INTRODUCTION

The ability to coordinate the expression of virulence factors in response to specific environmental stimuli is an important facet of bacterial pathogenesis. Alterations in patterns of virulence gene expression have been observed in vitro in response to a variety of environmental parameters such as changes in temperature, pH, O$_2$ concentrations, nutrient conditions and essential metal ion deficiency (Datta & Kothary, 1993; Bohne et al., 1996; Garcia Vescovi et al., 1996; Soncini et al., 1996; Cotter & DiRita, 2000; Swanson & Hammer, 2000; Wosten et al., 2000; Kreft & Vazquez-Boland, 2001). Regulation of pathogen virulence gene expression appears to be finely coordinated with environmental cues in that multiple stimuli may be required to trigger full activation of expression (Cotter & Miller, 1997). The patterns of regulation of bacterial gene expression observed for pathogens in vitro presumably reflect the ability of the bacteria to adapt to specific host cell and tissue environments in vivo. Experimental models of host infection indicate that bacterial virulence genes are regulated in a manner that allows the differential expression of virulence factors at specific phases of infection or within defined host cell compartments (Slauch et al., 1994; Mahan et al., 1995; Cotter & Miller, 1997; Bubert et al., 1999; Freitag & Jacobs, 1999; Lee & Camilli, 2000; Slauch & Camilli, 2000; Stanley et al., 2000).

We have been using the facultative intracellular bacterial pathogen Listeria monocytogenes as a model system to define the mechanisms used by a prokaryotic parasite to regulate virulence gene expression within host cells. L. monocytogenes is responsible for serious infections in immunocompromised individuals and pregnant women (Gray & Killinger, 1966; Gellin & Broome, 1989; Vazquez-Boland et al., 2001). The bacterium expresses a variety of virulence factors that enable it to invade many host cell types and to gain entry into the cell cytosol where bacterial replication occurs. L. monocytogenes regulates the expression of virulence genes in response to specific host cell compartment environments, including those of the phagosome and cytosol (Bubert et al., 1999; Freitag & Jacobs, 1999; Moors et al., 1999; Kreft &
Vazquez-Boland, 2001). The majority of the *L. monocytogenes* virulence determinants that have been thus far identified are regulated by a transcriptional activator known as PrfA, a 27 kDa site-specific DNA binding protein that is essential for *L. monocytogenes* pathogenesis (Mengaud *et al*., 1991; Chakraborty *et al*., 1992; Freitag *et al*., 1993).

While it is clear that PrfA is a key regulatory element required for the control of virulence gene expression in *L. monocytogenes*, it is not clear what controls its activity or how prfA expression is regulated. Three distinct promoters contribute to prfA expression. Two promoters, prfAP1 and prfAP2, are located immediately upstream of the prfA coding region (Freitag & Portnoy, 1994) (Fig. 1). The third promoter is contributed by the upstream plcA gene through the generation of a bicistronic plcA–prfA transcript (Camilli *et al*., 1993; Freitag *et al*., 1993). PrfA positively regulates its own expression through the activation of plcA transcription, and the increase in PrfA synthesis resulting from the generation of the prfA–plcA transcript is essential for spread of the bacteria from the initial infected cell to adjacent cells and for full virulence (Camilli *et al*., 1993; Freitag *et al*., 1993; Freitag & Portnoy, 1994). PrfA also appears to negatively regulate its own expression from the prfAP1 and prfAP2 promoters, as transcripts directed by these two promoters are significantly increased in the absence of functional PrfA (Freitag *et al*., 1993). It has been proposed that *L. monocytogenes* regulates the amounts of available PrfA protein through the use of a negative feedback loop that involves PrfA binding to the −35 region of prfAP2 and inhibition of transcriptional initiation at that promoter (and possibly at prfAP1) by an unknown mechanism (Freitag & Portnoy, 1994). The existence of this negative feedback loop has been postulated to be an important facet of *L. monocytogenes* virulence gene expression.

To begin to address the role of negative regulation of prfA expression in *L. monocytogenes* pathogenesis, we describe here the introduction of several defined prfA promoter mutations that result in loss of prfA negative regulation and significantly increased synthesis of PrfA. Levels of PrfA synthesis in broth-grown cultures of the promoter mutants were found to be greater than the amounts of PrfA reported to be induced following entry of the bacteria into the host cell cytosol (Renzoni *et al*., 1999). High level PrfA production resulted in increased levels of PrfA-regulated gene expression in broth-grown cultures; however the apparent loss of negative prfA regulation had no deleterious effects on growth and spread of the bacteria within infected tissue culture cells or on virulence in mouse models of infection.

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Fig. 1. Construction of *L. monocytogenes* strains containing prfA promoter mutations. (a) Organization of the plcA–prfA operon. The stem–loop structure denotes the rho-independent transcriptional terminator located downstream of plcA. The asterisks indicate the region shown in detail in (b). (b) Detailed depiction of the mutations introduced into the prfA upstream region. The stop codon for plcA and the start codon for prfA are underlined. The stem of the plcA terminator is indicated by the dashed line, and boxed sequences represent the sequences deleted for the plcA–ΔT and prfAΔP-35 mutants. Sequence within the heavy black box represents the PrfA DNA binding site from the hly promoter introduced into the prfA promoter region. Small arrows indicate the start of transcription for transcripts originating from the plcA, prfAP1 and prfAP2 promoters. All mutations were introduced in single copy into the *L. monocytogenes* chromosome by allelic exchange and verified by DNA sequencing.
Our results suggest that although negative regulation of prfA expression provides a feedback system to control PrfA levels, this feedback system is dispensable for virulence.

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 LD<sub>50</sub> for mice of 2 × 10<sup>4</sup> (Freitag et al., 1993). L. monocytogenes was stored at −70 °C in brain–heart infusion broth (BHI, Difco Laboratories) containing 20% (v/v) glycerol. Escherichia coli HB101 or DH5a was used as the host strain for recombinant plasmids. Antibiotics were used at the following concentrations unless otherwise noted: carbenicillin, 50 μg ml<sup>−1</sup>; chloramphenicol, 10 μg ml<sup>−1</sup>; streptomycin, 200 μg ml<sup>−1</sup>.

The thermo-sensitive plasmid vectors pKSV7 (Smith & Youngman, 1992) and pCON1-prfA7973 (Behari & Youngman, 1998) have been described previously.

Construction of L. monocytogenes prfA promoter mutants. The introduction of targeted deletion and substitution mutations within the prfA promoter region was accomplished using the Kunkel method of site-directed DNA mutagenesis with M13 bacteriophage-derived vectors as previously described (Ausubel et al., 1991; Freitag & Portnoy, 1994). prfA mutant constructs were sequenced to verify desired mutations and then fragments containing the mutations were subcloned into the thermo-sensitive shuttle plasmid vector pKSV7 (Smith & Youngman, 1992). Mutations were introduced into the L. monocytogenes chromosome of strain NL-1476 [containing a transcriptional fusion of actA with the gus reporter gene (Shetron-Rama et al., 2001)] by allelic exchange as previously described (Freitag & Portnoy, 1994) to generate strains NL-1623 (containing plcA-ΔT + prfAΔP2-35); NL-1625 (containing plcA-ΔT); NL-1627 (containing prfAΔP2(hly)); and NL-1629 (containing prfAΔP2-35). Chromosomal mutations were confirmed by sequencing of PCR amplified products derived from genomic DNA isolated from each mutant strain.

Introduction of prfA-7973 (prfA<sup>+</sup>) into L. monocytogenes mutant strains. L. monocytogenes strain NCTC 7973, a natural isolate, has been described (Park & Kroll, 1993; Millenbachs et al., 1997; Behari & Youngman, 1998). The NCTC 7973 prfA allele, which contains a serine in place of a glycine at position 145, produces increased expression of virulence genes in NCTC 7973 in comparison to wild-type L. monocytogenes and is thought to represent a transcriptionally active, cofactor-independent form of PrfA protein (PrfA<sup>+</sup>) (Ripio et al., 1997). The NCTC 7973 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 (Behari & Youngman, 1998). pCON1-prfA7973 was used to insertionally inactivate the wild-type prfA allele and to introduce the prfA<sup>+</sup> allele under the control of the wild-type prfA promoter (Behari & Youngman, 1998). pCON1-prfA7973 conjugated into 10403S derivatives NL-1476, NL-1623, NL-1625, NL-1627 and NL-1629 to generate NL-1657 (prfA<sup>+</sup> actA–gus–plcB); NL-1653 (prfA<sup>+</sup>ΔP2(hly) actA–gus–plcB); NL-1654 (plcA-ΔT prfA<sup>+</sup> actA–gus–plcB); NL-1655 (plcA-ΔT+prfAΔP2-35 actA–gus–plcB); and NL-1656 (prfA<sup>+</sup>ΔP2-35 actA–gus–plcB). Transconjugants were isolated as described by

### Table 1. Bacterial strains and relevant characteristics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
<th>Haemolytic activity&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Plaque size (%)&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;</th>
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<td>10403S</td>
<td>Wild-type</td>
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<td>100</td>
<td>2 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>Shetron-Rama et al. (2000)</td>
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<td>90 ± 7</td>
<td>&lt;10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>NF-1629</td>
<td>prfAΔP2-35</td>
<td>This work</td>
<td>60</td>
<td>93 ± 7</td>
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<td>This work</td>
<td>40</td>
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<td>&lt;10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
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<td>This work</td>
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<td>99 ± 6</td>
<td>&lt;10&lt;sup&gt;5&lt;/sup&gt;</td>
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<sup>†</sup>Expressed as the reciprocal of the dilution at which 50% lysis of erythrocytes was observed.

<sup>‡</sup>Diameter of plaques formed in mouse L2 fibroblast tissue culture cells expressed as a percentage of wild-type.

*Integrative replacement of prfA with prfA7973 allele (prfA<sup>+</sup>) using pCON1-ΔprfA7973 (Behari et al., 1998).
Behari & Youngman (1998) with the following modifications: trans-conjugants were selected on BHI agar containing chloramphenicol (5 µg ml⁻¹) after a 24 h incubation at 30 °C. Selected colonies were used to inoculate BHI media containing chloramphenicol and streptomycin, and grown overnight at 40 °C with shaking to force chromosomal integration of the vector. Overnight 40 °C cultures were then diluted 1:1000 into fresh BHI containing chloramphenicol and streptomycin, and again incubated at 40 °C overnight with shaking. Appropriate dilutions of overnight cultures were plated on BHI agar containing 5 µg chloramphenicol ml⁻¹ and incubated at 40 °C. Integration of the vector into the chromosome and confirmation of the prfA7973 (prfA*) sequences were verified by PCR amplification of prfA from genomic DNA and sequencing of the PCR products.

β-Glucuronidase (GUS) assays of bacteria grown in liquid culture. For experiments using LB with and without added glucose or cellobiose, overnight cultures of bacteria grown in LB medium were diluted 1 to 10 into fresh LB buffer with 100 mM MOPS (pH 7-4) with or without glucose or cellobiose at the indicated concentrations and grown for 5 h with shaking at 37 °C. Bacterial pellets from 1 ml culture aliquots were collected following centrifugation and were quickly frozen on dry ice. Optical density (at 595 nm) was measured for each culture using a Spectronic 20 spectrophotometer (Milton Roy). For GUS enzymic 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 % (v/v) Triton X-100] and resuspended in 200 µl ABT buffer. GUS activity was measured as described by Youngman (1987) with the substitution of 4-methylumbelliferyl β-D-glucuronide in place of 4-methylumbelliferyl β-D-galactoside.

Western analysis of PrfA protein levels. Polyclonal rabbit antisera was generated against amino-terminal histidine-tagged PrfA protein produced using the Qiagen pQE30 vector in E. coli and purified by nickel column chromatography as recommended by the manufacturer (Qiagen). For Western analysis, overnight cultures of L. monocytogenes grown in LB broth at 37 °C without shaking were diluted 1:100 into fresh LB broth buffered to pH 7-4 with 100 mM MOPS buffer and grown for 6 h at 37 °C with shaking. For experiments designed to measure PrfA protein levels in LB broth buffered to pH 5-8 or in the presence of glucose or cellobiose, overnight cultures grown in LB were diluted 1:100 into fresh LB broth buffered with 100 mM MES to a pH of 5-8, or into LB broth buffered with 100 mM MOPS to pH 7-4 plus 25 mM glucose or 25 mM cellobiose. A 20 µl aliquot of each culture was briefly centrifuged to recover the cell pellet; the pellet was resuspended in 500 µl Cell Lysis Buffer (50 mM Tris/HCl, pH 7-5, 1 mM DTT, 0-1 % (v/v) Triton X-100). Bacterial suspensions were disrupted using a Mini-Beadbeater (BioSpec Products) with 1 min cycles followed by 30 s cooling on ice, for a total of 3 cycles. Samples were then centrifuged at 13 000 g for 5 min to recover supernatants. Protein concentration was determined using the DC Protein Assay Kit as recommended by the manufacturer (Bio-Rad Laboratories). Equivalent amounts of total protein from each culture lysate were mixed with equal volumes of 2× SDS-PAGE buffer [60 mM Tris/HCl (pH 6-8), 2 % (w/v) SDS, 10 % (v/v) glycerol, 10 % 2-mercaptoethanol, 0-01 % (w/v) bromophenol blue]. Samples were heated to 90 °C for 5 min prior to being subjected to SDS-PAGE on 10 % polyacrylamide gels. Proteins were visualized by staining the gel with Coomassie brilliant blue prior to transfer. Proteins were transferred to nitrocellulose membranes and incubated with a 1:200 dilution of polyclonal antibody generated against purified PrfA protein, followed by incubation with an alkaline phosphatase-coupled goat anti-rabbit antibody (Sigma). PrfA protein antigen was detected using the Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets, a colorimetric alkaline phosphate substrate.

For quantitative comparison of PrfA protein levels as visualized by Western analysis, 2 µg, 8 µg and 15 µg purified PrfA protein were compared with serial dilutions of protein extracts derived from the wild-type and prfA promoter mutant strains to determine the relative amount of PrfA produced by each mutant.

**Assay for haemolytic activity.** Stationary phase bacteria were diluted 1:10 into BHI and grown at 37 °C for 5 h with shaking. The supernatant fluid was assayed for haemolytic activity as previously described (Camilli et al., 1989).

**Plaque formation in L2 cells.** Plaque assays were performed as previously described by Sun et al. (1990). Plaque size was measured using a micrometer and the mean diameter of at least 10 plaques from three independent experiments was determined.

**LD₉₀ determinations.** The LD₉₀ were determined in BALB/c mice by intravenous injection as previously described (Portnoy et al., 1988).

**RESULTS**

**Construction of L. monocytogenes strains containing mutations within prfA promoter regulatory regions**

Mutations designed to alleviate negative regulation of prfA expression were introduced into the chromosome of L. monocytogenes by homologous recombination (Fig. 1 and Table 1). Mutations introduced were as follows. (1) A deletion of the motif resembling a degenerate PrfA binding site in the −35 region of the prfA promoter, previously shown to contribute to down-regulation of prfA1 expression (prfAAp2-35 mutation) (Freitag & Portnoy, 1994). (2) Replacement of the prfA2 promoter −35 motif with a high affinity PrfA binding site derived from the hly promoter in an attempt to introduce a positive PrfA regulatory element in place of a negative one [prfAAp2(hly)]. (3) A 14 bp deletion designed to facilitate transcription through the plcA rho-independent terminator and to increase prfA expression through the augmented generation of plcA−prfA transcripts (plcA−ΔT mutation). The plcA−ΔT deletion also removed a small 7 bp motif that resembles one half of a PrfA binding site in the plcA promoter. The plcA−ΔT deletion was introduced alone and in combination with the prfAAp2-35 deletion to determine if loss of both motifs resulted in levels of prfA expression that were higher than those observed for strains lacking a single motif. All mutations were introduced in single copy into the L. monocytogenes chromosome.

**Analysis of the effects of prfA promoter mutations on PrfA protein levels**

The prfAAp2-35 mutation has been previously demonstrated to increase levels of transcripts initiating at the prfA1 promoter (Freitag & Portnoy, 1994). However, PrfA protein levels were not examined in this strain, and it was important to determine whether increased mRNA levels directly correlated with an increase in protein levels. To directly compare the levels of PrfA present in each of the prfA promoter mutant strains, extracts derived from wild-type L. monocytogenes and the promoter mutants were...
L. monocytogenes encodes a gene product necessary for cell-to-cell spread of intracellular bacteria via the synthesis of PrfA protein in broth-grown cultures to levels equivalent to those observed for wild-type L. monocytogenes (Fig. 3). The prfAΔP2(hly) mutation resulted in the highest levels of actA expression, approximately eightfold greater than the levels observed for wild-type bacteria grown in broth culture. These results suggest that increased synthesis of PrfA protein influences a subset of PrfA-regulated genes (such as actA) and does not result in increased levels of all PrfA-dependent gene products (such as LLO).

Expression of virulence genes in L. monocytogenes has been shown to be dramatically influenced by available carbon sources (Park & Kroll, 1993; Millenbachs et al., 1997) and by low pH (Behari & Youngman, 1998). Readily metabolized carbon sources, such as glucose, fructose and cellobiose, repress the expression of hly and actA, as does media buffered to pH 5–8. We examined the effects of overexpression of prfA on catabolite and pH-mediated repression of actA gene expression (Fig. 3). Similar to wild-type expression patterns, expression of actA was subject to repression by low pH in all of the mutant strains; however, the absolute level of actA expression remained above wild-type levels for mutants prfAΔP2-35, plcA-ΔT+prfAΔP2-35 and prfAΔP2(hly). All of the mutants were extremely sensitive to catabolite repression of gene expression by both glucose and cellobiose, although again the absolute level of actA expression remained above wild-type levels for mutants prfAΔP2-35, plcA-ΔT+prfAΔP2-35 and prfAΔP2(hly) (Fig. 3b). The prfAΔP2(hly) mutant exhibited the highest levels of repression in the presence of glucose with a 34-fold reduction in actA expression (compared to a ninefold reduction for wild-type). Interestingly, Western analysis of L. monocytogenes prfAΔP2(hly)-derived extracts still showed higher levels of PrfA protein in comparison to the amounts observed for the wild-type strain following growth in media containing glucose or cellobiose (Fig. 4). These data are consistent with previously reported observations that indicate that PrfA protein can be present but inactive (Millenbachs et al., 1997; Renzoni et al., 1997). It further demonstrates that PrfA-dependent gene expression remains sensitive to repression by low pH and readily metabolized sugars despite the presence of high levels of PrfA.

Introduction of PrfA*, a constitutively activated form of PrfA, into L. monocytogenes prfA promoter mutant strains

A growing body of evidence strongly suggests that PrfA requires post-translational modification or the presence of infection, or bacterial virulence in murine models of infection (Shetron-Rama et al., 2002).

Despite the high levels of PrfA protein observed in each of the mutant strains, none of the mutants produced significantly increased levels of LLO (Table 1). In contrast, significant increases in the levels of actA expression were observed for all prfA promoter mutant strains following growth in LB broth, with the sole exception of mutant plcA-ΔT which had levels of expression equivalent to those observed for wild-type L. monocytogenes (Fig. 3). The prfAΔP2(hly) mutation resulted in the highest levels of actA expression, approximately eightfold greater than the levels observed for wild-type bacteria grown in broth culture. These results suggest that increased synthesis of PrfA protein influences a subset of PrfA-regulated genes (such as actA) and does not result in increased levels of all PrfA-dependent gene products (such as LLO).

In vitro expression of PrfA-regulated genes by prfA promoter mutant strains

To examine the effects of increased synthesis of PrfA protein on PrfA-dependent gene expression, two differentially regulated virulence genes were selected. hly, encoding the haemolysin listeriolysin O (LLO), is normally expressed during bacterial growth in standard broth culture and LLO activity can be easily detected in culture supernatants. actA encodes a gene product necessary for cell-to-cell spread of L. monocytogenes and in contrast to hly, is expressed at low to undetectable levels in broth-grown bacterial cultures but is highly induced following entry of L. monocytogenes into the host cell cytosol (Brundage et al., 1993; Bubert et al., 1999; Freitag & Jacobs, 1999; Moors et al., 1999). The prfA promoter mutations were introduced into L. monocytogenes strains containing actA transcriptional fusions to the gus reporter gene to facilitate measurement of actA expression. The actA–gus fusions do not affect intracellular growth or cell-to-cell spread of L. monocytogenes in tissue culture.
of a cofactor for full activity (Ripio et al., 1997; Vega et al., 1998; Kreft & Vazquez-Boland, 2001; Vazquez-Boland et al., 2001). Thus, the effects of increased prfA expression in L. monocytogenes are likely to be reduced in broth culture where the protein is not in its fully activated state. Ripio et al. (1997) have reported that L. monocytogenes strains that constitutively express high levels of several virulence gene products contain a prfA allele that encodes a serine in place of a glycine at position 145 within the protein. PrfA shares significant homology with the cAMP receptor protein (CRP) of E. coli (Lampidis et al., 1994; Sheehan et al., 1995; Vega et al., 1998) and the prfA G145S mutant allele, also known as prfA*, appears analogous to crp* mutants in which CRP functions as a transcriptional activator in the absence of its cAMP cofactor (Garges & Adhya, 1985; Harman et al., 1986; Kolb et al., 1993). To test if the effects of high level PrfA synthesis would be amplified by the presence of a constitutively activated form of the protein, the prfA* allele was introduced into the prfA promoter mutants via the integration of a temperature-sensitive plasmid (see Table 1). Integration of the prfA*-containing plasmid resulted in the inactivation of the wild-type prfA allele and the expression of prfA* under the control of the wild-type prfA promoter or the mutant prfA promoters. PrfA* levels were measured in

![Fig. 3. Measurement of actA expression levels in prfA promoter mutants containing transcriptional actA–gus reporter gene fusions. GUS activity was measured following 5 h growth in LB broth buffered to pH 7-4 in the presence and absence of either 25 mM glucose or 25 mM cellobiose, or in LB broth buffered to pH 5-8. Units of GUS activity were normalized for optical density at 595 nm as described by Youngman (1987) for the measurement of β-galactosidase activity but with the appropriate substrate substitution of 4-methylumbelliferyl β-D-glucuronide. Each assay was done in duplicate, and the data represents the mean and SE for three separate experiments. Part (b) provides an enhanced view of the data obtained in the presence of cellobiose or glucose shown in (a). Black bars, WT; dark grey bars, plcA-ΔT; bars shaded with straight lines, prfAΔP2-35; light grey bars, plcA-ΔT + prfAΔP2-35; bars shaded with curved lines, prfAΔP(hly).]

![Fig. 4. Western analysis of PrfA protein levels produced by L. monocytogenes wild-type and prfAΔP2(hly) promoter mutant strains. Soluble bacterial cell extracts were prepared from L. monocytogenes cultures grown in LB broth buffered to pH 7-4 in the presence of either 25 mM glucose or 25 mM cellobiose, or in LB broth buffered to pH 5-8. Equal amounts (121 μg) of total protein in SDS-PAGE sample buffer were run for each sample, and PrfA was detected using a rabbit polyclonal antibody directed against purified His-tagged PrfA. The arrow indicates the position of PrfA.]

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each of the mutant backgrounds using Western analysis with polyclonal rabbit antiserum directed against PrfA (Fig. 5). PrfA* levels in the plcA-DT (prfA*), prfA*ΔP2-35 and prfA*ΔP2(hly) mutant strains were found to be approximately fivefold higher than the levels observed in the presence of the wild-type promoter or in the plcA-DT+ prfA*ΔP2-35 mutant. The overall levels of PrfA* protein were similar to the levels observed in the wild-type and mutant strains containing the wild-type prfA allele (see Fig. 2), indicating that the mere presence of PrfA* did not dramatically increase levels of protein expression.

**Analysis of the effects of the L. monocytogenes prfA* promoter mutations on virulence gene expression**

Supernatants derived from prfA* promoter mutant strain cultures were assayed for secreted LLO activity. While the introduction of the prfA* allele did lead to a significant increase in LLO activity in comparison to wild-type strains, no additional increase was observed for the prfA* promoter mutants (Table 1). Similarly, the introduction of the prfA* allele yielded an overall increase in actA expression for all strains examined, but the presence of the prfA promoter mutations did not significantly increase actA expression above the levels observed for the wild-type promoter (Fig. 6). Levels were increased over wild-type for several of the mutants at pH 5-8 and in the presence of cellobiose. All of the prfA* strains were significantly more resistant to glucose-mediated repression. The prfA*ΔP2(hly) mutant, for example, had a twofold decrease in actA expression in the presence of glucose, in contrast to the 34-fold decrease observed for the prfAΔP2(hly) strain. The relative resistance of strains containing PrfA* to glucose-mediated repression was observed at glucose concentrations at least 10 times higher than those found to repress wild-type gene expression (Fig. 7). Taken together, these results indicate that no significant increase in actA expression results from an increase in synthesis of PrfA*, suggesting that for actA transcriptional activation, saturating amounts of PrfA* are already present in strains containing the wild-type promoter. Strains containing the prfA* allele were much less sensitive to repression mediated by low pH, cellobiose or glucose (as previously reported by Ripio et al., 1997; Behari & Youngman, 1998; Vega et al., 1998) and this reduction in sensitivity was only slightly augmented by the presence of the prfA promoter mutations.

**Loss of negative prfA regulation does not affect L. monocytogenes virulence**

To assess the effects of loss of prfA negative regulation within infected host cells, the prfA promoter mutant strains were examined for their ability to replicate within infected tissue culture cell monolayers. The capacity of L. monocytogenes to escape from a vacuole and spread from cell to cell can be measured by the ability of the bacteria to form plaques on monolayers of mouse L2 cells (Sun et al., 1990). Mutations that interfere with the function of virulence-related gene
products can be identified by the inability of the mutants to form plaques equal in size to those formed by wild-type bacteria (Sun et al., 1990; Camilli et al., 1993; Freitag & Portnoy, 1994; Jones & Portnoy, 1994; Marquis et al., 1995; Smith et al., 1995). All of the prfA promoter mutant strains were found to form plaques of approximately the same size and with the same efficiency as wild-type L. monocytogenes (Table 1), indicating that the high level PrfA synthesis observed in broth culture did not adversely affect intracellular growth or cell-to-cell spread in vitro. Interestingly, strains containing the prfA* allele formed plaques that were smaller in size than those formed by wild-type L. monocytogenes, but the strains containing the prfA* promoter mutations did not differ in size from the prfA* parent strain. These results may indicate that the presence of the constitutively activated prfA* allele reduced either intracellular growth or cell-to-cell spread of L. monocytogenes in L2 cells; however it should be noted that the mere presence of pKSV7 integrated within the L. monocytogenes chromosome can reduce plaque size by 30–40% (Camilli et al., 1993).

Intracellular growth of the prfA promoter mutants was also examined in a variety of host cell types, including mouse macrophage-like tissue culture cell lines. No defect in intracellular growth or cell-to-cell spread was detected (S. Greene & N. Freitag, unpublished data). Loss of prfA negative regulation also resulted in no significant defect in virulence following intravenous injection of mice for any of the mutant strains (LD50 2 × 10^4 c.f.u. for parent strain NF-L476, and <10^5 c.f.u. for each mutant).

**DISCUSSION**

The regulation of virulence gene expression by the PrfA transcriptional activator is a complex but essential process for L. monocytogenes virulence. It has been demonstrated that an increase in PrfA protein levels is necessary for the full induction of virulence gene expression within the host (Camilli et al., 1993; Freitag et al., 1993; Freitag & Portnoy, 1994), and previous reports have demonstrated that a threefold increase in PrfA protein synthesis occurs during infection of mammalian cells (Renzoni et al., 1999). Negative regulation of prfA expression has been implicated as an important feedback regulatory tool, but its role in L. monocytogenes virulence has never been examined. In this report, we show that it is possible to increase PrfA protein levels more than fivefold via the introduction of specific promoter mutations that interfere with negative regulation of prfA expression. Strains lacking prfA negative regulation had significantly increased levels of actA expression, but surprisingly no significant effects were observed for intracellular growth, cell-to-cell spread or virulence in mouse models of infection. These results confirm the existence of negative regulation of prfA expression, but show that this feedback system is dispensable for bacterial virulence.

We had anticipated that the loss of prfA negative regulation would be amplified in the presence of the constitutively activated PrfA* form of the protein. The G145S substitution within PrfA* has been postulated to produce a conformational change in the protein that results in activation of PrfA in the absence of cofactor binding (Ripio et al., 1997; Vega et al., 1998). As it has been demonstrated that an approximately threefold induction of PrfA synthesis occurs...
during infection of mammalian cells (Renzoni et al., 1999), the fivefold increase in PrfA* observed for the promoter mutants might have been sufficient to induce actA expression to the levels observed in cytosolic bacteria (Shetron-Rama et al., 2002). However, the prfA* promoter mutants did not show a significant increase in actA expression in comparison to strains containing prfA* in the presence of the native promoter, and the approximately tenfold induction of actA expression measured for the prfA* strains was still well below the greater than 200-fold induction observed for bacteria located within the host cytosol (Moors et al., 1999; Shetron-Rama et al., 2002). These results strongly support the premise that other factors besides PrfA are required for optimal induction of actA expression in L. monocytogenes and that the expression of these factors is likely to be PrfA-independent. Work in progress has indicated that it is possible to isolate L. monocytogenes mutants with increased actA expression in broth culture which contain mutations mapping outside of the prfA regulon (L. Shetron-Rama & N. Freitag, unpublished results). The participation of additional regulatory factors in the induction of L. monocytogenes gene expression within infected host cells further illustrates the resources used by the bacterium to ensure that specific gene products are synthesized within the proper host cell environment.

Why does negative regulation of prfA expression exist if it does not contribute to virulence in mammals? L. monocytogenes is a ubiquitous bacterium that occupies a variety of habitats. PrfA has been reported to negatively influence the expression of genes required for environmental stress, such as cplC (Ripio et al., 1998) and has been implicated in the down-regulation of stress resistance mechanisms during exponential growth (Herbert & Foster, 2001). Genes required for bacterial motility, such as motA, have also been reported to be downregulated by PrfA (Michel et al., 1998). It is possible that negative regulation of prfA expression plays an important role in facilitating bacterial survival outside host cells by ensuring that gene products required for survival in the extracellular environment are expressed. It is tempting to speculate, therefore, that while positive regulation of prfA expression is of paramount importance during mammalian infection, negative regulation has evolved to sustain bacterial survival in the outside environment. Confirmation of this hypothesis awaits detailed studies of factors contributing to L. monocytogenes survival within its ubiquitous habitats.

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