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Intracellular Induction of *Listeria monocytogenes actA* Expression

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Following entry into the host cytosol, the bacterial pathogen *Listeria monocytogenes* dramatically increases the expression of several key virulence factors. The expression of *actA*, whose protein product is required for *L. monocytogenes* actin-based intracellular motility, is increased by more than 200-fold in cytosolic bacteria in comparison to broth-grown cultures. Two distinct promoter elements have been reported to regulate *actA* expression. One promoter is located immediately upstream of *actA* coding sequences, while the second promoter is contributed by the upstream *mpl* gene via the generation of an *mpl-actA-plcB* transcript. A series of *L. monocytogenes* mutants were constructed to define the contributions of individual promoter elements to *actA* expression. The intracellular induction of *actA* expression was found to be dependent upon the *actA* proximal promoter; the *mpl* promoter appeared to contribute to the extracellular induction of *actA* but did not affect intracellular levels of expression. The *actA* promoter is dependent upon a regulatory factor known as PrfA for transcriptional activation; however, no increase in *actA* expression was detected following the introduction of a high-affinity PrfA binding site within the *actA* promoter. The presence of a mutationally activated form of PrfA, known as PrfA*, increased overall *actA* expression in broth-grown cultures of both wild-type and *actA* promoter mutant strains, but the levels of induction observed were still approximately 50-fold lower than those observed for intracellularly grown *L. monocytogenes*. Collectively, these results indicate that the dramatic induction of *actA* expression that occurs in the host cell cytosol is mediated through a single promoter element. Furthermore, intracellular induction of *actA* appears to require additional steps or factors beyond those necessary for the activation and binding of PrfA to the *actA* promoter.

Listeria monocytogenes is a gram-positive facultative intracellular pathogen that is responsible for serious infections in immunocompromised individuals, pregnant women, and neonates (25, 26). The bacteria infect a wide variety of host cells, and a number of gene products that participate in the processes of invasion, intracellular replication, and cell-to-cell spread have been identified (reviewed in references 46 and 56). Following internalization, *L. monocytogenes* escapes from the host cell phagosome through the activity of a pore-forming hemolysin known as listeriolysin O, encoded by *hly* (11, 16, 31, 41, 47). Once within the host cell cytosol, the bacteria begin to replicate and to express a surface-associated protein, ActA, that is required for host cell actin polymerization-based bacterial movement and for spread of the bacteria into adjacent cells (12, 17, 34, 44, 52).

L. monocytogenes has been demonstrated to specifically induce the expression of selected genes within host cells (7, 8, 18, 21, 22, 24, 33, 43). For example, the expression of *actA* is highly induced in the mammalian cell cytosol (226-fold), whereas *hly* shows more moderate levels of intracellular induction (20-fold) (43). The host environmental signals that lead to the induction of intracellular bacterial gene expression have not been identified, and the mechanisms by which that induction occurs are unknown. To begin to define the mechanisms that govern the intracellular induction of bacterial gene expression, we have chosen to focus on the transcriptional activation of *actA*, as the product of this gene is one of the most abundant bacterial proteins expressed within the cytosol but is expressed minimally in broth cultures (6). Two promoters appear to contribute to *actA* expression, a proximal promoter located 198 bp upstream from the start of *actA* translation and a promoter from the upstream *mpl* gene (Fig. 1). Northern analysis with RNA isolated from *L. monocytogenes* grown in broth cultures detected the presence of an approximately 3-kb transcript corresponding to *actA-plcB* and a 5.4-kb transcript corresponding to *mpl-actA-plcB* (5).

The *actA* and *hly* genes are both members of a 10-kb gene cluster that is positively regulated by a 27-kDa transcriptional activator known as PrfA (38, 40). PrfA belongs to the cyclic AMP receptor protein (CRP)-FNR family of transcriptional activators and binds to a conserved 14-bp region of dyad symmetry present within the −40 region of target promoters (2, 15, 23, 36, 55). Certain PrfA-regulated promoters, such as *actA* and *mpl*, contain PrfA DNA binding sites that are imperfect palindromes, and the activation of transcription by PrfA at these promoters is less efficient than the PrfA-dependent activation of *hly* and *plcA*, which share a perfect palindromic PrfA binding site (54, 60).

In this report, the contributions of the *actA* and *mpl* promoter elements to the intracellular induction of *actA* expression are further investigated. Mutational analysis of *actA* and *mpl* promoter and coding regions indicates that the *actA* proximal promoter is the primary element that contributes to the intracellular induction of *actA* expression, whereas the *mpl* promoter contributes to patterns of expression of *actA* outside

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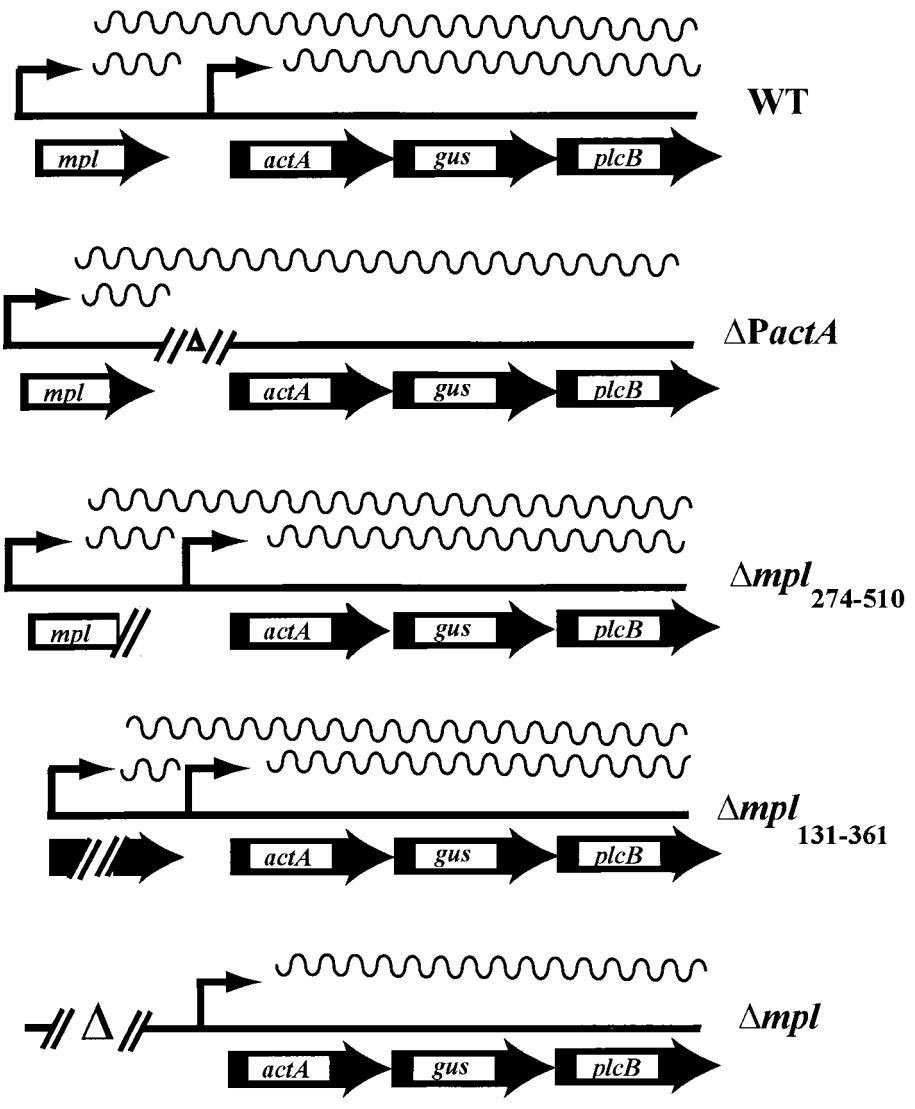

FIG. 1. Chromosomal *actA* and *mpl* deletion mutants in the presence of *actA-gus-plcB* transcriptional reporter gene fusions in *L. monocytogenes*. The *mpl* and *actA* promoters are shown as thin arrows with associated transcripts (wavy lines). WT, wild type. ΔP *actA* contains a complete deletion of the *actA* proximal promoter but maintains the ribosome binding site immediately upstream of the *actA* coding sequences. $\Delta mpl_{274–510}$ contains a frameshift mutation at codon 274 of *mpl* resulting in the loss of the protease active site and approximately 50% of the protein. $\Delta mpl_{131–361}$ contains an in-frame deletion of amino acids 131 through 361 of Mpl resulting in the loss of the protease active site. Δ*mpl* contains a complete deletion of the *mpl* promoter and coding regions. A promoterless copy of *gus* was inserted between the *actA* and *plcB* coding regions in the chromosomes of all mutants via homologous recombination.

host cells. Additional analysis of the *actA* proximal promoter, which has been reported to be completely dependent on PrfA activation (2), indicates that no increase in *actA* expression occurs following the introduction of a high-affinity PrfA DNA binding site within the *actA* promoter. The failure of a high-affinity PrfA DNA binding site to alter *actA* expression patterns is also seen in the presence of the mutationally activated *prfA** allele (49, 59), suggesting that the modified form of the protein does not discriminate between high-affinity and low-affinity binding sites. The intracellular induction of *actA* expression must therefore require additional factors or events beyond those leading to the activation and binding to the *actA* promoter of PrfA. Finally, our results suggest that the Mpl protease contributes an additional role to *L. monocytogenes* pathogenesis that is distinct from the activation of the PlcB lecithinase.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *L. monocytogenes* (serotype 1/2a) is resistant to streptomycin and has a 50% lethal dose (LD_{50}) for mice of 2×10^4 (23). *L. monocytogenes* was stored at −70°C in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) broth containing 20% glycerol. *Escherichia coli* HB101 or DH5α was used as the host strain for recombinant plasmids. Antibiotics were used at the following concentrations, unless otherwise noted: carbinicillin, 50 μg/ml; chloramphenicol, 10 μg/ml; and streptomycin, 200 μg/ml.

The thermosensitive plasmid vectors pKSV7 (56) and pCON1-Δ*prfA*7973 (1) were described previously.

Construction of *L. monocytogenes actA* and *mpl* promoter and gene deletion

TABLE 1. Bacterial strains and relevant characteristics

Strain[a]	Relevant characteristic(s)	Reference or source
10403S	Wild type	23
DP-L1942	$\Delta actA_{164-465}$[b]	6
DP-L1465	$\Delta mpl_{274-510}$[c]	Dan Portnoy
DP-L2296	$\Delta mpl_{131-361}$[d]	39
DP-L2343	Δmpl[e]	Dan Portnoy
NF-L559	$P_{(hly)}$-*actA*	This work
NF-L476	*actA-gus-plcB*	This work
NF-L767	Δ*PactA-gus-plcB*	This work
NF-L707	$\Delta mpl_{274-510}$ *actA-gus-plcB*	This work
NF-L861	$\Delta mpl_{131-361}$ *actA-gus-plcB*	This work
NF-L862	Δ*mpl actA-gus-plcB*	This work
NF-L572	$P_{(hly)}$-*actA-gus-plcB*	This work
NF-L753	*actA-gus-plcB prfA**	This work
NF-L754	$P_{(hly)}$-*actA-gus-plcB prfA**	This work
NF-L904	Δ*PactA-gus-plcB comK*::*actA*	This work

[a] All DP and NF strains were derived from *L. monocytogenes* 10403S.
[b] In-frame deletion of *actA*.
[c] *mpl* frameshift mutation.
[d] In-frame deletion of *mpl*.
[e] Deletion of *mpl* promoter and coding region.

mutants. An internal out-of-frame *mpl* deletion was introduced into strain 10403S by allelic exchange with vector pDP1434 (39). Vector pDP1434 contains a copy of the *mpl* gene that has been digested with *Bam*HI and *Xba*I, treated with the Klenow fragment to produce blunt ends, and religated, resulting in a deletion of 83 bp followed by three new codons and a stop codon. The resulting open reading frame encoded a product that was 274 amino acids long, corresponding to approximately 50% of the length of the original protein and lacking the putative active site of the protease. Strain DP-L2296 contains a 684-bp in-frame deletion of *mpl* in 10403S, encompassing 45% of the open reading frame, including the putative propeptide cleavage site and the putative active site of Mpl (39). A complete deletion of the *mpl* coding region and promoter sequences was generated by ligating the 3′ end of the *hly* structural gene and terminator to the 5′ end of the *actA* promoter and structural gene. A 637-bp DNA fragment encompassing the *actA* promoter region and an N-terminal portion of the structural gene was amplified by PCR with primers DP-2276 (5′GGGGTACCTTA ACAAATGTTAGAGAAAAA3′) and DP-2277 (5′GGAATTCCGCTGCGCT ATCC3′) (the underlined sequences designate the introduced *Kpn*I and *Eco*RI restriction sites, respectively). The resulting PCR fragment was ligated into vector pKSV7 (56) digested with *Kpn*I and *Eco*RI. A 638-bp fragment encompassing the 3′ end of *hly* and its terminator was amplified by PCR with primers DP-2295 (5′-GGCTCTAGACTTACGCGATATTTTG-3′) and DP-2275 (5′-G GGGTACCTTCTTCTAAAAAAATTAAAAAAT-3′) (the underlined sequences designate the introduced *Xba*I and *Kpn*I restriction sites, respectively). The resulting PCR fragment was ligated into the *Xba*I and *Kpn*I cloning sites of vector pKSV7 containing the 5′-end *actA* fragment described above. The new construct was designated DP-2308 and was verified by DNA sequencing. The 10403S chromosomal allele of *hly-mpl-actA* was replaced with the *hly-actA* allele (or Δ*mpl*) of DP-2308 by allelic exchange (20) to generate strain DP-L2343. The Δ*mpl* mutation of DP-L2343 was verified by Southern analysis.

Deletion of the *actA* promoter region was achieved by using the gene splicing by overlap extension (SOEing) method of PCR (27) with the following primers: actAΔ1 (5′-GGCGAATTCCATGCGCCAAAACTATTGTTG-3′), actAΔ4 (5′-GGCCTGCAGAGCCGCATTCTCAGTTTGTTC-3′), actAΔ5 (5′-TTAATCC CACTTATACTCCCTCCTTCAGTTAACCCCAACTG-3′), and actAΔ6 (5′-T TAATCCCACTTATACTCCCTCCTTCAGTTAACCCCAACTG-3′). Primers actAΔ1 and actAΔ4 contained *Eco*RI and *Pst*I sites, respectively (underlined), to facilitate cloning of the final PCR product into allelic exchange shuttle vector pKSV7 (57). Initial PCRs were carried out with separate reaction mixtures, primers actAΔ1 and actAΔ5, and primers actAΔ4 and the actAΔ6; the products were purified and combined in a second PCR with primers actAΔ1 and actAΔ4. The resulting PCR product contained a deletion of the *actA* promoter region beginning directly downstream of the *mpl* stop codon and extending to the Shine-Delgarno region of *actA*, with a flanking sequence on either side to facilitate homologous recombination. The final PCR product was digested with *Eco*RI and *Pst*I and subcloned into vector pKSV7 to yield vector pNF765. Transfer of the *actA* promoter deletion to the *L. monocytogenes* chromosome in a single copy was carried out by allelic exchange as previously described (20) to generate strain NF-L767, and the deletion was verified by Southern analysis and DNA sequencing of PCR products derived from the *L. monocytogenes* chromosome.

Construction of *L. monocytogenes actA-gus-plcB* transcriptional gene fusion mutants. Primers GUS-XBA (5′-GCTCTAGAAGGAGGAAAAATATGTTTA CGTCCTGTAGAAA-3′) and GUS-PST (5′-GGCTGCAGTCATTGTTTGCC TCCCTGC-3′) were designed to amplify *gus* coding sequences from plasmid pMLK100 (30) by PCR (28) and to introduce a gram-positive ribosome binding site derived from Shine-Delgarno sequence 1 (SD1) of *ermC* (14) (underlined sequence of GUS-XBA) upstream of *gus*. Plasmid pNF333, containing a transcriptional fusion of *actA* to the wild-type *gfp* allele of *Aquorea victoria*, was described previously (21). Plasmid pNF333 was digested with *Xba*I and *Pst*I to remove the wild-type copy of *gfp*, and the appropriately digested *gus* PCR product was subcloned into pNF333 to generate plasmid pNF470. Plasmid pNF470 thereby contains a transcriptional fusion of *actA* to *gus* as well as flanking *L. monocytogenes* regions for introduction of the *actA-gus-plcB* fusion into the *L. monocytogenes* chromosome. Transfer of the *actA-gus-plcB* transcriptional fusion to the *L. monocytogenes* chromosome in a single copy was carried out by allelic exchange as previously described (20) with the following *L. monocytogenes* strains (Table 1): 10403S, to generate NF-L476 (*actA-gus-plcB*); DP-L1465, to generate NF-L707 ($\Delta mpl_{274-510}$ *actA-gus-plcB*); DP-L2296, to generate NF-L861 ($\Delta mpl_{131-361}$ *actA-gus-plcB*); and DP-L2343, to generate NF-L862 (Δ*mpl actA-gus-plcB*). Introduction of the *actA-gus-plcB* reporter gene fusion into *L. monocytogenes* parent strain 10403S did not affect extracellular growth, intracellular growth, cell-to-cell spread, or virulence of the strain in mice (N. Freitag and H. Bouwer, unpublished data).

Complementation analysis of Δ*PactA* and Δ*mpl* deletion mutants with *actA* supplied in a single copy in *trans*. The introduction of the complete *actA* promoter and coding sequence in a single copy within the *comK* locus of the *L. monocytogenes* chromosome in strain NF-L767 to generate strain NF-L904 was accomplished with the assistance of Richard Calendar, Peter Lauer, and Daniel Portnoy, University of California at Berkeley, by using a method based on bacteriophage integration (unpublished data) (see also reference 37).

Metabolic labeling of ActA in infected J774 cells. J774 mouse macrophage-like cells were infected with *L. monocytogenes* as described previously (39). At 2.5 h postinfection, the infected cells were starved for methionine and cysteine, host protein synthesis was blocked with anisomycin (30 μg/ml) and cycloheximide (22.5 μg/ml), and cell-to-cell spread was inhibited with cytochalasin D (0.25 μM). Twenty minutes later, the infected cells were pulse-labeled for 5 min with ^{35}S-methionine as described previously (39). The cells were rapidly chilled on ice, washed four times in cold phosphate-buffered saline, and lysed in 200 μl of 2× sample buffer (0.06 M Tris HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue). The lysates were rapidly frozen in dry ice-ethanol and stored at −80°C for 1 day. Bacterial counts were determined in triplicate for each bacterial strain tested as described previously (39). Samples were resolved on an SDS–8% polyacrylamide gel. The amount of sample loaded per lane was normalized for the number of bacteria. After electrophoresis, the gel was processed for phosphorimaging analysis.

Substitution of the *actA* promoter PrfA binding site with that of *hly*. *L. monocytogenes* genomic DNA was isolated and used in conjunction with PCR (28) to amplify a fragment containing the *actA* promoter and a portion of the N-terminal coding region of *actA* with the following primers: ActA-hlyP (5′-G CGGATCCTGATTAACATTTGTTAAAGAAAAATT-3′), designed to substitute the PrfA box of *actA* with that of *hly* (underlined nucleotides), and ActA-2 (5′-GCTCTAGAGTGTTTTTAATTATTTTTTC-3′). The resulting PCR product was digested with *Xba*I and *Bam*HI and subcloned into pKSV7 (57) to generate plasmid pNF468. Primers mpl-12/97 (5′-GCAGATCTTCAGTTAACC CCAACTGC-3′) and mpl-4 (5′-GCGAATTCGCCTGGTAACGCGAGAAA-3′) were used in conjunction with PCR and *L. monocytogenes* genomic DNA to amplify a product containing the C-terminal coding region of *mpl*. This PCR product was digested with *Eco*RI and *Bgl*II and subcloned into pNF468 to produce plasmid pNF517. Plasmid pNF517 thus contains the modified *actA* promoter with the *hly* PrfA box substitution [designated $P_{(hly)}$-*actA*] and regions of flanking homology for introduction of the $P_{(hly)}$-*actA* promoter mutation into the *L. monocytogenes* chromosome via homologous recombination.

Plasmid pNF517 was introduced into *L. monocytogenes* strain NF-L476 by electroporation, and transformants were isolated following growth at 30°C on BHI agar containing chloramphenicol. *L. monocytogenes* strains containing the $P_{(hly)}$-*actA* promoter mutation in a single copy in the chromosome were isolated following allelic exchange as previously described (20) to generate strain NF-L572 [$P_{(hly)}$-*actA-gus-plcB*] (Table 1). Southern analysis (51) was used to verify the presence of the $P_{(hly)}$-*actA* promoter in a single copy in the correct *L. monocytogenes* chromosomal location by confirmation of a novel *Bst*YI restric-

tion site. The sequence of the $P_{(hly)}$-*actA* mutant promoter was confirmed by DNA sequencing of PCR products amplified from chromosomal DNA by using Thermosequenase (Amersham Life Science, Arlington Heights, Ill.).

Introduction of *prfA7973* (*prfA) into NF-L476 and NF-L572.** *L. monocytogenes* strain NCTC7973, a natural isolate, was described elsewhere (1, 42, 45). The NCTC7973 *prfA* allele, which encodes a serine in place of a glycine at position 145, produces increased expression of virulence genes in NCTC7973 in comparison to wild-type *L. monocytogenes* and is thought to encode a transcriptionally active, cofactor-independent form of PrfA (PrfA*) (49). NCTC7973 PrfA also has a second amino acid change (in comparison to 10403S PrfA), a Cys to Tyr change at position 229; however, this substitution has not been demonstrated to influence PrfA-dependent gene expression (1). pCON1-Δ*prfA*7973 was conjugated into 10403S derivatives NF-L476 and NF-L572 to generate NF-L753 [*prfA* actA-gus-plcB*] and NF-L754 [*prfA** $P_{(hly)}$-*actA-gus-plcB*] as described by Behari and Youngman (1), with the following modifications. Transconjugants were selected on BHI agar containing chloramphenicol (5 μg/ml) after 24 h of incubation at 30°C. Selected colonies were inoculated into BHI medium containing chloramphenicol and streptomycin and grown overnight at 40°C with shaking to force chromosomal integration of the vector. Overnight 40°C cultures were diluted 1:1,000 into fresh BHI medium containing chloramphenicol and streptomycin and incubated overnight at 40°C with shaking. Appropriate dilutions were then plated on BHI agar containing chloramphenicol and incubated at 40°C. Integration of the vector into the chromosome was verified and the *prfA7973* (*prfA**) sequences were confirmed by PCR amplification of *prfA* and sequencing of the PCR products.

Assay for hemolytic activity. Stationary-phase bacteria were diluted 1:10 into BHI medium and grown at 37°C for 5 h with shaking. The supernatant fluid was assayed for hemolytic activity as previously described (9).

Plaque formation in L2 cells. Plaque assays were performed as previously described by Sun et al. (58). Plaque size was measured by using a micrometer, and the average diameter of at least 10 plaques from three independent experiments was determined.

Intracellular growth assays. The cell line used in these studies was the J774 mouse macrophage-like cell line maintained as previously described (6). Intracellular growth in J774 cells and in mouse bone marrow-derived macrophages was monitored by using cell monolayers grown on acid-washed glass coverslips in tissue culture dishes as previously described (20, 48, 58).

β-Glucuronidase (GUS) assays of bacteria grown in liquid cultures. For experiments with BHI broth, BHI broth treated with 0.2% charcoal (charcoal removed by filtration of autoclaved medium), or Luria broth (LB), overnight cultures of bacteria grown in the indicated broth media were diluted 1:10 into fresh media and grown for 5 h with shaking at 37°C. For cultures incubated in Dulbecco's minimal essential medium (MEM; GIBCO-BRL, Rockville, Md.), overnight cultures of bacteria were diluted into BHI broth as described above and grown for 3 h with shaking at 37°C. Aliquots (3 ml) of cultures were removed and centrifuged briefly to recover bacteria, and then the bacterial pellets were washed in phosphate-buffered saline and resuspended in 3 ml of MEM. Bacterial suspensions in MEM were incubated at 37°C for 2 h. Bacterial pellets from 1-ml culture aliquots were collected following centrifugation and quickly frozen on dry ice. The optical density at 595 nm was measured for each culture by using a Spectronic 20 spectrophotometer (Milton Roy, Rochester, N.Y.). In some experiments, aliquots of bacterial cultures were diluted and plated on LB agar plates to determine CFU per milliliter.

For GUS enzymatic assays, bacterial cell pellets were thawed, washed once with ABT buffer (0.1 M potassium phosphate [pH 7.0], 0.1 M NaCl, 0.1% Triton) and resuspended in 200 μl of ABT buffer. GUS activity was measured as described by Youngman (61), with the substitution of 4-methylumbilliferyl-β-D-glucuronide in place of 4-methylumbilliferyl-β-D-galactoside. Units were normalized to bacterial CFU to enable direct comparison of GUS activity between broth-grown cultures and bacteria isolated from infected tissue culture cells. In some experiments, in which units of GUS activity were compared for broth-grown cultures, the units were normalized to the optical density at 595 nm. Background activity from negative control samples was subtracted from all samples.

Intracellular GUS assays. Measurement of intracellular GUS activity was carried out by using the protocol described by Moors et al. (43) and J774 cells, with the substitution of 4-methylumbelliferyl-β-D-glucuronide as a substrate in place of 4-methylumbilliferyl-β-D-galactoside.

Virulence phenotype. LD_{50}s were determined by intravenous injection of BALB/c mice as previously described (47).

RESULTS

Measurement of extracellular and intracellular *actA* expression levels.

To better define the promoter elements that contribute to the intracellular induction of *actA* expression, a series of *actA* and *mpl* promoter and coding region mutants were constructed and introduced into the *L. monocytogenes* chromosome. The constructs were designed such that the loss of promoter function (promoter deletions) could be phenotypically contrasted with the loss of gene function (coding sequence deletions). To facilitate the measurement of *actA* expression levels, transcriptional fusions between *actA* and the reporter gene *gus* were constructed and introduced into each *L. monocytogenes* mutant strain (Fig. 1). *gus* encodes the enzyme β-glucuronidase (GUS) (29) and has been used successfully to generate reporter gene fusions in a variety of bacteria (19, 30, 53), including *L. monocytogenes* (1).

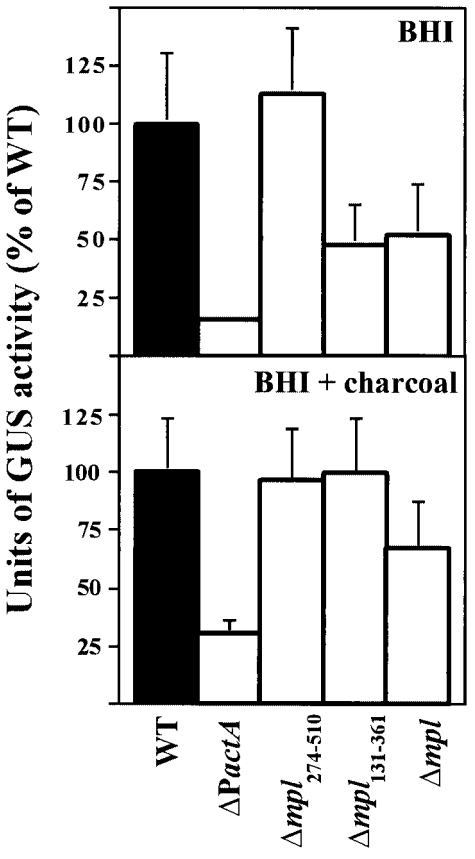

FIG. 2. Examination of *actA* expression in *L. monocytogenes mpl* and *actA* mutant strains grown in broth media. GUS activity was measured following 5 h of growth in BHI broth or BHI broth treated with 0.2% charcoal. Units of GUS were normalized for optical density at 595 nm as described by Youngman (61) for the measurement of β-galactosidase activity but with the appropriate substrate substitution of 4-methylumbilliferyl-β-D-glucuronide. Units are expressed as a percentage of NF-L476 (wild-type [WT]) activity in BHI broth or BHI broth treated with 0.2% charcoal. Each assay was done in triplicate, and the data represent the mean and SE for at least three individual experiments.

As shown in Fig. 2, the Δ*PactA* mutant strain lacking the *actA* proximal promoter showed a significant, sixfold decrease compared to the wild type in the levels of *actA* expression in standard broth media (BHI) (Fig. 2). The $\Delta mpl_{131-361}$ and Δ*mpl* mutants also showed reduced expression levels, but to a

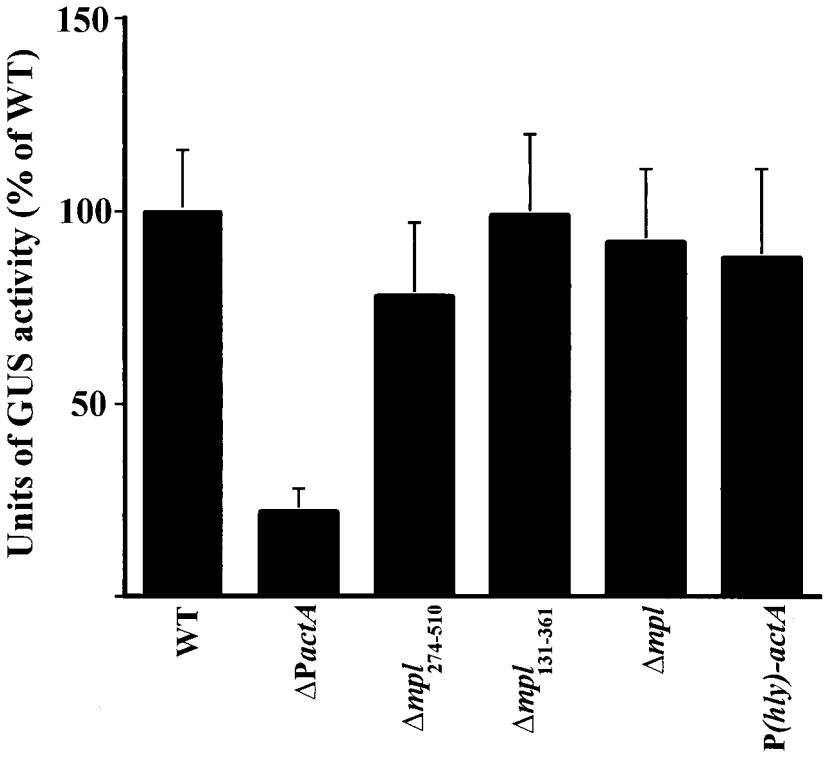

FIG. 3. Intracellular *actA* expression in *mpl* and *actA* mutant strains. Tissue culture dishes containing monolayers of J774 cells were infected with the indicated *L. monocytogenes* strains for 5 h as described in Materials and Methods. GUS activity was determined following lysis of the monolayers. The number of CFU per dish was determined by lysing infected J774 cells grown on coverslips in duplicate dishes and plating a portion of the lysates on LB agar. The total number of CFU per dish was extrapolated by multiplication by a factor that corrected for the area of the coverslip relative to that of the 60-mm dish. Units of GUS are as described by Youngman (61) for the measurement of β-galactosidase activity but with the appropriate substrate substitution of 4-methylumbilliferyl-β-D-glucuronide. Units are expressed as a percentage of NF-L476 (wild-type [WT]) activity. Each assay was done in triplicate, and the data represent the mean and SE for at least three individual experiments. Background activities from 10403S-infected monolayers and from uninfected cells were equivalent and never represented greater than 2% of the activity detected for NF-L476.

lesser degree (approximately twofold lower than the wild-type expression level). Levels of *actA* expression were not reduced in the *mpl* frameshift mutant, Δ*mpl*$_{274–510}$. It was previously demonstrated that *actA* expression is up-regulated following the growth of *L. monocytogenes* in BHI broth treated with 0.2% activated charcoal (5, 50) (Fig. 2). The Δ*actA* mutant demonstrated lower overall levels of *actA* expression in comparison to the wild type and the *mpl* deletion mutants following growth in BHI broth treated with 0.2% charcoal; however, expression was still induced approximately 11-fold over the levels in BHI broth (mean and standard error [SE], 3 ± 1 U in BHI broth versus 33 ± 6 U in BHI broth-charcoal). The remaining mutants did not differ significantly from the wild type in *actA* expression levels. Taken together, these data suggest that the *actA* proximal promoter is the major regulatory element contributing to the levels of *actA* expression observed in broth cultures. A modest contribution from the *mpl* promoter could be observed in BHI broth but was not evident following treatment of BHI broth with 0.2% activated charcoal. Interestingly, the induction of *actA* expression in BHI broth–0.2% charcoal occurred in all of the mutant strains, but the highest level of induction (11-fold) was observed in the Δ*actA* mutant, which contains the complete *mpl* promoter and coding sequences.

ActA is one of the most abundant surface proteins expressed by *L. monocytogenes* within the host cell cytosol (5, 6). Moors et al. (43), using an *actA-lacZ* reporter gene fusion, recently demonstrated that *actA* expression is highly induced (226-fold) in bacteria grown in the mouse macrophage-like cell line J774. To compare *actA* expression by use of *gus* transcriptional fusions in cytosolic bacteria, J774 cells were infected with *L. monocytogenes*; at 5 h postinfection, the cells were lysed, aliquots were removed to determine the numbers of viable bacteria, and GUS activity was measured (Fig. 3). We found that the amount of *actA-gus* expression observed for wild-type *L. monocytogenes* following intracellular growth was approximately 150 times the amount produced by bacteria grown in LB (mean and SE, 61.0 ± 1.1 U/10^6 CFU in J774 cells and 0.4 ± 0.1 U/10^6 CFU in LB); this high-level induction of *actA* expression agrees well with the 226-fold level of induction reported by Moors et al. (43). The amount of *actA* expression observed in the host cytosol was 47 times the amount produced by bacteria grown in BHI broth treated with 0.2% charcoal (1.3 ± 0.5 U/10^6 CFU in BHI broth treated with 0.2% charcoal). The levels of *actA* expression were approximately fourfold lower in the Δ*actA* mutant than in the wild type; all other mutants showed no statistically significant difference in *actA* expression following cytosolic growth.

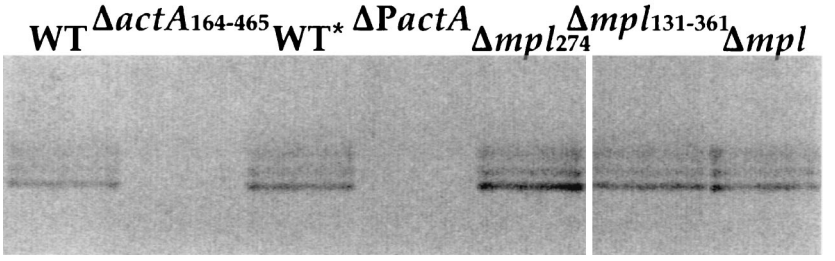

FIG. 4. Intracellular expression of ActA by *L. monocytogenes* in J774 cells. J774 mouse macrophage-like cells were infected with *L. monocytogenes*; at 2.5 h postinfection, the infected cells were starved for methionine and cysteine, host protein synthesis was blocked, and cell-to-cell spread was inhibited as described in Materials and Methods. Infected cells were pulse-labeled with ^{35}S-methionine prior to host cell lysis, and SDS-extractable bacterial proteins were resolved on an SDS-8% polyacrylamide gel. The amount of sample loaded per lane was normalized for the number of bacteria. The three species of ActA, representing different phosphorylation states of the protein, are the major protein bands observed by using this approach (6). WT and WT*, proteins derived from 10403S- and NF-L476-infected cells, respectively.

To confirm the *actA* expression patterns observed for the *L. monocytogenes* mutant strains with GUS reporter assays, metabolic labeling of bacteria grown in the macrophage-like cell line J774 was carried out, and ActA was extracted and detected as described in Materials and Methods (Fig. 4). In agreement with the reporter gene fusion data, the Δ*PactA* mutant demonstrated a significant reduction in ActA protein levels in comparison to the wild type, as little to no protein could be detected. With the exception of the *actA* coding region deletion mutant, Δ$actA_{164–465}$, the remaining mutants produced ActA at levels comparable to those seen with the wild type. These results indicate that the primary promoter responsible for the induction of *actA* expression within the cytosol of infected host cells is the *actA* proximal promoter and that the *mpl* promoter contributes little, if any, to the intracellular induction of *actA* expression.

To assess the effects of the *actA* and *mpl* deletion mutations on the capacity of *L. monocytogenes* to grow and spread within infected host cells, the mutants were examined for their ability to form plaques in monolayers of mouse L2 cells (Table 2). All of the *actA* and *mpl* deletion mutants formed smaller plaques than wild-type *L. monocytogenes*, indicative of defects in intracellular growth and/or cell-to-cell spread. The most severe defects were observed for the Δ*PactA* and Δ$actA_{164–465}$ mutants, which formed tiny plaques representing a 93% reduction in plaque size in comparison with the plaques formed by wild-type *L. monocytogenes*. The defect in the plaque formation of Δ*PactA* could be partially complemented by the introduction of a single copy of *actA* into the *comK* locus of *L. monocytogenes* (Table 2). The degree of complementation of the Δ*PactA* mutant (78%) was similar to that observed for the Δ$actA_{164–465}$ mutant following the introduction of *actA*::*comK* (37). The Δ$actA_{164–465}$ mutant was previously demonstrated to be defective for the nucleation of actin filaments and impaired for cell-to-cell spread (6). The Δ*PactA* mutant was also defective for cell-to-cell spread within monolayers of J774 cells and formed microcolonies within infected cells that were similar in appearance to those formed by the Δ$actA_{164–465}$ mutant (H. Marquis, unpublished data). It therefore appears that the loss of the *actA* proximal promoter results in a phenotype equivalent to that observed for mutants that lack a functional ActA protein.

TABLE 2. Plaque formation in L2 cells and virulence phenotype analysis

Strain	Plaque size (% of wild type)	Virulence phenotype (LD_{50})
10403S	100	1×10^4–3×10^4[a]
NF-L476	100	1×10^4–3×10^4
DP-L1942	7	2×10^7[b]
NF-L767	6	$\sim 5 \times 10^6$
DP-L1465	53	2×10^6
DP-L2296	71	$<5 \times 10^4$[c]
DP-L2343	60	7×10^6
NF-L904	78	ND[d]
NF-L572	108	ND

[a] Reference 47.
[b] Reference 6.
[c] Reference 39.
[d] ND, not determined.

Deletion of the *mpl* promoter and coding region resulted in plaques that were 60% the size of wild-type *L. monocytogenes* plaques (Table 2). The Δ*mpl* mutant defect in plaque formation was less severe than that observed for the Δ*PactA* or Δ$actA_{164–465}$ mutant; however, the Δ*mpl* mutant did exhibit a dramatic decrease in virulence in mice (2 orders of magnitude), similar to the decreases observed for the Δ*PactA* and Δ$actA_{164–465}$ mutants. In contrast, deletion of the Mpl protease active site (Δ$mpl_{131–361}$) did not reduce virulence in mice (LD_{50}, $<5 \times 10^4$), although plaque size was moderately reduced (70%). The reduction in virulence for the Δ*mpl* mutant does not appear to result from decreased expression of *actA*, as ActA protein levels were not significantly reduced in this mutant (Fig. 3 and 4). Interestingly, the Δ$mpl_{274–510}$ mutant, which contains a functional *mpl* promoter but lacks the C-terminal half of the protein, shows a 2-log-unit reduction in virulence in mice (LD_{50}, 2×10^6). The virulence defect observed for Δ$mpl_{274–510}$ but not for Δ$mpl_{131–361}$ suggests that the C-terminal portion of the Mpl protein, beyond the active site for the protease, contributes a significant role toward the function of Mpl and its role in *L. monocytogenes* pathogenesis.

Introduction of a high-affinity PrfA DNA binding site within the *actA* promoter. The experiments described above defined the *actA* proximal promoter as the major regulatory element contributing to the induction of *actA* expression within the cytosol of infected host cells. To further investigate what nucleotide motifs are important for promoter activation, we di-

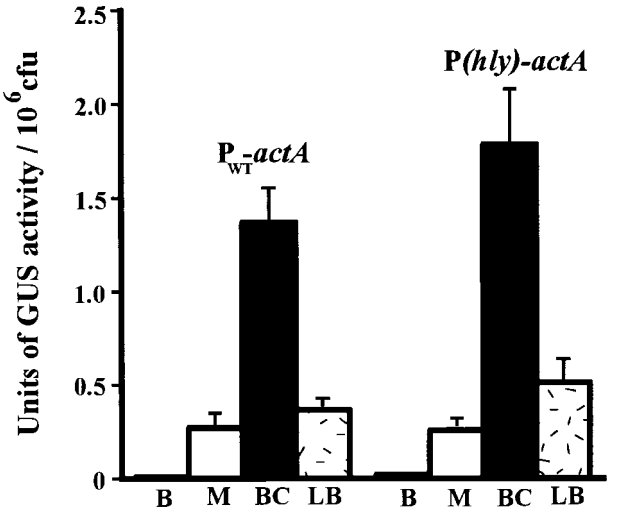

FIG. 5. Examination of *actA* expression in $P_{(hly)}$-*actA* and P_{WT}-*actA* *L. monocytogenes* strains grown in various broth media. GUS activity was measured following 5 h of growth in BHI broth (B), MEM (M), BHI broth treated with 0.2% charcoal (BC), and LB. Units of GUS are as described by Youngman (61) for the measurement of β-galactosidase activity but with the appropriate substrate substitution of 4-methylumbilliferyl-β-D-glucuronide. Each assay was done in triplicate, and the data represent the mean and SE for at least three individual experiments.

rected our focus on the PrfA binding site of the *actA* promoter. *actA* is strictly dependent upon PrfA for expression (2). The *actA* proximal promoter contains a PrfA DNA binding site in the −40 region that is an imperfect palindrome and that is thought to represent a low-affinity binding site for the transcriptional activator (22, 23, 54). To determine if the introduction of a high-affinity PrfA DNA binding site within the *actA* promoter would result in an increased level of *actA* expression, the PrfA box of the *actA* promoter was replaced with a high-affinity site derived from *hly* by allelic exchange as described in Materials and Methods. The *actA* promoter mutation [$P_{(hly)}$-*actA*] was introduced in a single copy into the chromosome of *L. monocytogenes* strain NF-L476, containing the *actA-gus-plcB* transcriptional reporter gene fusion (Table 1). The wild-type *actA* gene and promoter are referred to from this point on as P_{WT}-*actA* to distinguish this construct from the $P_{(hly)}$-*actA* promoter mutant.

Previous studies demonstrated that *hly* transcription occurs at levels 10-fold higher than those for *actA* following bacterial growth in LB cultures (43). To determine if *actA* transcriptional activation in broth cultures was increased by the introduction of the $P_{(hly)}$-*actA* promoter mutation, we measured *actA* expression in *L. monocytogenes* strains containing chromosomal *actA-gus* transcriptional reporter gene fusions following growth under a variety of broth culture conditions (Fig. 5). Consistent with results reported in previous studies (5, 6, 50), only low levels of *actA* expression were detected for wild-type *L. monocytogenes* following 5 h of growth in BHI broth (Fig. 5). Significantly higher levels of *actA* expression were seen following growth in LB or after 2 h of incubation in tissue culture medium (MEM), as first reported by Bohne et al. (4, 5). The highest levels of *actA* expression were achieved following 5 h of growth in BHI broth treated with 0.2% activated charcoal (Fig. 5). For all culture media tested, there was no significant difference in *actA* expression levels between *L. monocytogenes* strains containing either P_{WT}-*actA* or $P_{(hly)}$-*actA*. In agreement with data obtained from the *gus* reporter gene fusion experiments, similar amounts of ActA were detected in the P_{WT}-*actA* and $P_{(hly)}$-*actA* strains by SDS-polyacrylamide gel electrophoresis analysis of surface proteins after 5 h of bacterial growth in LB (L. Shetron-Rama and N. Freitag, unpublished data). These data indicate that substitution of the *actA* promoter PrfA binding site with that of the *hly* promoter produced no significant changes in the levels of extracellular *actA* expression.

It was demonstrated previously that multiple copies of the *hly* PrfA DNA binding site may reduce the transcription of PrfA-dependent genes in *L. monocytogenes* by titrating available PrfA (4, 10). However, the P_{WT}-*actA* and $P_{(hly)}$-*actA* strains produced similar levels of *hly*-encoded listeriolysin O, as measured by secreted-hemolysin assays [mean and SE, 18 ± 2 U for 10403S, 20 ± 1 U for the P_{WT}-*actA* strain, and 25 ± 5 U for the $P_{(hly)}$-*actA* strain], thus indicating that the single extra copy of the *hly* PrfA box resulted in no significant titration effects.

To compare P_{WT}-*actA* and $P_{(hly)}$-*actA* expression by using *gus* transcriptional fusions in cytosolic bacteria, J774 cells were infected with *L. monocytogenes*; at 5 h postinfection, the cells were lysed and GUS activity was measured (Fig. 3). As observed for bacteria grown in broth cultures, the expression of *actA* in the *L. monocytogenes* $P_{(hly)}$-*actA* promoter mutant was not significantly different from wild-type *actA* expression (Fig. 3). Furthermore, no defect was observed for this mutant with regard to intracellular growth and/or cell-to-cell spread in mouse L2 cells, macrophage cell lines, or primary mouse bone marrow-derived macrophages (Table 2) (Shetron-Rama and Freitag, unpublished). Thus, the introduction of a high-affinity PrfA binding site into the *actA* promoter resulted in no change in either extracellular or intracellular patterns of *actA* expression.

***actA* promoter activation in *L. monocytogenes* strains containing PrfA*.** The *prfA** mutant allele appears to encode a constitutively activated form of the PrfA protein (PrfA*) which is thought to resemble CRP*, which is active in the absence of cofactor binding (32, 35, 49). PrfA* has been demonstrated to have an increased binding affinity for target DNA (59). To quantitate and compare the effects of PrfA* on P_{WT}-*actA* and $P_{(hly)}$-*actA* promoter activation, the *prfA** allele from *L. monocytogenes* strain NCTC7973 was introduced into the chromosome in place of wild-type *prfA* in strains NF-L476 and NF-L572 as described in Materials and Methods. NCTC7973 PrfA also has a second amino acid change (in comparison to 10403S PrfA), a Cys-to-Tyr change at position 229; however, this substitution has not been demonstrated to influence PrfA-dependent gene expression (1). *actA-gus* expression was measured following bacterial growth in BHI broth and BHI broth treated with 0.2% charcoal (Fig. 6). The introduction of *prfA** resulted in an 11-fold stimulation of *actA* expression for bacteria grown in BHI broth (mean and SE, 0.01 ± 0.004 U/10^6 CFU for NF-L476 versus 0.11 ± 0.02 U/10^6 CFU for NF-L752) and an approximately 3-fold stimulation for bacteria grown in BHI broth treated with 0.2% activated charcoal. No significant difference was seen in expression levels between P_{WT}-*actA* and

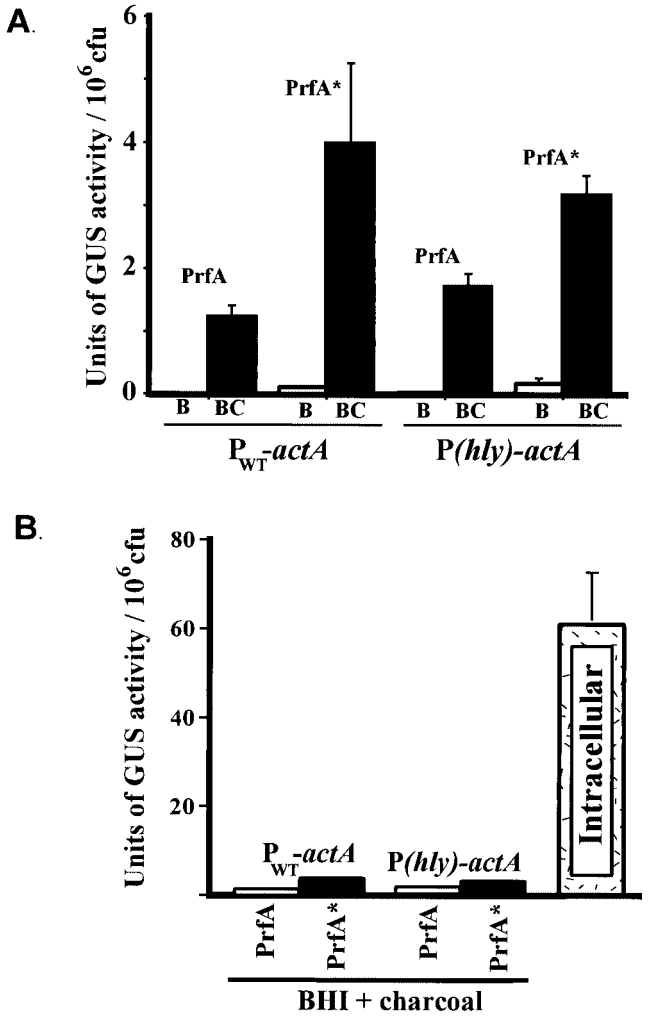

FIG. 6. Comparison of P_{WT}-*actA* and $P_{(hly)}$-*actA* promoter activation in *L. monocytogenes* strains containing PrfA or PrfA*. GUS activity was measured following 5 h of growth in BHI broth or BHI broth treated with 0.2% charcoal or following bacterial growth in J774 cells. Units of GUS are as described by Youngman (61) for the measurement of β-galactosidase activity but with the appropriate substrate substitution of 4-methylumbilliferyl-β-D-glucuronide. Each assay was done in triplicate, and the data represent the mean and SE for at least three individual experiments. (A) Measurement of *actA-gus* expression in cultures following growth in either BHI broth (B) or BHI broth treated with 0.2% charcoal (BC). (B) Comparison of the levels of *actA-gus* expression achieved under optimal extracellular conditions versus the level of activity observed for *L. monocytogenes* located within the host cell cytosol (intracellular). The intracellular value shown is for NF-L476. Background activities from 10403S-infected monolayers and from uninfected cells were equivalent and never represented greater than 2% of the activity detected for NF-L476.

$P_{(hly)}$-*actA L. monocytogenes* mutant strains, indicating that the transcriptional activation of *actA* by PrfA* was not influenced by the introduction of a high-affinity PrfA binding site within the *actA* promoter (Fig. 6A). Interestingly, the introduction of the *prfA** allele resulted in levels of *actA* expression following growth in BHI broth that were 555-fold lower than those observed for intracellular bacteria (0.11 ± 0.02 U/10^6 CFU versus 61.0 ± 10.6 U/10^6 CFU) (Fig. 6B). The expression of *actA* in *prfA**-containing bacteria grown in BHI broth-charcoal was highly induced in comparison to the results for strains grown in BHI broth, yet the absolute level of induction was still approximately 20-fold lower than the level achieved by *L. monocytogenes* within the host cytosol (Fig. 6B). No significant difference was seen between intracellular levels of either P_{WT}-*actA* or $P_{(hly)}$-*actA* expression for *L. monocytogenes* strains containing wild-type *prfA* or *prfA** (data not shown).

DISCUSSION

L. monocytogenes provides an excellent model system for defining the responses of an intracellular pathogen to the host cell environment. In this study, we have chosen to focus on identifying the promoter regions that contribute to the intracellular induction of *actA*, a gene that is highly induced once *L. monocytogenes* reaches the cytosol of infected host cells (5, 6, 21, 43). Although two promoters have been reported to contribute to *actA* expression (5), the data presented in this study indicate that only the *actA* proximal promoter is required for intracellular induction. The increase in *actA* expression mediated by the *actA* proximal promoter is impressive, as cytosolic expression levels were found to be 150 times greater than the levels of expression observed for cultures grown in LB and 46 times greater than the levels observed for cultures grown in BHI broth treated with activated charcoal. The large increase observed in *actA* expression for intracellular bacteria could not be duplicated in broth cultures by the introduction of a higher-affinity PrfA binding site within the *actA* promoter or the mutationally activated form of PrfA (PrfA*), suggesting that additional steps or cofactors are required for the intracellular induction of *actA* expression.

The upstream *mpl* promoter functions as a minor contributor to patterns of *actA* expression, but its presence is required for full virulence. Comparative studies of the $\Delta mpl_{274–510}$, $\Delta mpl_{131–361}$, and Δmpl mutant strains indicate that the Mpl protein may play an additional role in *L. monocytogenes* pathogenesis beyond the processing of proPC-PLC to mature PC-PLC (a broad-spectrum phospholipase C). It has been demonstrated that the processing of proPC-PLC to the mature form in infected cells occurs via both Mpl-dependent and Mpl-independent pathways, the latter being mediated by host cell cysteine proteases (39). The mutant strain containing the in-frame deletion of the protease active site ($\Delta mpl_{131–361}$) was not attenuated for virulence in a mouse model of infection (38) (Table 2). In contrast to the $\Delta mpl_{131–361}$ mutant, a mutant lacking the C-terminal half of Mpl ($\Delta mpl_{274–510}$) or containing a complete deletion of the *mpl* coding sequences (Δmpl) exhibited a dramatic 2-log-unit decrease in virulence. Complementation of the *mpl* promoter and gene deletion mutant could not be achieved by providing a copy of *actA* in *trans*, suggesting that the defect in virulence was not due to insufficient synthesis of *actA* (Shetron-Rama and Freitag, unpublished). These results were further supported by experiments demonstrating that *actA* expression occurs at levels similar to those in wild-type strains in the absence of the *mpl* promoter (Fig. 3 and 4). The results presented here therefore suggest that the functional roles of Mpl in *L. monocytogenes* pathogenesis have yet to be completely defined. Experiments designed to more closely examine the contributions of

Mpl to *L. monocytogenes* intracellular growth and cell-to-cell spread are currently in progress.

The Δ*PactA* promoter deletion mutant was found to closely resemble the Δ$actA_{164–465}$ gene deletion mutant in tissue culture infection assays, and both mutants were highly attenuated for virulence in mice (Table 2). However, the LD_{50} for the *actA* promoter deletion mutant was approximately fourfold lower than that for the Δ$actA_{164–465}$ mutant. It is possible that the low level of intracellular *actA* expression contributed by the upstream *mpl* promoter (Fig. 3) accounts for the difference in virulence between the Δ$actA_{164–465}$ and Δ*PactA* mutants. Low-level *actA* expression may provide enough functional ActA for some cell-to-cell spread, although this was not apparent in L2 cell plaque assays (Table 2). Alternatively, it is possible that PlcB activity is toxic for host cells in the absence of cell-to-cell spread of the bacteria. Attenuation of virulence as a result of host cell toxicity has been reported for *L. monocytogenes hly* mutants lacking a PEST-like sequence (13). It is likely that normal levels of PlcB are expressed in the Δ$actA_{164–465}$ mutant strain, whereas the loss of the *actA* promoter should greatly diminish *plcB* expression.

Previous studies suggested that the timing of PrfA-dependent promoter activation and the levels of transcriptional induction achieved are both closely tied to the binding affinity of PrfA for specific target promoters (22, 23, 54). The data presented here, in agreement with a recent study carried out with *Bacillus subtilis* (60), suggest that the presence of a high-affinity PrfA DNA binding site does not significantly influence the PrfA-dependent activation of all target promoters. Intracellular induction of virulence gene expression in *L. monocytogenes* therefore appears to be a complex process requiring multiple events, including increased production of PrfA protein, activation of PrfA, and induction or activation of a second bacterial factor (or factors) within the host cytosol. The existence of a PrfA-activating factor has been reported (3, 15), but thus far, other than RNA polymerase (2), no other cofactors that participate in *L. monocytogenes* virulence gene regulation have been identified. It is, of course, possible that the product of the mutationally activated *prfA** allele does not fully mimic the environmentally activated form of the wild-type protein. Work in progress, however, indicates that it is possible to isolate *L. monocytogenes* mutants which have increased *actA* expression in broth culture and which contain mutations mapping outside the *prfA* regulon (Shetron-Rama and Freitag, unpublished). Characterization of these mutants will aid in the identification of additional factors that contribute to the regulation of *L. monocytogenes* virulence gene expression. Indeed, it is possible that the induction of *actA* expression within the host cell cytosol does not require PrfA but instead relies on the activity of other factors. This possibility will be explored in future studies.

It has been shown that *hly* and *actA* differ in their patterns of expression within infected host cells (8, 43). The *hly* and *plcA* promoters have been reported to be predominantly activated in the phagosomal compartment, while those for *actA* and *inlC* are predominantly activated in the host cell cytosol (8). We investigated whether the $P_{(hly)}$-*actA* promoter mutation resulted in altered patterns of *L. monocytogenes actA* expression with respect to cell compartment location by introducing the mutation into *L. monocytogenes* strains containing a transcriptional fusion of *gfp* to *actA* in the chromosome. *gfp* was successfully used in previous studies as a reporter gene system to monitor the timing and intracellular location of *actA* expression through the use of fluorescence microscopy (8, 21). No significant difference was observed for either the timing or the location of intracellular *actA* expression with the $P_{(hly)}$-*actA* mutant (Shetron-Rama and Freitag, unpublished).

We have focused our attention on the mechanisms of induction of *actA* expression as a means of characterizing the events that lead to intracellular gene expression in *L. monocytogenes*. The high-level induction of *actA* expression that occurs within the host cytosol is impressive, and it accents the ability of *L. monocytogenes* to respond to specific host cell environments. By isolating and analyzing *L. monocytogenes* mutants with altered patterns of intracellular gene expression, we hope to be able to identify additional components of the regulatory machinery and perhaps to gain insight into the nature of the intracellular signals used by this pathogen to guide its infectious processes.

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