Functional impact of mutational activation on the *Listeria monocytogenes* central virulence regulator PrfA

Maurine D. Miner,^{1,2} Gary C. Port^{2,3†} and Nancy E. Freitag^{1,2,3,4}

Correspondence

Nancy E. Freitag
nfreitag@uic.edu

¹Program in Pathobiology, University of Washington, Seattle, WA, USA

²Seattle Biomedical Research Institute, Seattle, WA, USA

³Molecular and Cellular Biology Program, University of Washington, Seattle, WA, USA

⁴Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL, USA

The transcriptional activator PrfA is required for the expression of virulence factors necessary for *Listeria monocytogenes* pathogenesis. PrfA is believed to become activated following *L. monocytogenes* entry into the cytosol of infected host cells, resulting in the induction of target genes whose products are required for bacterial intracellular growth and cell-to-cell spread. Several mutations have been identified that appear to lock PrfA into its highly activated cytosolic form (known as *prfA** mutations). In this study PrfA and five *PrfA** mutant proteins exhibiting differing degrees of activity were purified and analysed to define the influences of the mutations on distinct aspects of PrfA activity. Based on limited proteolytic digestion, conformational changes were detected for the *PrfA** mutant proteins in comparison to wild-type PrfA. For all but one mutant (*PrfA* Y63C), the DNA binding affinity as measured by electrophoretic mobility shift assay appeared to directly correlate with levels of PrfA mutational activation, such that the high-activity mutants exhibited the largest increases in DNA binding affinity and moderately activated mutants exhibited more moderate increases. Surprisingly, the ability of PrfA and *PrfA** mutants to form dimers in solution appeared to inversely correlate with levels of PrfA-dependent gene expression. Based on comparisons of protein activity and structural similarities with PrfA family members Crp and CooA, the *prfA** mutations modify distinct aspects of PrfA activity that include DNA binding and protein–protein interactions.

Received 2 June 2008

Revised 21 August 2008

Accepted 23 August 2008

INTRODUCTION

The transcriptional regulator PrfA (positive regulatory factor A) is responsible for regulating the gene expression of nearly all known virulence factors of *Listeria monocytogenes* (Chakraborty *et al.*, 1992; Gray *et al.*, 2006; Leimeister-Wachter *et al.*, 1990; Miner *et al.*, 2008; Pizarro-Cerda & Cossart, 2006; Scotti *et al.*, 2007). PrfA is a 27 kDa protein that recognizes and binds a 14 bp DNA palindrome present in the promoters of its target genes (Freitag *et al.*, 1992; Mengaud *et al.*, 1989). PrfA regulates the expression of gene products required for *L. monocytogenes* invasion of host cells, intracellular growth, and cell-to-cell spread, and is absolutely essential for bacterial virulence (Freitag, 2006; Scotti *et al.*, 2007).

Based on sequence and structural homology, PrfA has been identified as a member of the Crp/Fnr family of transcriptional activators (Eiting *et al.*, 2005; Korner *et al.*, 2003; Ripio *et al.*, 1997). Proteins within this family generally become activated following the binding of small-molecule cofactors. Crp, for example, undergoes an allosteric change after binding cAMP and becomes a site-specific DNA binding protein that recognizes target promoters and interacts with RNA polymerase (RNAP) (Busby & Ebright, 1999; Kim *et al.*, 1992; Kolb *et al.*, 1993; Lawson *et al.*, 2004). Crp appears to exist in an equilibrium between an active form that efficiently binds DNA target sequences and an inactive form that does not. Cofactor cAMP binding by Crp shifts the equilibrium toward the active form, either by stabilizing this form or by destabilizing the inactive form of the protein (Youn *et al.*, 2007). PrfA may exist in an analogous equilibrium state such that binding of a cofactor is required to shift PrfA to a high-activity form capable of high-affinity DNA binding. Although it is generally believed that a PrfA cofactor exists, this cofactor has not yet been identified.

†Present address: Department of Molecular Microbiology, Washington University, St. Louis, MO 63110, USA.

Abbreviations: EMSA, electrophoretic mobility shift assay; GUS, β -glucuronidase; RBC, red blood cell; RNAP, RNA polymerase; X-gluc, 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid.

Mutations in *crp* have been identified that result in an active form of Crp in the absence of cAMP cofactor (Garges & Adhya, 1985; Harman *et al.*, 1986; Kim *et al.*, 1992; Youn *et al.*, 2006, 2007). Structural and functional studies of these mutants (known as Crp* mutants) have led to the identification of regions of Crp that are important for activity, and it has been observed that Crp* mutants exhibit a conformation that resembles that of wild-type Crp bound to cofactor (Harman *et al.*, 1986). Similar to *crp**, several *prfA* mutations have been identified that appear to result in activation of PrfA in the absence of cofactor (known as *prfA** mutants) (Miner *et al.*, 2008; Mueller & Freitag, 2005; Ripio *et al.*, 1997; Shetron-Rama *et al.*, 2003; Vega *et al.*, 2004; Wong & Freitag, 2004). Strains with *prfA** mutations express high levels of PrfA-dependent gene products under conditions in which gene expression is usually repressed. The *prfA** mutations identified thus far are not functionally equivalent, and significant differences in bacterial virulence have been reported for *L. monocytogenes* strains containing different *prfA** alleles (Miner *et al.*, 2008; Mueller & Freitag, 2005; Scortti *et al.*, 2007; Shetron-Rama *et al.*, 2003). This study describes a biochemical comparison of wild-type PrfA with five different PrfA* mutants (including a novel *prfA** mutation) to elucidate the effects of specific amino acid substitutions on distinct aspects of PrfA function.

METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *L. monocytogenes* strains were grown in brain heart infusion (BHI) medium and *Escherichia coli* strains were grown in Luria broth (LB) at 37 °C with shaking. Strains containing high-activity *prfA** alleles such as *prfA* L140F and *prfA* G145S have previously proven difficult to construct using standard allelic-exchange techniques (Port & Freitag, 2007; Wong & Freitag, 2004; M. D. Miner and others, unpublished observations). However, a modified approach for generating isogenic mutants was developed and used successfully as follows: *prfA* L140F and *prfA* G145S were introduced into *L. monocytogenes* NF-L1124 containing a transcriptional fusion of *gus* and *neo* (Karow & Piggot, 1995) downstream of *actA* in the bacterial chromosome. Selection for the mutant strains was then facilitated based on an increased level of neomycin resistance in the presence of the *prfA** allele, as conferred by the PrfA-dependent promoter *actA*. In addition, to prevent expression of the introduced *prfA** mutations from the plasmid vector used for allelic exchange, *prfA* coding sequences missing the ATG start codon were amplified by PCR and inserted into the temperature-sensitive plasmid shuttle vector pKSV7 (generating plasmid pNF1147), and the L140F and G145S mutations were then separately introduced via the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions with the primers listed in Table 2 [generating plasmids pNF1162 (*prfA* L140F) and pNF1161 (*prfA* G145S)]. To enrich for mutants containing the *prfA* L140F or *prfA* G145S chromosomal replacement, 5 µg neomycin ml⁻¹ was used to select for *prfA**-induced neomycin resistance on the final day of allelic exchange at 40 °C. Following allelic exchange, *L. monocytogenes* strains containing the desired mutations within *prfA* were confirmed by sequencing of PCR fragments derived from chromosomal DNA.

Genetic selection for *prfA* mutations that lead to enhanced *actA* expression following *prfA* plasmid mutagenesis in XL1 Red *E. coli*.

Table 1. Bacterial strains used in this work

Strain or plasmid	Brief description	Source or reference
Strains		
10403S	<i>L. monocytogenes</i>	Bishop & Hinrichs (1987)
NF-L476	10403S <i>actA-gus-plcB</i>	Shetron-Rama <i>et al.</i> (2002)
NF-L924	NF-L476 <i>prfA</i> E77K	Shetron-Rama <i>et al.</i> (2003)
NF-L1124	10403S <i>actA-gus-neo-plcB</i>	This work
NF-L1213	NF-L1124 <i>prfA</i> Y154C	Miner <i>et al.</i> (2008)
NF-L1214	NF-L1124 <i>prfA</i> Y63C	This work
NF-L1166	NF-L1124 <i>prfA</i> L140F	This work
NF-L1177	NF-L1124 <i>prfA</i> G145S	This work
BL21 Star (DE3)	<i>E. coli</i> protein expression strain	Invitrogen
Plasmids		
pET100	Protein expression vector	Invitrogen
pNF1280	pET100- <i>prfA</i> WT	This work
pNF1281	pET100- <i>prfA</i> G145S	This work
pNF1282	pET100- <i>prfA</i> L140F	This work
pNF1283	pET100- <i>prfA</i> Y63C	This work
pNF1284	pET100- <i>prfA</i> Y154C	This work
pNF1285	pET100- <i>prfA</i> E77K	This work

Plasmid pNF1019 containing *prfA* under the control of the *prfA*P1, *prfA*P2 and *plcA* promoters in the integrative plasmid vector pPL2 pNF1019 (Wong & Freitag, 2004) was transformed into chemically competent XL1 Red *E. coli* hypermutator bacterial cells (Stratagene). Selected transformants were inoculated into LB at 1:1000 dilution and grown with shaking to stationary phase at 37 °C. Cultures were repeatedly diluted and grown to stationary phase for a total of 10 cycles. The pNF1019 plasmid was then purified from XL1 Red and introduced via electroporation into conjugation-competent SM10 cells. Transfer of pNF1019 from SM10 into *L. monocytogenes* $\Delta prfA$ was carried out as described previously (Wong & Freitag, 2004). Transconjugant *prfA* mutants exhibiting enhanced *actA* expression were identified as blue colonies on BHI plates containing 7.5 µg chloramphenicol ml⁻¹, 200 µg streptomycin ml⁻¹, 50 µg 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-gluc) ml⁻¹ and 5 µg neomycin ml⁻¹.

Generation and purification of recombinant PrfA* proteins.

DNA fragments containing *prfA* and *prfA** ORFs were amplified using PCR of *L. monocytogenes* genomic DNA isolated from NF-L1124 (*prfA* WT), NF-L1177 (*prfA* G145S), NF-L1166 (*prfA* L140F), NF-L1214 (*prfA* Y63C), NF-L1213 (*prfA* Y154C) or NF-L924 (*prfA* E77K) using primers listed in Table 2. The PCR fragments were then cloned into pET100 using a Champion pET Directional TOPO Expression kit (Invitrogen) as per the manufacturer's instructions. Plasmids containing the *prfA* and *prfA** ORFs were transformed into BL21 Star (DE3) expression cells and PrfA/PrfA* protein production was induced by addition of 1 mM IPTG for 1.5 h. Protein extracts containing recombinant PrfA/PrfA* proteins were passed over a nickel-affinity column, and PrfA was eluted with 200–500 mM imidazole buffer and dialysed into PBS (137 mM NaCl, 10 mM potassium phosphate, 2.7 mM KCl, pH 7.4) with 10 % glycerol (v/v). Purified protein was visualized and assessed for purity following

Table 2. Oligonucleotides used in this study

Primer name	Sequence (5'–3')	Characteristics
5'hly	TCCTATCTTAAAGTGACTTTTATGTT	5' <i>hly</i> promoter
3'hly	GCTTCTAAAGATGAAACGCAATATTA	3' <i>hly</i> promoter
5'actA	GATGCTTCTAAAAAAGTTGCTGAAGC	5' <i>actA</i> promoter
3'actA	TATTCATGAATTATTTTAAGAATATCA	3' <i>actA</i> promoter
+ 374 <i>prfA</i>	GCGCTGCAGGAAACTTGT'TTTTGTAGGGTTTGG	3' Truncated <i>prfA</i> ORF
5' <i>prfA</i> orf	CACCATGAACGCTCAAGCAGAA	5' <i>prfA</i> ORF for protein expression
3' <i>prfA</i> orf	TCCTCATTGAGGAATACTGTT	3' <i>prfA</i> ORF for protein expression
<i>prfA</i> -trunc-nt3-F	GCGCTGCAGGAACGCTCAAGCAGAAGAATTC	5' Truncated <i>prfA</i>
<i>prfA</i> -trunc-nt841-R	GCGCTGCAGGAACAACATCTGTTGCAGCTC	3' Truncated <i>prfA</i> ORF
<i>prfA</i> -L140F-QkCh-F	GATTTTTCGATTAAACGGGAAGTTTGGCTCTATTTGCGGTCAAC	5' L140F Quikchange
<i>prfA</i> -L140F-QkCh-R	GTTGACCGCAAATAGAGCCAAACTTCCGTTAATCGAAAAATC	3' L140F Quikchange
<i>prfA</i> -G145S-QkCh-F	GGAAGCTTGGCTCTATTTGCAGTCAACTTTTAATCCTGACC	5' G145S Quikchange
<i>prfA</i> -G145S-QkCh-R	GGTCAGGATTAAGTTGACTGCAATAGAGCCAAGCTTCC	3' G145S Quikchange

separation on SDS-PAGE gels and Coomassie staining, and also confirmed by Western blot analysis using an anti-PrfA polyclonal antibody (Greene & Freitag, 2003).

Limited proteolysis. One microgram of purified wild-type PrfA and each *PrfA* mutant was incubated with 300 ng trypsin (Sigma) or 250 ng subtilisin (Sigma) in Sigma 10× Multicore buffer for the indicated times at 37 °C. Reactions were terminated by the addition of 1 µl PMSF, and samples were then boiled for 5 min, run on 12 % acrylamide gels in MES Buffer (50 mM MES, 50 mM Tris base, 0.1 % SDS, 1 mM EDTA, pH 7.3; Invitrogen) for small-band separation, and visualized by Coomassie stain.

Electrophoretic mobility shift assays (EMSAs). Primers used to PCR-amplify DNA fragments (~100 bp) containing the *hly* and *actA* promoters from *L. monocytogenes* genomic DNA are listed in Table 2. Primers labelled with cy5.5 label on the 5' ends were purchased from Operon Biotechnologies. To generate a DNA fragment for use as a non-specific competitor for DNA binding assays, primers were used to amplify the *prfA* ORF (~370 bp), as this region lacks *PrfA* binding sites (primer sequences listed in Table 2). Extracts from NF-L890 ($\Delta prfA$) were made as follows: bacteria from 1 l cultures grown to mid-exponential phase in BHI were collected using centrifugation for 10 min at 6000 g and resuspended in 20 ml ice-cold PBS, and bacterial cells were disrupted by a triple passage through a French press. EMSA were performed as described elsewhere (Böckmann *et al.*, 2000). EMSA reaction mixtures consisted of the following: 40 ng labelled DNA probe (hlycy5.5 or actAcy5.5), *PrfA* protein (as indicated), 1 µg BSA ml⁻¹, and 50 mM dIdC in TB buffer [10 mM Tris-HCl, pH 8, 10 mM MgCl₂, 5 mM CaCl₂, 1 mM EDTA, 0.2 mM DTT, 10 % glycerol (v/v)] in a final reaction volume of 20 µl. For experiments including cell extracts, 1 µl (3 µg) extract was added to each reaction mixture. Sample reactions containing all components except labelled DNA were incubated for 15 min at room temperature. The labelled DNA probe was then added and samples were incubated for 3 min at 37 °C, followed by 27 min on ice. Samples were then loaded onto a 5 % acrylamide gel (0.5× Tris-boric acid-EDTA) and run at a constant current of 20 mA for approximately 3 h in the dark at 4 °C. Gels were then visualized as in-gel Western blots using the Odyssey Imager (Li-Cor Biosciences) with the cy5.5-labelled fluorescent probes visualized at 700 nm. The His- and Express-tags were found to have minimal effects on *PrfA* and *PrfA** protein function in comparison to purified *PrfA* protein without the tags (M. D. Miner,

unpublished data), as has been reported elsewhere (Böckmann *et al.*, 1996, 2000).

Measurement of β -glucuronidase (GUS) activity. GUS activity was measured as previously described (Shetron-Rama *et al.*, 2003) with minor changes. Briefly, *L. monocytogenes* cultures grown overnight at 37 °C in BHI were diluted 1:50 and grown with shaking at 37 °C for 8 h. OD₆₀₀ was measured for each time point and two 500 µl culture aliquots were collected for all strains, except for the *prfA* L140F (NF-L1166), *prfA* G145S (NF-L1177) and *prfA* Y63C (NF-L1214) mutant strains, for which two 50 µl aliquots were collected (reflective of the increased GUS activity present in these three highly activated *prfA** strains). Bacterial cells were recovered by microcentrifugation and the supernatants were removed. Bacterial pellets were resuspended in 100 µl (aliquot 1) or 1 ml (aliquot 2) ABT buffer (0.1 M potassium phosphate, pH 7.0, 0.1 M NaCl, 0.1 % Triton). GUS activity was measured as described elsewhere, with the substitution of 4-methylumbelliferyl- β -D-glucuronide in place of 4-methylumbelliferyl- β -D-galactoside (Sigma) (Youngman, 1987). Data were derived from duplicate samples taken from three independent experiments.

Measurement of haemolytic activity. Haemolytic activity was measured as described elsewhere, with minor modifications (Camilli *et al.*, 1989). Briefly, bacteria were grown without shaking overnight in BHI at 30 °C, the bacterial supernatants were recovered following centrifugation, and twofold serial dilutions of the supernatants were incubated with PBS-washed sheep red blood cells (RBCs; 0.3–10 %) for 30 min at 37 °C. After incubation, RBCs were recovered by centrifugation to measure 50 % lysis and supernatants were read in a spectrophotometer plate reader at A₄₅₀.

Protein chemical cross-linking. Purified proteins (500 ng) were incubated with either 10 µM sulfo-ethylene glycol bis[succinimidyl-succinate] (S-EGS) or bis[sulfosuccinimidyl] suberate (BS³) in 0.2 M triethylamine (TEA), pH 8.0, for 1 h at room temperature. Samples were then heated at 85 °C for 10 min in SDS sample buffer (1 % SDS, 10 % glycerol, v/v, 10 mM Tris-Cl, pH 6.8, 1 mM EDTA, and 0.05 mg ml⁻¹ bromophenol blue dye) containing 5 % β -mercaptoethanol, run on SDS-PAGE and transferred to nitrocellulose. Rabbit polyclonal antibody directed against *PrfA* was used for Western blot analysis at 1:4000 dilution followed by incubation with goat-anti-rabbit-IRDye 680 at 1:10 000 (Li-cor Biosciences). Membranes were visualized on an Odyssey Imager (Li-cor Biosciences).

RESULTS

Isolation of a novel mutationally activated *L. monocytogenes* *prfA** mutant (*prfA* Y63C)

As part of a study designed to identify mutations within *prfA* that result in increased PrfA-dependent virulence gene expression in *L. monocytogenes* strains grown in culture, promoterless copies of the genes encoding neomycin resistance (*neo*) and GUS (*gus*) were introduced as single copies into the bacterial chromosome downstream of *actA* in a $\Delta prfA$ strain. The *actA-gus-neo-plcB* transcriptional fusion within the *L. monocytogenes* chromosome was used for the identification of *prfA** mutations based on the enhanced expression of neomycin resistance and blue colony colour in the presence of the GUS substrate X-gluc on indicator plates. Plasmid pPL2-*prfA* containing a copy of wild-type *prfA* and its promoters was propagated in the *E. coli* mutator strain XL1 Red as described in Methods, and then transformed into conjugation-competent *E. coli* SM10 cells for conjugal transfer into $\Delta prfA/actA-gus-neo-plcB$ *L. monocytogenes*. Transconjugants with *prfA** mutations were selected based on enhanced neomycin resistance and dark-blue colony colour on plates containing neomycin and X-gluc. Out of approximately 40 000 transconjugants screened, two mutants were identified with approximately 200-fold and 185-fold higher levels of *actA* expression (based on GUS activity in broth culture) in comparison with the wild-type *prfA* strain. DNA sequencing of the mutant *prfA* alleles revealed a leucine to phenylalanine substitution at amino acid position 140 [*prfA* L140F, a previously described mutation (Wong & Freitag, 2004)], and a novel tyrosine to cysteine amino acid substitution at residue 63 (PrfA Y63C). The position of the Y63C substitution with respect to the 3D structure of PrfA and in relation to other identified *prfA** mutations is shown in Fig. 1.

*prfA** mutations are not equivalent in their ability to activate PrfA

Direct comparison of *L. monocytogenes* *prfA** mutants has proven challenging for strains containing high-activity *prfA* alleles (*prfA* L140F and *prfA* G145S), as it has not previously been possible to construct isogenic chromosomal mutant strains using allelic exchange. Both *prfA* L140F and *prfA* G145S strains exhibit subtle fitness defects during bacterial growth in broth culture that have hampered efforts to introduce these mutations into the *L. monocytogenes* chromosome (Port & Freitag, 2007; Wong & Freitag, 2004; M. D. Miner and others, unpublished observations). However, the use of the NF-L1124 strain containing the *actA-gus-neo-plcB* transcriptional fusion enabled selection of isogenic chromosomal mutants using enhanced *neo* expression to select for *prfA** alleles (see Methods). As a result, it was possible to directly compare *in vitro* expression of PrfA-dependent genes and gene products for all five *prfA** strains along with the wild-type

Fig. 1. Location of *prfA** mutations on the PrfA crystal structure. Crystal structure of the PrfA dimer (monomers in black and grey) adapted from Eiting *et al.* (2005). Locations of the five *PrfA** mutations used in this study are shown on each monomer and colour-coded: Y63C (pink), E77K (green), L140F (light blue), G145S (darker blue) and Y154C (orange). Structural motifs discussed in the text are indicated with white letters (α C and α D) or yellow numbers (β 5, β 6 and β 7) and derived from Eiting *et al.* (2005).

parent strain. For the *actA* gene product, wild-type *L. monocytogenes* is known to express low-to-undetectable levels of *actA* during growth in BHI broth culture, whereas expression levels increase over 200-fold upon PrfA activation in the host cell cytosol (Bubert *et al.*, 1999; Freitag & Jacobs, 1999; Moors *et al.*, 1999; Shetron-Rama *et al.*, 2002). High-level *actA* expression in broth culture was observed for *L. monocytogenes* strains containing the *prfA* G145S, *prfA* L140F and *prfA* Y63C alleles (~200-fold higher than the levels expressed by strains containing wild-type *prfA*) (Fig. 2a); in comparison, moderate *actA* expression was observed for the *prfA* E77K and *prfA* Y154C mutant strains (~10-fold increase in expression over wild-type).

To compare levels of activation at an additional PrfA-regulated promoter, *hly*, the expression of listeriolysin O (LLO, encoded by *hly*) was measured. Bacterial supernatants derived from broth cultures of each mutant strain were incubated with red blood cells and cell lysis was measured (Fig. 2b). Consistent with the observed increases in *actA* expression, the *prfA* G145S, *prfA* L140F and *prfA* Y63C strains exhibited the highest levels of LLO activity, followed by *prfA* E77K and *prfA* Y154C (Fig. 2b). Taken together, these data indicate a hierarchy of *prfA** mutant activity based on the patterns of PrfA-dependent gene expression: high-activity mutants (*prfA* G145S, *prfA* L140F and *prfA* Y63C) and moderate-activity mutants (*prfA* E77K and *prfA* Y154C).

Fig. 2. *prfA** strains exhibit increased expression of PrfA-dependent gene products in broth culture. (a) Levels of *actA* expression measured by monitoring GUS activity. Each time point represents the mean $\pm$ SEM of duplicate samples, and the data are representative of three independent experiments. (b) Secreted haemolytic activity as measured by erythrocyte (RBC) lysis. Serial dilutions of bacterial supernatants were incubated with RBCs and cell lysis was determined by measuring A_{450} . Each point represents the mean $\pm$ SD, and the data are derived from three independent experiments.

PrfA* mutants are conformationally distinct from the wild-type protein

Limited proteolytic digestion of proteins serves as a useful tool for rapid detection of protein conformational changes, and it has been used to distinguish between active and inactive forms of Crp (Harman *et al.*, 1986; Tan *et al.*, 1991). Limited protease digestion of Crp* mutants results in cleavage patterns that resemble those observed for Crp when bound to cAMP (Harman *et al.*, 1986; Tan *et al.*, 1991). To detect any conformational alterations associated with PrfA* mutations, each mutant protein was purified and subjected to limited trypsin digestion (Fig. 3). PrfA G145S protein served as a positive control for the assay, as a conformational change in this protein has already been demonstrated by crystal structure analysis (Eiting *et al.*, 2005). As anticipated, PrfA G145S was found to exhibit enhanced susceptibility to protease digestion in comparison with wild-type PrfA (Fig. 3). Similar to PrfA G145S, PrfA Y63C and PrfA E77K exhibited similar patterns of enhanced susceptibility to proteolysis. Interestingly, the highly activated PrfA L140F did not exhibit enhanced susceptibility to proteolysis, but the substitution of phenylalanine for leucine in this mutant occurs adjacent to a trypsin cleavage site, and may thus interfere with protease digestion. These results strongly suggest that the presence of the *prfA** mutations alters PrfA conformation.

PrfA* mutants appear to exhibit reduced dimer formation *in vitro*

PrfA has been shown to crystallize as a homodimer by two independent groups (Eiting *et al.*, 2005; Velge *et al.*, 2007), and it was recently shown that wild-type PrfA (27 kDa) migrates in SDS-PAGE gels at a molecular mass of approximately 60 kDa following incubation with a chemical cross-linking agent (Velge *et al.*, 2007). To assess the ability of the different PrfA* proteins to form dimers, purified proteins were incubated with two distinct chemical cross-linking agents and analysed on SDS-polyacrylamide gels. Two cross-linkers were used: S-EGS and BS³, both of which react with free amine groups but differ in the lengths of the linker arms [16 Å (1.6 nm) for S-EGS and 11 Å (1.1 nm) for BS³]. Interestingly, wild-type PrfA was found to form dimers more readily than any of the PrfA* mutants, with the ratio of dimer to monomer for S-EGS and BS³ being 0.27 and 0.44 respectively (Fig. 4). The moderately active PrfA Y154C and PrfA E77K showed reduced dimer formation in comparison to wild-type, with dimer to monomer ratios of approximately 0.05 for S-EGS and 0.11 for BS³ (Fig. 4). Unexpectedly, the most highly active PrfA* mutants PrfA G145S, PrfA L140F and PrfA Y63C exhibited the lowest ratio of dimer to monomer following chemical cross-linking (Fig. 4).

Fig. 3. Protease sensitivity of WT PrfA and PrfA* mutants. (a) Wild-type (W) and PrfA G145S (G) recombinant proteins were digested for 10, 30, 60, 90 and 120 min with trypsin, subjected to SDS-PAGE and visualized by Coomassie stain. –, Protein without trypsin; +, trypsin digestion of denatured protein. Lane M contains molecular mass markers. (b) Wild-type and PrfA* proteins were treated with trypsin for 10, 30, 60 and 120 min. W, wild-type; L, L140F; Y1, Y63C; Y2, Y154C; E, E77K. –, Samples without trypsin; +, trypsin-treated denatured protein. Numbers on the left represent molecular mass in kDa. The gel is representative of three similar experiments.

High-activity PrfA* mutant proteins exhibit enhanced binding to the *hly* and *actA* promoters

It has been demonstrated by EMSA that the PrfA G145S mutant protein binds to the *hly* promoter with higher affinity than wild-type PrfA (Mauder *et al.*, 2006; Vega *et al.*, 2004). Subsequent to these studies, Eiting *et al.*

(2005) demonstrated by surface plasmon resonance that PrfA G145S binds to the *hly* promoter with an 18-fold higher affinity than wild-type PrfA. Consistent with these observations, both of the highly activated PrfA* mutants PrfA G145S and PrfA L140F were observed to readily bind DNA fragments containing the *hly* promoter (Fig. 5). Wild-type PrfA was found to bind only weakly to the *hly* promoter, with detectable binding observed in the presence of 2 µg PrfA protein and no binding evident in the presence of 500 ng protein (Fig. 5). In contrast, the highly active PrfA* mutants PrfA G145S and PrfA L140F bound target DNA with very high affinity, such that all available DNA was bound at low protein concentrations (500 ng) (Fig. 5). PrfA Y63C, PrfA Y154C and PrfA E77K also demonstrated higher-affinity DNA binding in comparison with wild-type protein, but to a lesser degree than either PrfA G145S or PrfA L140F (Fig. 5). Binding in all cases was specific, as the addition of cold specific competitor DNA but not non-specific competitor DNA eliminated the PrfA-dependent mobility shift (M. D. Miner, unpublished data).

Purified PrfA protein has been previously shown to bind the *hly* promoter with higher affinity than the *actA* promoter (Böckmann *et al.*, 1996). Consistent with the promoter preference previously observed for the wild-type protein, the mutant PrfA proteins were observed to bind the *actA* promoter with a lower apparent affinity than the *hly* promoter; however, the relative binding affinity hierarchy for the proteins was similar to that observed for *hly* DNA (Fig. 5). Binding was detectable with significantly lower amounts of both PrfA G145S and PrfA L140F protein in comparison to wild-type PrfA.

Examination of PrfA*–DNA–RNA polymerase complex formation in bacterial cell extracts

Activation of target gene expression requires both binding of PrfA to target promoter sites and recruitment of RNAP. To examine the ability of PrfA* mutants to form complexes with target promoter DNA fragments and RNAP, purified PrfA and PrfA* mutant proteins were incubated with DNA in the presence of cell extracts derived from an *L. monocytogenes* *prfA* deletion strain. As mentioned above for purified protein incubated with DNA, wild-type PrfA exhibited weak binding of the *hly* promoter in comparison with PrfA* mutants (Eiting *et al.*, 2005; Mauder *et al.*, 2006; Vega *et al.*, 2004) (Fig. 6a, CIII complexes). However, in the presence of bacterial cell extracts, wild-type PrfA formed DNA–RNAP complexes with an affinity apparently equal to that of the PrfA* proteins (Fig. 6a, CI complexes). These results suggest that PrfA binding to the *hly* promoter is enhanced by the presence of RNAP and/or other components within bacterial cell extracts. The PrfA* mutants appeared to form PrfA–DNA–RNAP complexes that were roughly equivalent in amount to those formed using wild-type PrfA (Fig. 6a, CI complexes), and the absence of visible CII bands (RNAP–DNA complexes) suggests that RNAP is limiting under these assay

Fig. 4. Dimer formation of PrfA and PrfA* proteins. (a) Purified wild-type and PrfA* proteins (500 ng) were incubated with 10 μ M EGS (E) or BS³ (B), or without cross-linking agent (–) for 1 h at room temperature and subjected to SDS–PAGE and Western blot analysis with polyclonal antibody directed against PrfA (Greene & Freitag, 2003). (b) Ratio of dimer to monomer as determined from three independent experiments. Ratios were calculated by measuring the intensity of the peptide bands and dividing the monomer values by the dimer values, and include SD (error bars).

conditions. Similar results were observed with the *actA* promoter (Fig. 6b).

DISCUSSION

Despite its critical role in promoting the pathogenesis of *L. monocytogenes*, the mechanism by which PrfA becomes activated in the host cell cytosol remains undefined. However, the isolation and characterization of mutationally activated *prfA* alleles has helped to define the consequences of PrfA activation on *L. monocytogenes* physiology and pathogenesis (Mauder *et al.*, 2006; Miner *et al.*, 2008; Mueller & Freitag, 2005; Ripio *et al.*, 1997; Scortti *et al.*, 2007; Shetron-Rama *et al.*, 2003; Vega *et al.*, 2004; Wong & Freitag, 2004). This study represents, to our knowledge, the first biochemical comparison of multiple PrfA* mutant proteins with differing levels of activation. Our results suggest that *prfA** mutations have the capacity to activate PrfA via a variety of structural and functional modifications.

Overall, the expression levels of PrfA-dependent gene products *in vitro* appeared in this study to correlate most strongly with the binding affinity of PrfA for target DNA (Fig. 5). *prfA** mutations that conferred the highest levels of PrfA-dependent gene expression *in vitro* exhibited the highest affinity of DNA binding as detected by EMSA, with moderately active *prfA* mutant alleles correspondingly exhibiting more moderate increases in DNA binding. Interestingly, although wild-type PrfA formed very low amounts of protein–DNA complexes with either the *hly* or *actA* promoter fragments in comparison with PrfA* proteins (Fig. 5), PrfA–RNAP–DNA complexes were readily formed for both promoter fragments with RNAP present in cell extracts (Fig. 6). Earlier studies by Mauder

et al. (2006) have suggested that the efficiency of binding of PrfA to its binding site alone (CIII formation) does not necessarily indicate its potential to initiate transcription at a PrfA-dependent promoter. Their conclusions were based on *in vitro* transcription assays using purified PrfA proteins (including PrfA G145S) and partially purified RNAP with linear DNA templates. However, substantially less PrfA G145S is required to form either PrfA–DNA or PrfA–RNAP–DNA complexes than wild-type PrfA (Mauder *et al.*, 2006; Fig. 5); thus, it seems reasonable to speculate that under conditions in which PrfA concentrations are limiting, activated PrfA or PrfA* mutants with increased DNA binding affinity would be better able to stimulate the formation of active transcription complexes with RNAP.

Mutations that enhance PrfA-dependent gene expression *in vitro* have been isolated in multiple regions of the protein (Fig. 1). PrfA G145S and PrfA L140F map within the α D helix of PrfA, with G145S positioned near the centre of the helix and L140F located at one end (Eiting *et al.*, 2005; Fig. 1). The mutations are positioned near what corresponds to the hinge region of Crp, a region believed to mediate communication between the C- and N-terminal domains of the protein (Garges & Adhya, 1985; Harman *et al.*, 1986; Kim *et al.*, 1992; Youn *et al.*, 2006). Selected mutations in the Crp hinge region lead to the constitutive activation of Crp in the absence of cAMP via a change in secondary structure that enhances the solvent exposure of the DNA binding helix. Eiting *et al.* (2005) reported a similar structural change that occurs in PrfA G145S mutants. Based on the functional similarities of the L140F mutant with G145S, most notably a large increase in DNA binding affinity, the PrfA L140F mutation may mediate a similar structural change. While the conformational changes imparted by the L140F mutation as detected by limited proteolysis indicated that the PrfA L140F

Fig. 5. *In vitro* DNA binding activity of wild-type PrfA and PrfA* proteins. (a) Purified protein was incubated with labelled *hly* or *actA* DNA fragments. All reactions contained 500 ng protein, except those indicated by *, which contained 10 ng. (b) Increasing amounts of purified protein were incubated with *hly* DNA fragments. Amounts of protein are given above the lanes. Gels are representative of three experiments. fp, free probe; CIII, PrfA-bound DNA (CIII designation derived from Böckmann *et al.*, 2000).

protease susceptibility was most similar to that of the wild-type protein (Fig. 3), this result may be misleading, as the L140F mutation is located near a trypsin cleavage site (K139), which could influence the efficiency of trypsin cleavage at this position.

Other *prfA** mutations with the potential for distinct structural influences include the PrfA E77K, Y63C and Y154C mutations. The E77K mutation lies between $\beta 6$ and $\beta 7$ in a region near the central C helices (Eiting *et al.*, 2005; Fig. 1). This mutation enhanced PrfA DNA binding to a lesser extent than that of the G145S and L140F mutations, which suggests either that E77K has a more modest effect on the repositioning of the central C helices or that E77K enhances PrfA-dependent gene expression through a different mechanism. The E77K mutation is located near

a region of PrfA that corresponds to an area of Crp and CooA known to interact with RNAP (AR2) (Leduc *et al.*, 2001; Niu *et al.*, 1996). AR2 comprises a patch of positively charged residues that contact an acidic patch on the N-terminal domain of the α -subunit of RNAP (α -NTD). As the PrfA E77K substitution adds a positively charged lysine residue within a similarly located potential AR2 region, it is possible that the additional positive charge enhances PrfA interactions with RNAP.

Y154C and Y63C map within regions of PrfA (αD and $\beta 5$, respectively) that are associated with a structural tunnel that may serve as a binding pocket for PrfA co-factor (Eiting *et al.*, 2005). Y154C is located at the very end of the αD helix, whereas Y63C is located within the $\beta 5$ domain (Fig. 1). Despite the similar chemical nature of the

Fig. 6. PrfA and PrfA* formation of DNA–RNAP complexes. Purified wild-type or PrfA* proteins (500 ng) were incubated with *hly* (a) or *actA* (b) DNA fragments in the presence of cell extracts derived from a $\Delta prfA$ strain (NF-L890). Gels are representative of three experiments. fp, free probe; CI, PrfA+DNA; CII, RNAP+DNA; CIII, PrfA–RNAP–DNA (CI, CII and CIII designations are derived from Böckmann *et al.*, 2000).

substitutions, these mutations have dramatically different effects on PrfA function. The Y154C mutation slightly enhanced PrfA-dependent gene expression in broth culture and exhibited a modest but reproducible increase in apparent DNA binding affinity (Fig. 5). Interestingly, this mutation impedes PrfA-dependent gene expression in cytosolic *L. monocytogenes*, suggesting that the Y154C mutation may interfere with the shift of PrfA to a fully activated state (Miner *et al.*, 2008). In contrast, Y63C dramatically increased PrfA-dependent gene expression in broth culture but did not dramatically increase DNA binding affinity (Figs 2 and 5). Several possibilities exist that could account for the effects of these mutations on PrfA function. The mutations could: (1) inhibit (Y154C) or enhance (Y63C) PrfA cofactor binding; (2) stabilize the low- (Y154C) or high- (Y63C) activity form of PrfA; or (3) result in the formation of disulfide bridges that serve to lock PrfA in either a low-activity (Y154C) or a high-activity (Y63C) state. Although we cannot differentiate between these possibilities at this time, we favour the Y63C mutation enhancing cofactor binding for the simple reason that no significant increase in DNA binding was observed for this mutant *in vitro*, suggesting that its high activity is not due to increased accessibility of the PrfA DNA binding helix-turn domain.

The apparent inverse correlation that was found to exist between the ability of the PrfA* mutants to form dimers and their ability to activate gene expression was unexpected. Crp has long been known to form dimers as an active transcription factor, and Fnr is believed to form dimers when active and to be monomeric when inactive (Lazazzera

et al., 1993). While the chemical cross-linking experiments presented here suggest that PrfA dimerization inversely correlates with DNA binding and activation of target gene expression, the cross-linking agents used were specifically reactive for amine groups and it is possible that these moieties are less available as a result of conformational changes resulting from the *prfA** mutations.

Multiple *prfA** mutations have been isolated in *L. monocytogenes* using a variety of approaches (Miner *et al.*, 2008; Shetron-Rama *et al.*, 2003; Vega *et al.*, 2004), and the reconstruction of these mutations in isogenic backgrounds has been highly desirable for unambiguous comparison of the effects of the *prfA** mutations on *L. monocytogenes* physiology and pathogenesis. While the moderately active *prfA** alleles have been easily introduced into isogenic strains using allelic exchange (Miner *et al.*, 2008; Shetron-Rama *et al.*, 2003; Vega *et al.*, 2004), this approach has not proven feasible for the higher-activity *prfA** mutants *prfA* G145S and *prfA* L140F (Port & Freitag, 2007; Wong & Freitag, 2004; M. D. Miner, unpublished observations). The *prfA* G145S and *prfA* L140F mutations appear to confer a subtle fitness defect upon *L. monocytogenes* that is not evident in pure cultures of bacteria but which can be detected in mixed cultures when the mutant strains are grown in the presence of wild-type bacteria (J. Bruno and N. E. Freitag, unpublished data). A fitness defect has also been reported for high-activity *crp** mutants (Youn *et al.*, 2006). To our knowledge, until now, the *prfA* G145S had never been reintroduced into its correct chromosomal location by allelic exchange in any *L. monocytogenes* strain, including EGD and 10403S. This

work therefore represents a novel method enabling the reconstruction of *prfA** isogenic strains with highly active *prfA** mutations without the use of plasmids and with *prfA** in the proper chromosomal location.

In summary, *prfA** mutations appear to activate PrfA through a variety of structural and functional modifications. In general, the *prfA** mutations that most dramatically enhanced the binding of PrfA to its DNA recognition sequences resulted in the highest levels of PrfA-dependent gene expression in bacterial cultures. Surprisingly, an inverse correlation appears to exist between the level of PrfA activation conferred by a *prfA** mutation and the ability of the purified mutant protein to form a dimer. Future studies focused on 3D structural analyses of the mutant proteins will help to further clarify the influences of individual *prfA** mutations on PrfA activation.

ACKNOWLEDGEMENTS

We thank Dr Patrick Piggot, Temple University School of Medicine, for the pBEST501 plasmid, Dr Hao Shen, University of Pennsylvania School of Medicine, and Dr Jeff Miller, University of California at Los Angeles, for the *L. monocytogenes* Δ *prfA* strain, and members of the Freitag laboratory for helpful discussions. This work was supported by Public Health Service grants AI41816 (N.E.F.) from NIAID, by a NIAID Bacterial Pathogenesis training grant fellowship AI55396 (M.D.M.), a National Science Foundation Graduate Research Fellowship (NSF-GRF) (G.C.P.), and by the M. J. Murdock Trust. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the funding sources.

REFERENCES

- Bishop, D. K. & Hinrichs, D. J. (1987). Adoptive transfer of immunity to *Listeria monocytogenes*: the influence of in vitro stimulation on lymphocyte subset requirements. *J Immunol* **139**, 2005–2009.
- Böckmann, R., Dickneite, C., Middendorf, B., Goebel, W. & Sokolovic, Z. (1996). Specific binding of the *Listeria monocytogenes* transcriptional regulator PrfA to target sequences requires additional factor(s) and is influenced by iron. *Mol Microbiol* **22**, 643–653.
- Böckmann, R., Dickneite, C., Goebel, W. & Bohne, J. (2000). PrfA mediates specific binding of RNA polymerase of *Listeria monocytogenes* to PrfA-dependent virulence gene promoters resulting in a transcriptionally active complex. *Mol Microbiol* **36**, 487–497.
- Bubert, A., Sokolovic, Z., Chun, S.-K., Papatheodorou, L., Simm, A. & Goebel, W. (1999). Differential expression of *Listeria monocytogenes* virulence genes in mammalian host cells. *Mol Gen Genet* **261**, 323–336.
- Busby, S. & Ebright, R. H. (1999). Transcription activation by catabolite activator protein (CAP). *J Mol Biol* **293**, 199–213.
- Camilli, A., Paynton, C. R. & Portnoy, D. A. (1989). Intracellular methicillin selection of *Listeria monocytogenes* mutants unable to replicate in a macrophage cell line. *Proc Natl Acad Sci U S A* **86**, 5522–5526.
- Chakraborty, T., Leimeister-Wachter, M., Domann, E., Hartl, M., Goebel, W., Nichterlein, T. & Notermans, S. (1992). Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. *J Bacteriol* **174**, 568–574.
- Eiting, M., Hagelken, G., Schubert, W. D. & Heinz, D. W. (2005). The mutation G145S in PrfA, a key virulence regulator of *Listeria monocytogenes*, increases DNA-binding affinity by stabilizing the HTH motif. *Mol Microbiol* **56**, 433–446.
- Freitag, N. E. (2006). From hot dogs to host cells: how the bacterial pathogen *Listeria monocytogenes* regulates virulence gene expression. *Future Microbiol* **1**, 89–101.
- Freitag, N. E. & Jacobs, K. E. (1999). Examination of *Listeria monocytogenes* intracellular gene expression by using the green fluorescent protein of *Aequorea victoria*. *Infect Immun* **67**, 1844–1852.
- Freitag, N. E., Youngman, P. & Portnoy, D. A. (1992). Transcriptional activation of the *Listeria monocytogenes* hemolysin gene in *Bacillus subtilis*. *J Bacteriol* **174**, 1293–1298.
- Garges, S. & Adhya, S. (1985). Sites of allosteric shift in the structure of cyclic AMP receptor protein. *Cell* **41**, 745–751.
- Gray, M. J., Freitag, N. E. & Boor, K. J. (2006). How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infect Immun* **74**, 2505–2512.
- Greene, S. L. & Freitag, N. E. (2003). Negative regulation of PrfA, the key activator of *Listeria monocytogenes* virulence gene expression, is dispensable for bacterial pathogenesis. *Microbiology* **149**, 111–120.
- Harman, J. G., McKenney, K. & Peterkofsky, A. (1986). Structure–function analysis of three cAMP-independent forms of the cAMP receptor protein. *J Biol Chem* **261**, 16332–16339.
- Karow, M. L. & Piggot, P. J. (1995). Construction of *gusA* transcriptional fusion vectors for *Bacillus subtilis* and their utilization for studies of spore formation. *Gene* **163**, 69–74.
- Kim, J., Adhya, S. & Garges, S. (1992). Allosteric changes in the cAMP receptor protein of *Escherichia coli*: hinge reorientation. *Proc Natl Acad Sci U S A* **89**, 9700–9704.
- Kolb, A., Busby, S., Buc, H., Garges, S. & Adhya, S. (1993). Transcriptional regulation by cAMP and its receptor protein. *Annu Rev Biochem* **62**, 749–795.
- Korner, H., Sofia, H. J. & Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp–Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol Rev* **27**, 559–592.
- Lawson, C. L., Swigon, D., Murakami, K. S., Darst, S. A., Berman, H. M. & Ebright, R. H. (2004). Catabolite activator protein: DNA binding and transcription activation. *Curr Opin Struct Biol* **14**, 10–20.
- Lazazzera, B. A., Bates, D. M. & Kiley, P. J. (1993). The activity of the *Escherichia coli* transcription factor FNR is regulated by a change in oligomeric state. *Genes Dev* **7**, 1993–2005.
- Leduc, J., Thorsteinsson, M. V., Gaal, T. & Roberts, G. P. (2001). Mapping CoaA–RNA polymerase interactions. Identification of activating regions 2 and 3 in CoaA, the co-sensing transcriptional activator. *J Biol Chem* **276**, 39968–39973.
- Leimeister-Wachter, M., Haffner, C., Domann, E., Goebel, W. & Chakraborty, T. (1990). Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. *Proc Natl Acad Sci U S A* **87**, 8336–8340.
- Mauder, N., Ecker, R., Mertins, S., Loeffler, D. I., Seidel, G., Sprehe, M., Hillen, W., Goebel, W. & Müller-Altrick, S. (2006). Species-specific differences in the activity of PrfA, the key regulator of listerial virulence genes. *J Bacteriol* **188**, 7941–7956.
- Mengaud, J., Vicente, M. F. & Cossart, P. (1989). Transcriptional mapping and nucleotide sequence of the *Listeria monocytogenes* *hlyA* region reveal structural features that may be involved in regulation. *Infect Immun* **57**, 3695–3701.
- Miner, M., Port, G., Bouwer, H., Chang, J. & Freitag, N. (2008). A novel *prfA* mutation that promotes *Listeria monocytogenes* cytosol

- entry but reduces bacterial spread and cytotoxicity. *Microb Pathog* **45**, 273–281.
- Moors, M. A., Levitt, B., Youngman, P. & Portnoy, D. A. (1999).** Expression of listeriolysin O and ActA by intracellular and extracellular *Listeria monocytogenes*. *Infect Immun* **67**, 131–139.
- Mueller, K. J. & Freitag, N. E. (2005).** Pleiotropic enhancement of bacterial pathogenesis resulting from the constitutive activation of the *Listeria monocytogenes* regulatory factor PrfA. *Infect Immun* **73**, 1917–1926.
- Niu, W., Kim, Y., Tau, G., Heyduk, T. & Ebright, R. H. (1996).** Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. *Cell* **87**, 1123–1134.
- Pizarro-Cerda, J. & Cossart, P. (2006).** Subversion of cellular functions by *Listeria monocytogenes*. *J Pathol* **208**, 215–223.
- Port, G. C. & Freitag, N. E. (2007).** Identification of novel *Listeria monocytogenes* secreted virulence factors following mutational activation of the central virulence regulator, PrfA. *Infect Immun* **75**, 5886–5897.
- Ripio, M.-T., Dominguez-Bernal, G., Lara, M., Suarez, M. & Vazquez-Boland, J.-A. (1997).** A Gly145Ser substitution in the transcriptional activator PrfA causes constitutive overexpression of virulence factors in *Listeria monocytogenes*. *J Bacteriol* **179**, 1533–1540.
- Scortti, M., Monzo, H. J., Lacharme-Lora, L., Lewis, D. A. & Vazquez-Boland, J. A. (2007).** The PrfA virulence regulon. *Microbes Infect* **9**, 1196–1207.
- Shetron-Rama, L. M., Marquis, H., Bouwer, H. G. A. & Freitag, N. E. (2002).** Intracellular induction of *Listeria monocytogenes* *actA* expression. *Infect Immun* **70**, 1087–1096.
- Shetron-Rama, L. M., Mueller, K., Bravo, J. M., Bouwer, H. G., Way, S. S. & Freitag, N. E. (2003).** Isolation of *Listeria monocytogenes* mutants with high-level *in vitro* expression of host cytosol-induced gene products. *Mol Microbiol* **48**, 1537–1551.
- Tan, G. S., Kelly, P., Kim, J. & Wartell, R. M. (1991).** Comparison of cAMP receptor protein (CRP) and a cAMP-independent form of CRP by Raman spectroscopy and DNA binding. *Biochemistry* **30**, 5076–5080.
- Vega, Y., Rauch, M., Banfield, M. J., Ermolaeva, S., Scortti, M., Goebel, W. & Vazquez-Boland, J. A. (2004).** New *Listeria monocytogenes* *prfA** mutants, transcriptional properties of PrfA* proteins and structure–function of the virulence regulator PrfA. *Mol Microbiol* **52**, 1553–1565.
- Velge, P., Herler, M., Johansson, J., Roche, S. M., Témoïn, S., Fedorov, A. A., Gracieux, P., Almo, S. C., Goebel, W. & Cossart, P. (2007).** A naturally occurring mutation K220T in the pleiotropic activator PrfA of *Listeria monocytogenes* results in a loss of virulence due to decreasing DNA-binding affinity. *Microbiology* **153**, 995–1005.
- Wong, K. K. & Freitag, N. E. (2004).** A novel mutation within the central *Listeria monocytogenes* regulator PrfA that results in constitutive expression of virulence gene products. *J Bacteriol* **186**, 6265–6276.
- Youn, H., Kerby, R. L., Conrad, M. & Roberts, G. P. (2006).** Study of highly constitutively active mutants suggests how cAMP activates cAMP receptor protein. *J Biol Chem* **281**, 1119–1127.
- Youn, H., Kerby, R. L., Koh, J. & Roberts, G. P. (2007).** A C-helix residue, Arg-123, has important roles in both the active and inactive forms of the cAMP receptor protein. *J Biol Chem* **282**, 3632–3639.
- Youngman, P. (1987).** Plasmid vectors for recovering and exploiting Tn917 transpositions in *Bacillus* and other Gram-positive bacteria. In *Plasmids: a Practical Approach*, pp. 79–103. Edited by K. Hardy. Oxford: IRL Press.

Edited by: H. Ingmer