DP1570 (5′-GGGGATCCTAACCTAATAATGCCAAATA C-3′) and DP1703 (5′-TTTATGTGGATCCATTAACATTT GT-3′), were used for PCR amplification (29) of a 178-bp DNA fragment containing the *hly* promoter region. The amplified fragment was purified following electrophoresis through a low-melting-point agarose gel, suspended in TE (10 mM Tris, 1 mM EDTA [pH 8.0]), and quantitated by measuring the optical density at 260 nm (27). The purified *hly* promoter fragment was end labeled with [γ-^{32}P]ATP as described previously (27).

Gel mobility shift reaction mixes (1) contained 5.4 μg of purified PrfA protein, 2.9 ng of ^{32}P-labeled *hly* promoter fragment, 500 μg of bovine serum albumin per ml, 500 ng of poly(dI-dC) DNA, 6.7 mM Tris acetate (pH 7.5), 3.3 mM sodium acetate, 1 mM EDTA, 20 mM $MgSO_4$, and 2 mM dithiothreitol in a final volumes of 20 μl. Reactions were preincubated in the absence of labeled *hly* promoter fragment for 1 min at 37°C. After addition of the fragment, the reactions were incubated for an additional 8 min at 37°C, and then 3.5 μl of gel-loading buffer (15% Ficoll, 0.25% xylene cyanol, 0.25% bromophenol blue) was added and samples were loaded onto a 5% polyacrylamide gel in low-ionic-strength buffer (6.7 mM Tris acetate [pH 7.5], 3.3 mM sodium acetate, 1 mM EDTA). Following electrophoresis, the portion of the gel below the bromophenol blue marker dye was removed and the upper portion of the gel was dried and subjected to autoradiography.

Mapping sites of Tn*917*-LTV3 insertions. The exact sites of transposon insertion within DP-L973, DP-L1303, and DP-L1075 were determined by DNA sequencing of the transposon-chromosome junction which had been cloned as previously described (7). Dideoxy sequencing of double-stranded plasmid DNA was performed as recommended by the manufacturer with a Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio), using an oligonucleotide primer complementary to a sequence 83 bp from the *lacZ*-proximal end of Tn*917*-LTV3.

Cloning of *prfA* into plasmid vector pAM401. *prfA* promoter and coding sequences were PCR amplified with primers prfA-4 (5′-GGTCTAGAGCATTTCTTTTGCGAA-3′) and prf-2 (5′-GGGTCGACCAGCTCTTCTTGGTGAAG-3′), which create *Xba*I and *Sal*I sites, respectively, at respective 5′ and 3′ ends of the gene. The amplified product was cloned into *Xba*I-*Sal*I-digested pAM401 (36) as described by Sambrook et al. (27) and transformed by electroporation (24) into wild-type *L. monocytogenes* and strains DP-L1075 and DP-L973 to produce strains DP-L1502, DP-L1613, and DP-L1611, respectively.

Assay for hemolytic and phospholipase activity. Stationary-phase bacteria were diluted 1:10 into LB supplemented with 2.5 mM $CaCl_2$ and 20 mM $MgCl_2$ and grown for 5 h at 37°C with shaking. The supernatant fluid was assayed for hemolytic activity (6) and phosphatidylinositol-specific phospholipase C activity (5).

Determination of LD_{50}s. The 50% lethal doses (LD_{50}s) were determined after intravenous injection of the strains into BALB/c mice (Simonsen Laboratories, Gilroy, Calif.) as described previously (26).

Plaque formation by *L. monocytogenes* in L2 cells. The plaque sizes of strains 10403S, DP-L1075, DP-L973, DP-L1502, DP-L1611, and DP-L1613 were determined as previously described (31).

Intracellular growth assay. Intracellular growth in J774 cells and bone marrow-derived macrophages has been described previously (26). For visualization, coverslips were stained with Diff-Quik (American Scientific Products, McGaw Park, Ill.) and mounted in Permount mounting medium (Fisher Scientific, Philadelphia, Pa.). The stained coverslips were observed under oil immersion with a Nikon Microphot-FXA.

Fluorescence labeling of F-actin. The fluorescence labeling of F-actin with NBD-phallacidin was performed as previously described (11).

Primer extension assays. RNA was purified by centrifugation through CsCl as described previously (15). *L. monocytogenes* strains were grown in buffered LB or buffered LB plus 0.5% glucose by inoculating 50 ml of culture medium with 1 ml of an overnight culture grown in the same medium at 37°C. Cultures were incubated at 37°C with shaking for 5 h and before harvest. High-pressure liquid chromatography-purified primer PE-PRFA2 (5′-TGGTTTTATCCCGTTAG TT/TCTAAATA-3′; 10 pmol; OPERON Technologies) was end labeled with 50 μCi of [γ-^{32}P]ATP (>3,000 Ci/mmol) and phage T4 polynucleotide kinase (Bethesda Research Laboratories) and used in primer extension assays as previously described (15).

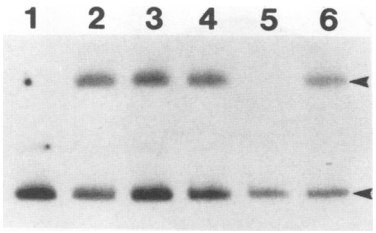

FIG. 1. Gel shift assays measuring the binding of PrfA protein to DNA fragments containing the *hly* promoter. A DNA *hly* promoter fragment (178 bp) was end labeled with [γ-^{32}P]ATP as described in Materials and Methods. Binding of PrfA to the fragment was monitored following electrophoresis through 5% polyacrylamide gels and autoradiography. All reaction mixes contained 2.9 ng of ^{32}P-labeled *hly* fragment and 500 ng of poly(dI-dC); additional poly(dI-dC) or unlabeled *hly* fragment was added as indicated. Lanes: 1, no PrfA protein; 2, PrfA protein; 3, PrfA protein and 2,000 ng of poly(dI-dC); 4, PrfA protein plus 1,000 ng of poly(dI-dC); 5, PrfA protein plus 910 ng of unlabeled *hly* fragment; 6, PrfA protein plus 315 ng of unlabeled *hly* fragment. The bottom arrowhead points to the unbound *hly* fragment, and the top arrowhead points to the PrfA-bound *hly* fragment.

RESULTS

PrfA protein is a site-specific DNA-binding protein. Results of previous in vivo experiments have suggested that PrfA functions as a transcriptional activator by recognizing a 14-bp DNA palindromic sequence present in the promoter regions of its target genes (15, 20, 21). Gel mobility shift assays were used with recombinant PrfA protein to examine its DNA-binding capacity (Fig. 1). PrfA protein specifically retarded the mobility of DNA fragments containing the 14-bp DNA palindrome. Binding was dependent on the presence of Mg^{2+} (data not shown). Nonspecific DNA did not compete for PrfA protein binding with labeled palindrome-containing fragments; however, unlabeled specific competitor containing the 14-bp palindrome eliminated the PrfA-dependent shift. These experiments directly demonstrate that PrfA is a site-specific DNA-binding protein.

The *prfA* gene product is an essential determinant of *L. monocytogenes* pathogenicity. Previously, several classes of *L. monocytogenes* transposon-insertion mutants were isolated by screening for mutants which formed small plaques in monolayers of mouse L2 cells (31). These mutants were shown to be defective for either intracellular growth or cell-to-cell spread. Two classes of mutants demonstrated the pleiotropic phenotype predicted for *prfA* mutations; i.e., they were completely avirulent (2) and had greatly reduced levels of hemolysin and phosphatidylinositol-specific phospholipase C activity (31). DNA sequence analysis of the transposon-chromosome junctions indicated that the first class (class 4) of insertion mutants (represented by DP-L1075) contained transposon insertions within the *prfA* structural gene, whereas the second class (class 6) of insertion mutants (represented by DP-L973 and DP-L1303) contained insertions within the 5′ upstream region of *prfA* (Fig. 2).

Complementation analysis was performed with PCR-amplified *prfA* and its promoter region cloned into plasmid pAM401. *L. monocytogenes* wild type (10403S) and *prfA* insertion mutants DP-L1075 and DP-L973 were transformed with the *prfA*-containing plasmid and assayed for virulence in mice and the ability to form plaques in mouse L2 fibroblast monolayers. The presence of cloned *prfA* restored full virulence and the ability of both classes of *prfA* insertion mutants to form wild-type-size plaques (Fig. 3; Table 1). Consistent with this observation, hemolysin and phosphatidylinositol-specific phospholipase C activity were also restored. Therefore, introduction of a wild-type copy of *prfA* into both classes of *prfA* mutant is sufficient to restore full virulence.

Characteristics of *prfA* insertion mutants in cultured macrophages. The two classes of *prfA* transposon mutants demonstrated similar phenotypes with respect to LD_{50}s, reduction in hemolysin and phospholipase activities, and the ability to be fully complemented by introduction of wild-type copies of *prfA*. In contrast, DP-L1075 and DP-L973 differed dramatically in their abilities to grow within macrophages (Fig. 4). The *prfA* structural gene insertion mutant, DP-L1075, was completely defective in intracellular growth, presumably because of its inability to escape from the phagosome. However, the promoter insertion mutant, DP-L973, had the same intracellular doubling time as did the wild type during 8 h of growth. The ability of DP-L973 to grow in macrophages does not appear to be the result of *prfA* expression by a cryptic promoter provided by the transposon, since the second class 6 mutant, DP-L1303, which contains a transposon insertion located 2 bp 5′ to the site of the DP-L973 insertion but in the opposite orientation, was

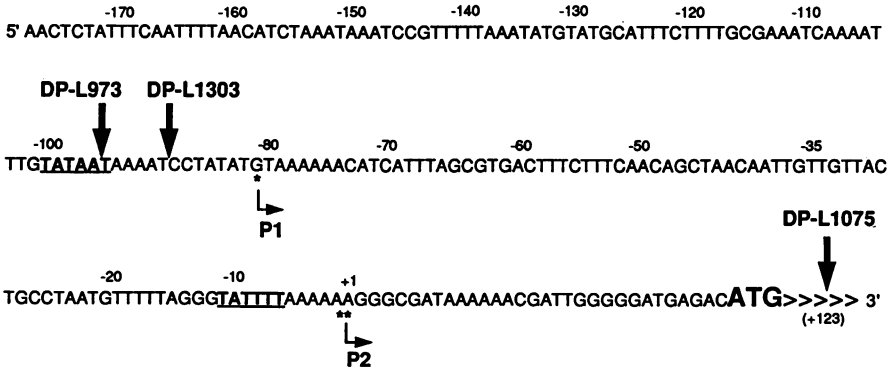

FIG. 2. Nucleotide sequence of the *prfA* upstream region and location of transposon insertions. The sequence of the *prfA* promoter is from Leimeister-Wachter et al. (20). The vertical arrows point to the precise site of Tn*917*-LTV3 insertions in the indicated strains. P1 and P2 denote the two promoters and direction of transcription as identified in Fig. 7. The −10 regions of P1 and P2 are underlined. The DP-L1075 insertion within the *prfA* structural gene is located at nucleotide 123 (31 amino acids from the N-terminal methionine).

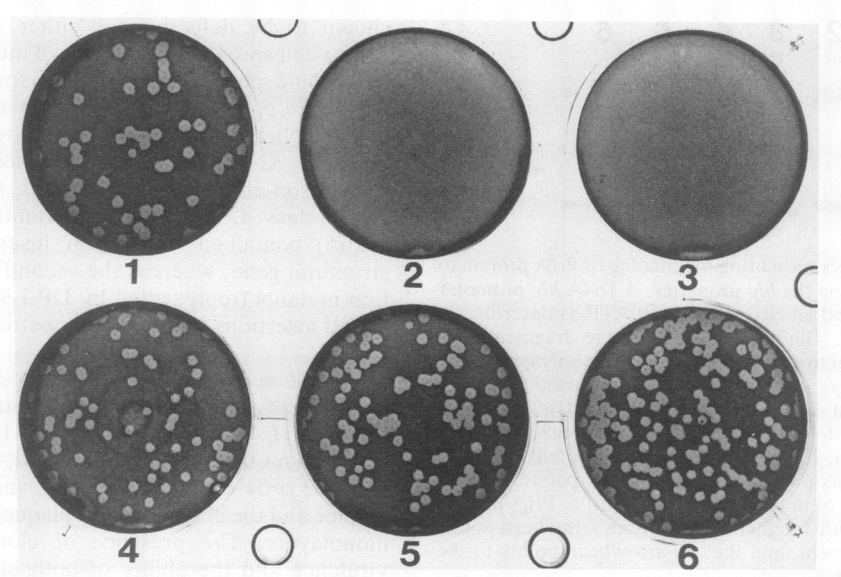

FIG. 3. Plaque formation by *L. monocytogenes* strains. 1, 10403S; 2, DP-L1075; 3, DP-L973; 4, DP-L1502; 5, DP-L1611; 6, DP-L1613. Plaques were stained with neutral red after 4 days. DP-L1075 shows no visible plaques, whereas DP-L973 forms very tiny plaques.

phenotypically indistinguishable from DP-L973 (data not shown). Examination of the DP-L973 mutant by light microscopy revealed that it fails to spread to adjacent cells even though it grows in the cytoplasm (Fig. 5).

As previously reported, intracytoplasmic *L. monocytogenes* organisms utilize host actin filaments to mediate their spread within a cell and from cell to cell (11, 23, 34). Actin filaments associated with the bacteria can be indirectly visualized by fluorescence microscopy with NBD-phallacidin, a reagent which binds F-actin and not G-actin. Intracytoplasmic DP-L973 stained poorly with NBD-phallacidin (Fig. 6), indicating that DP-L973 is deficient in mediating actin polymerization. In addition, video microscopy of intracellular bacteria (32) revealed that far fewer DP-L793 organisms were moving in the cytoplasm than wild-type organisms did, although an occasional bacterium did move like the wild type (33).

Identification of differentially regulated *prfA* promoters. The site of the transposon insertion in DP-L973 would eliminate the *prfA* promoter identified by Mengaud et al. (21) 113 bp upstream of the AUG initiation codon. However, the phenotype of DP-L973 suggested that some *prfA* promoter activity still existed. Therefore, we used primer extension to examine transcriptional initiation sites of *prfA*. In the wild-type strain a transcript initiation site was identified at 113 bp upstream from the AUG initiation codon of *prfA* (Fig. 7, lanes 1 and 2). This transcript was approximately 20 times more abundant in bacteria grown in buffered LB plus glucose than in those grown in buffered LB alone (lane 2). The initiation site for this transcript, *prfAp*1, corresponded to the P1 transcript identified by Mengaud et al. (21). In addition, we detected another transcript, *prfAp*2, which initiated approximately 30 bp upstream from the AUG initiation codon of *prfA* (20). The *prfAp*2 transcript is the only detectable transcript present in DP-L973 (lanes 3 and 4), since the transposon blocks transcription from *prfAp*1 (Fig. 2). No transcripts corresponding to the P2 transcript identified by Mengaud et al. (21) and located 30 bp upstream of *prfAp*1 were detected.

The *prfA* structural gene mutant DP-L1075 had approximately 20-fold higher levels of both *prfAp*1 and *prfAp*2 transcripts (Fig. 7, lanes 5 and 6). Since DP-L1075 lacks a functional *prfA* gene product, this suggests that the PrfA protein may play a role in down-regulating its own expres-

TABLE 1. Bacterial strains and relevant characteristics

Strain[a]	Genotype	LD_{50}	% Hemolytic activity	% PI-PLC[b] activity	Intracellular growth[c]	Cell-to-cell spread[d]
10403S	Wild type	2×10^4[e]	100	100	+	+
DP-L1303	*prfA*::Tn*917*-LTV3[f]	ND[g]	ND	ND	+	−
DP-L973	*prfA*::Tn*917*-LTV3[f]	5×10^8[e]	20	4	+	−
DP-L1075	*prfA*::Tn*917*-LTV3[f]	$>3 \times 10^9$	0–0.6	2	−	−
DP-L1611	DP-L973(pAM401-*prfA*)	$<1 \times 10^5$	150	100	+	+
DP-L1613	DP-L1075(pAM401-*prfA*)	2×10^4	133	100	+	+

[a] All strains are derived from 10403S.
[b] PI-PLC, phosphatidylinositol-specific phospholipase C.
[c] Intracellular growth observed in J774 cells.
[d] Cell-to-cell spread observed following infection of bone marrow-derived macrophages.
[e] See reference 2.
[f] See Fig. 2.
[g] ND, not determined.

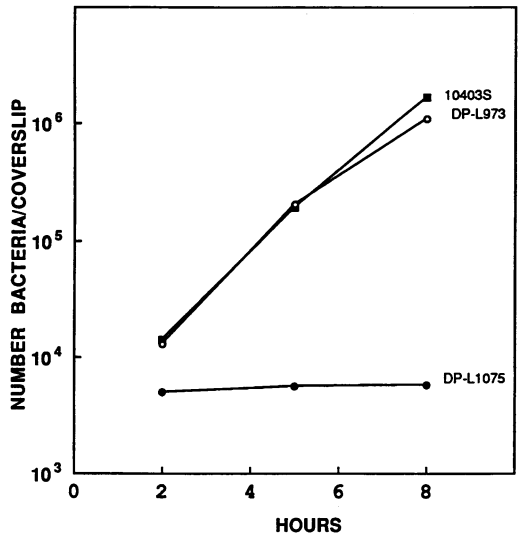

FIG. 4. Intracellular growth of *L. monocytogenes* strains in the mouse macrophage-like cell line J774. The data represent averages for three coverslips.

sion. In addition, the *prfAp*1 transcript was more abundant in DP-L1075 grown in LB plus glucose than in LB without glucose, indicating that functional PrfA protein is not necessary for this induction. Interestingly, *prfAp*2 expression in LB plus glucose decreased in comparison with the levels observed in LB alone.

DISCUSSION

The ability of *L. monocytogenes* to enter the mammalian cell cytoplasm, grow, and spread to adjacent cells requires the contributions of multiple gene products (25). The PrfA protein is the first regulatory factor identified in this pathogen that positively regulates the expression of several protein products necessary for pathogenicity (9, 20, 21). The present study demonstrated that PrfA is a DNA-binding protein which binds specifically to a sequence upstream of *hly*. The results of this study also showed that both *prfA* structural and promoter transposon insertion mutants are completely avirulent; however, they differ markedly in their behavior during the course of infection in macrophages in vitro. Whereas the structural mutant is unable to grow intracellularly, the promoter mutant grows in the cytoplasm but is incapable of spreading to adjacent cells. This suggests that the relative abundance of PrfA protein may govern events which occur in the cytoplasm.

Once *L. monocytogenes* escapes from the phagosome, it is capable of replicating in the cell cytoplasm, nucleating host actin filaments, and spreading within infected cells and into adjacent cells (11, 23, 28, 32, 34). In contrast, the *prfA* promoter mutant DP-L973 replicates in the cytoplasm but is

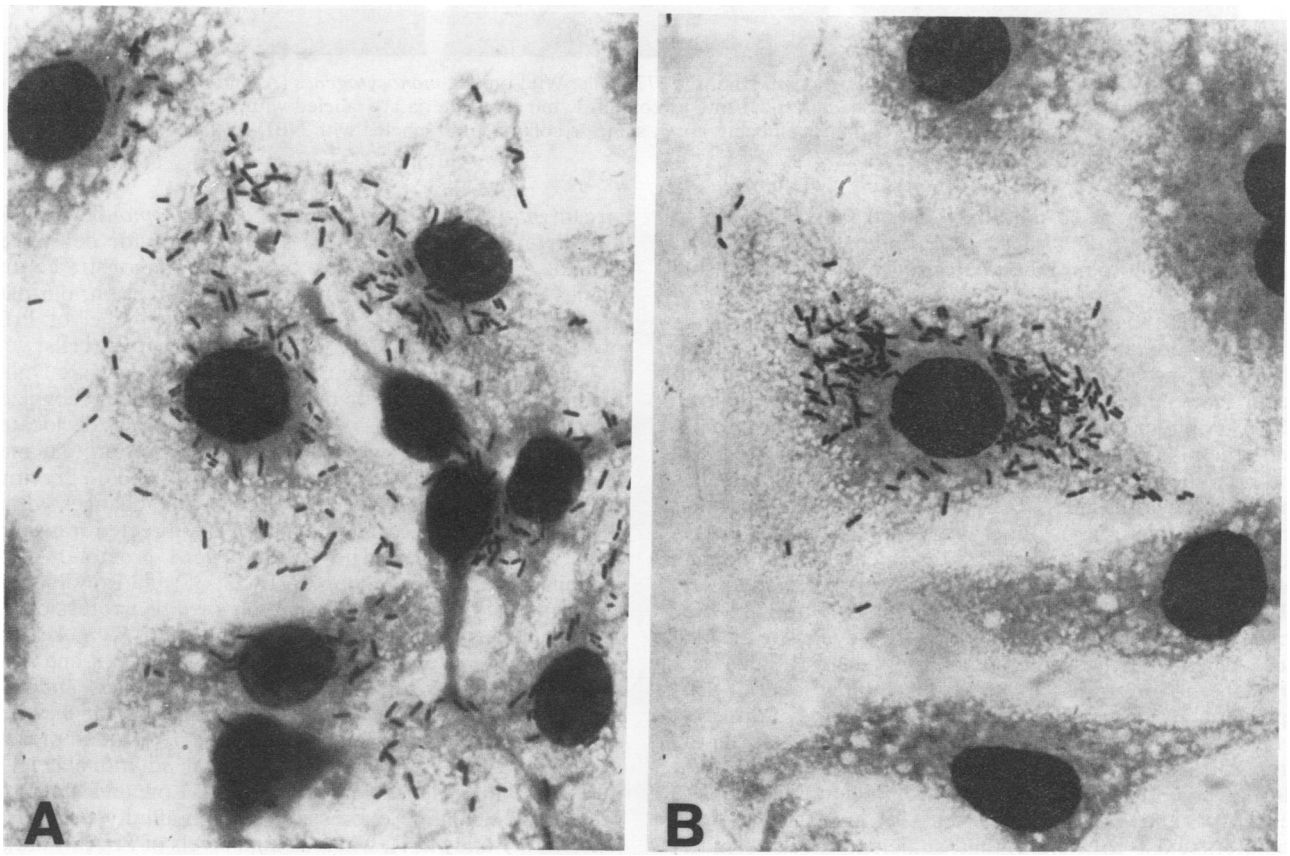

FIG. 5. Light micrographs of wild-type *L. monocytogenes* and DP-L973 grown in bone marrow-derived macrophages for 8 h. (A) Wild-type strain 10403S. Bacteria have spread from cell to cell. (B) DP-L973. A few bacteria have migrated within the infected cell but have not spread to adjacent cells.

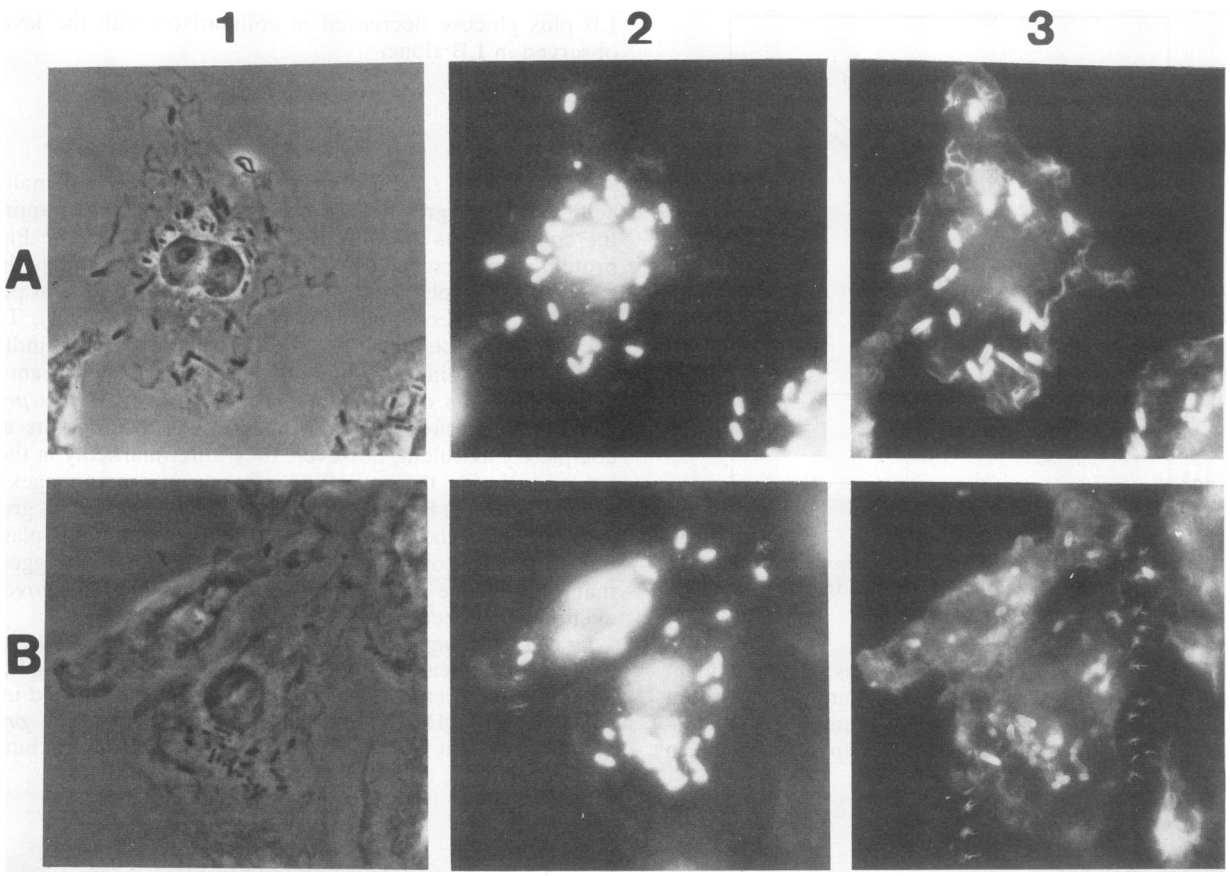

FIG. 6. NBD-phallacidin staining of *L. monocytogenes* strains in J774 cells. Wild-type *L. monocytogenes* (A) and DP-L973 (B) were used. Columns: 1, phase micrograph of intracellular bacteria; 2, same as column 1, but the bacteria are labeled with rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (labels all bacteria); 3, same as column 1 but labeled with NBD-phallacidin.

defective in actin-based motility (33) and fails to spread to adjacent cells (31). This observation indicates (i) that *prfA* expression is still required on entry into the host cytoplasm and (ii) that transcriptional initiation from the *prfAp*2 promoter must be insufficient in the cell cytoplasm to provide for gene products required for actin polymerization and cell-to-cell spread. Two hypotheses can be proposed to account for the behavior of DP-L973. It is possible that the *prfAp*2 promoter provides for expression of *prfA* specifically inside the phagosome and that expression of *prfA* from the *plcA* promoter or from *prfAp*1 occurs once the bacterium has entered the cytoplasm. This hypothesis would suggest that promoter elements controlling *prfA* expression may be responsive to specific host cell compartmental environments encountered during the infection process. Alternatively, only low levels of *prfA* expression may be required for escape from the phagosome and higher levels are needed once the bacteria has entered the cytoplasm. In this scenario, the *prfAp*2 transcript provides the low level of *prfA* expression necessary for escape but is incapable of directing levels high enough for the gene products required within the cytoplasm. Recent evidence suggests that this latter hypothesis may be correct. Data obtained from deletion analysis of the *prfAp*1 and *prfAp*2 promoters (14) suggest that both promoters exist to ensure that sufficient levels of PrfA protein are produced to enable growth and spread in the cytoplasm. Camilli et al. (8) have demonstrated that the *plcA-prfA* readthrough transcript originating from the upstream *plcA* promoter is not required for *L. monocytogenes* growth in the cytoplasm but is necessary for cell-to-cell spread. Although it has yet to be directly demonstrated that the levels of PrfA protein control gene expression, our data provide genetic evidence that the absolute level of PrfA protein is crucial for bacterial spread to adjacent cells.

There is direct evidence that PrfA is a site-specific DNA-binding protein. The 14-bp palindromic sequence recognized by PrfA in the *hly*, *plcA*, *mpl*, and *actA* promoters is located in the −35 region, suggesting that the PrfA protein may directly interact with RNA polymerase (10, 35). Previous experiments have indicated that single-base changes within the 14-bp palindrome can eliminate PrfA-directed transcriptional activation (15). The *mpl* and *actA* promoters each contain a single-base change (21, 22, 35), and preliminary experiments suggest that activation of *mpl* expression by PrfA is not as efficient as that observed for *hly* (14). It is possible that PrfA has a lower affinity for the *mpl* and *actA* palindromes because of the single-base changes and that greater amounts of PrfA protein are required for *mpl* and *actA* activation. The absolute levels of PrfA protein present in *L. monocytogenes* might provide for an additional level of regulation of virulence gene expression. Low levels of PrfA protein would allow expression of high-affinity promoters (such as *hly* and *plcA*), whereas high levels of PrfA would be required for expression of low-affinity promoters (such as *mpl*). In this model, escape of *L. monocytogenes* from the phagosome requires only low levels of PrfA protein and

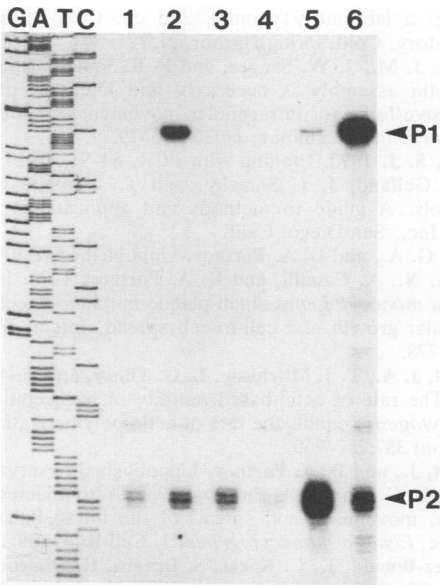

FIG. 7. Primer extension analysis of *prfA* transcripts in wild-type *L. monocytogenes* and strains DP-L1075 and DP-L973. A radiolabeled primer was incubated with RNA isolated from wild-type strain 10403S and strains DP-L1075 and DP-L973 grown in buffered LB with and without 0.5% glucose. Reverse transcriptase was added to produce a DNA copy of the transcript, as described in Materials and Methods. The same primer was used in dideoxy sequencing reactions with a DNA template containing the wild-type *prfA* gene. The letters above the first four lanes indicate the dideoxynucleotide used to terminate each reaction. Other lanes: 1, wild-type 10403S; 2, wild-type 10403S plus glucose; 3, DP-L973; 4, DP-L973 plus glucose; 5, DP-L1075; 6, DP-L1075 plus glucose.

expression from a subset of PrfA-activated promoters. Nucleation of actin filaments and cell-to-cell spread would then require increased levels of PrfA protein to activate transcription from the lower-affinity promoters.

As observed by Mengaud et al. (21), the *prfA* gene product appears to positively regulate its own synthesis by activating transcription from the *plcA* promoter. Primer extension analysis of the *prfA* structural-gene mutant DP-L1075 suggests that PrfA may also play a role in negatively regulating its own expression. Levels of transcripts directed from *prfAp*1 and *prfAp*2 were greatly increased in the absence of functional PrfA protein. The PrfA protein may therefore activate the expression of a distal gene, which in turn down-regulates *prfA* expression. Alternatively, PrfA protein may play a more direct role by binding a site (or sites) within its upstream region and shutting down expression from its own promoters. The presence of *prfA* on a multicopy plasmid appears to produce no deleterious effects with regard to virulence. This may be the result of negative regulation of the multicopy *prfA* promoters, or it may indicate that high levels of PrfA protein expression are tolerated by *L. monocytogenes*. Lastly, *prfA* expression directed from *prfAp*1 was induced in the presence of glucose. Preliminary experiments indicate that this induction was not due to the drop in the pH of the medium observed as glucose was metabolized (14). It is possible that glucose serves as an environmental signal for increased *prfA* expression.

The regulation of *prfA* expression is a key event in the course of *L. monocytogenes* pathogenesis, and it appears that both negative and positive controls exist. In addition, the present work demonstrates that *prfA* is controlled by multiple promoters. Future experiments should assess the contributions of each of the *prfA* promoters to *L. monocytogenes* virulence as well as determine the precise nature of environmental signals which influence *prfA* expression.

ACKNOWLEDGMENTS

We thank A. Bouwer and D. Hinrichs for performing the animal studies, H. Goldfine and C. Knob for phosphatidylinositol-specific phospholipase C assays, and members of the Portnoy laboratory for helpful discussions.

This research was supported by National Institute of Health grants AI-26919 and AI-27655 (to D.A.P.) and by National Research Service Award GM-13303 (to N.E.F.).

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