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

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SUMMARY

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 analyzed 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 electophoretic mobility shift assay (EMSA) 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.

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; Scortti *et al.*, 2007). PrfA is a 27 kD protein that recognizes and binds a 14 base pair DNA palindrome present in the promoters of its target genes (Freitag *et al.*, 1992; Mengaud *et al.*, 1989). PrfA regulates the expression of gene

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products required for *L. monocytogenes* invasion of host cells, intracellular growth, and cell-to-cell spread, and it is absolutely essential for bacterial virulence (Freitag, 2006; Scortti *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. Co-factor 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 co-factor is required to shift PrfA to a high activity form capable of high affinity DNA binding. Although it is generally believed that a PrfA co-factor exists, this co-factor has not yet been identified.

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; Youn *et al.*, 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 *E. 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 been proven difficult to construct using standard allelic exchange techniques [(Port & Freitag, 2007; Wong & Freitag, 2004) and 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.*

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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 QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions with 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*

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* Δ*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 ug chloramphenicol ml^{-1}, 200 ug streptomycin ml^{-1}, 50 ug 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc) ml^{-1}, and 5 ug 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 either 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 Supplementary Table S1. The PCR fragments were then cloned into pET100 using Champion pET Directional TOPO Expression Kit (Invitrogen) 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 hours. Protein extracts containing recombinant PrfA/PrfA* proteins were passed over a nickel column, and PrfA was eluted with 200–500 mM imidazole buffer and dialyzed into phosphate buffered saline (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) (PBS) with 10% glycerol (v/v). Purified protein was visualized and assessed for purity following separation on SDS-PAGE gels and

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Coomassie staining, and also confirmed by western analysis using a α-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 10X Multicore buffer for the indicated times at 37° C. Reactions were terminated by the addition of 1 ul phenylmethanesulfonylfluoride and samples were then boiled for 5 minutes and 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 (EMSA)

Primers used to amplify DNA fragments (~100 bp) containing the *hly* and *actA* promoters from *L. monocytogenes* genomic DNA using PCR are listed in Table 2. Primers were purchased labeled with cy5.5 label on the 5'ends (Operon Biotechnologies, Inc.) To generate a DNA fragment for use as a non-specific competitor for DNA binding assays, primers were used to amplify the *prfA* open reading frame (~370 bp), as this region lacks PrfA binding sites (primer sequences listed in Table 2.) Extracts from NF-L890 (*ΔprfA*) were made as follows: bacteria from 1 L cultures grown to mid-log phase in BHI were collected using centrifugation for 10 minutes at 6,000 x g, resuspended in 20 ml ice cold PBS, and bacterial cells were disrupted by a triple passage through a French Press. Electrophoretic mobility shift assays (EMSA) were performed as previously described (Bockmann *et al.*, 2000). EMSA reaction mixtures consisted of the following: 40 ng labeled DNA probe (hlycy5.5 or actAcy5.5), PrfA protein (as indicated),1 ug BSA ml^{-1}, and 50 mM dIdC in TB buffer (10mM Tris HCl pH 8, 10mM MgCl2, 5mM CaCl2, 1mM EDTA, 0.2mM DTT, 10% glycerol [v/v]) in a final reaction volume of 20 ul. For experiments including cell extracts, 1 ul (3 ug) extract was added to each reaction mixture. Sample reactions containing all components except labeled DNA were incubated for 15 minutes at room temperature. The labeled DNA probe was then added and samples were incubated for 3 minutes at 37°, followed by 27 minutes on ice. Samples were then loaded onto a 5% acrylamide gel (0.5X TBE, Tris-boric acid-EDTA) and run at a constant current of 20 mA for approximately 3 hours in the dark at 4° C. Gels were then visualized as in-gel westerns using the Odyssey Imager (Li-Cor Biosciences) with the cy5.5-labeled fluorescent probes visualized at 700 nm. The His- and Express-tags were found to have minimal impact on PrfA or PrfA* protein function in comparison to purified PrfA protein without the tags (M. Miner, unpublished data), as has been previously reported (Bockmann *et al.*, 2000; Böckmann *et al.*, 1996).

Measurement of β-glucoronidase activity

β-glucuronidase (GUS) activity was measured as previously described (Shetron-Rama *et al.*, 2003) with minor changes. Briefly, *L. monocytogenes* cultures grown overnight at 37º C in BHI were diluted 1:50 and grown with shaking at 37º C for 8 hours. 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

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microcentrifugation and the supernatants were removed,. Bacterial pellets were resuspended in 100 μl (aliquot 1) or 1 ml (aliquot 2) ABT buffer (0.1M potassium phosphate, pH 7.0, 0.1M NaCl, 0.1% Triton). GUS activity was measured as described with the substitution of 4-methylumbelliferyl-β-D-glucuronide in place of 4-methylumbilliferyl-β-D-galactoside (Sigma) (Youngman, 1987). Data were derived from duplicate samples taken from three independent experiments.

Measurement of hemolytic activity

Hemolytic activity was measured as previously described 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 two-fold serial dilutions of the supernatants were incubated with PBS-washed sheep red blood cells (0.3% to 10%) for 30 minutes at 37° C. After incubation, RBCs were recovered by centrifugation to measure 50% lysis and supernatants were read in a spectrophotometer plate reader at OD450.

Protein chemical crosslinking

500 ng of purified proteins were incubated with either 10uM sulfo-ethylene glycol bis[succinimidylsuccinate] (S-EGS) or *Bis*[sulfosuccinimidyl] suberate (BS^3) in 0.2M triethylamine (TEA), pH 8.0 for 1 hour at room temperature. Samples were then heated at 85° C for 10 minutes in SDS sample buffer (1% SDS, 10% glycerol, 10 mM Tris-Cl, pH 6.8, 1 mM ethylene diamine tetraacetic acid (EDTA), and 0.05 mg bromphenol blue dye ml^{-1}) containing 5% BME, run on SDS-PAGE and transferred to nitrocellulose. Rabbit polyclonal antibody directed against PrfA was used for western analysis at 1:4000 dilution followed by incubation with goat-anti-rabbit-IRDye 680 at 1:10000 (Li-cor Biosciences). Membranes were visualized on 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 for neomycin resistance (*neo*) and β-glucuronidase (*gus*) were introduced in single copy into the bacterial chromosome downstream of *actA* in a Δ*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 color in the presence of the GUS substrate 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (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 Δ*prfA/ actA-gus-neo-plcB L. monocytogenes*. Transconjugants with *prfA** mutations were selected based on enhanced neomycin resistance and dark blue colony color 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

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(based on GUS activity in broth culture) in comparison to 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 three dimensional 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 been previously 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 mutation into the *L. monocytogenes* chromosome [(Port & Freitag, 2007; Wong & Freitag, 2004) and 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 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 (approximately 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 (approximate 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).

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

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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 to 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 kD) migrates in SDS-PAGE gels at a molecular weight of approximately 60 kD following incubation with a chemical crosslinking 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 crosslinking agents and analyzed on SDS polyacrylamide gels. Two crosslinkers were used: sulfo-ethylene glycol bis[succinimidylsuccinate] (S-EGS) and Bis[sulfosuccinimidyl] suberate (BS^3), both of which react with free amine groups but differ in the lengths of the linker arms (16 angstroms for S-EGS and 11 angstroms for BS^3). 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 BS^3 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 BS^3 (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 crosslinking (Fig. 4).

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

It has been previously 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* (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. 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 ug 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 to wild-type protein, but to a lesser degree than either PrfA G145S or PrfA

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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. 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 RNA polymerase (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 a *L. monocytogenes prfA* deletion strain. As previously mentioned for purified protein incubated with DNA, wild-type PrfA exhibited weak binding of the *hly* promoter in comparison to 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 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 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.

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Interestingly, although wild type PrfA formed very low amounts of protein-DNA complexes with either the *hly* or *actA* promoter fragments in comparison to PrfA* proteins (Fig. 5), PrfA-RNAP-DNA complexes were readily formed for both promoter fragments with RNAP present in cell extracts (Fig. 6). Previous studies by Mauder *et al* (Mauder *et al.*, 2006) have suggested that the binding efficiency 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) and 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 αD α-helix of PrfA, with G145S positioned near the center of the helix and L140F located at one end [(Eiting *et al.*, 2005) and 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 (Eiting *et al.*, 2005) reported a similar structural change occurring 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 comparable structural change. While the conformational changes imparted by the L140F mutation as detected by limited proteolysis indicated that the PrfA L140F 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 likely 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 the Y154C mutations. The E77K mutation lies between β6 and β7 in a region near the central C helices [(Eiting *et al.*, 2005) and Fig. 1]. This mutation enhanced PrfA DNA binding to a lesser extent than that of the G145S and L140F mutations, which could suggest E77K has either 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 with an area of Crp and CooA known to interact with RNA polymerase (AR2) (Leduc *et al.*, 2001; Niu *et al.*, 1996). AR2 is comprised of a patch of positively charged residues that contact an acidic patch on α-NTD of RNAP. As the PrfA E77K substitution adds a positively charged lysine residue within a potential similarly located AR2 region, it is possible that the additional positive charge enhances PrfA interactions with RNAP.

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Y154C and Y63C map within regions of PrfA (αD and β5 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 β5 domain (Fig. 1). Despite the similar chemical nature of the substitutions, these mutations have dramatically different affects 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 (Fig. 2 and Fig. 5). Several possibilities exist that could account for the effects of these mutations on PrfA function. The mutations could either: (1) inhibit (Y154C) or enhance (Y63) PrfA co-factor 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 high activity (Y63) state. Whereas we cannot differentiate between these possibilities at this time, we favor the Y63C mutation enhancing co-factor 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 been long known to form dimers as an active transcription factor, and Fnr is believed to form dimers when active and be monomeric when inactive (Lazazzera *et al.*, 1993). While the chemical crosslinking experiments presented here suggest that PrfA dimerization inversely correlates with DNA binding and activation of target gene expression, the crosslinking 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) and 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. 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 current work therefore represents a novel method enabling the reconstruction of *prfA**

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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 apparent 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 three dimensional structural analyses of the mutant proteins will help to further clarify the influences of individual *prfA** mutations on PrfA activation.

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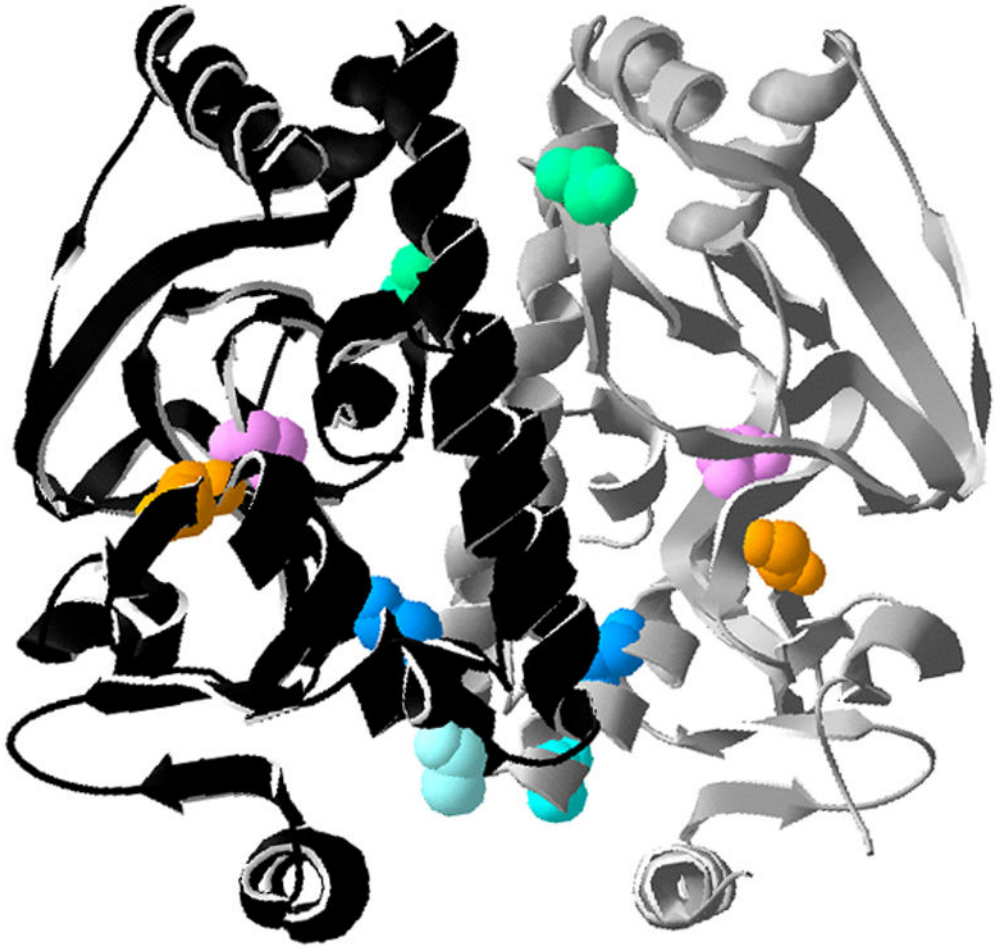

Figure 1. Location of *prfA mutations on the PrfA crystal structure**

Crystal structure of the PrfA dimer (monomers in black and grey) adapted from Eiting *et al* (Eiting *et al.*, 2005). Locations of the five PrfA* mutations used in this study are shown on each monomer and color-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 (αC and αD) or yellow numbers (β5, β6, and β7) and derived from Eiting *et al.*, 2005).

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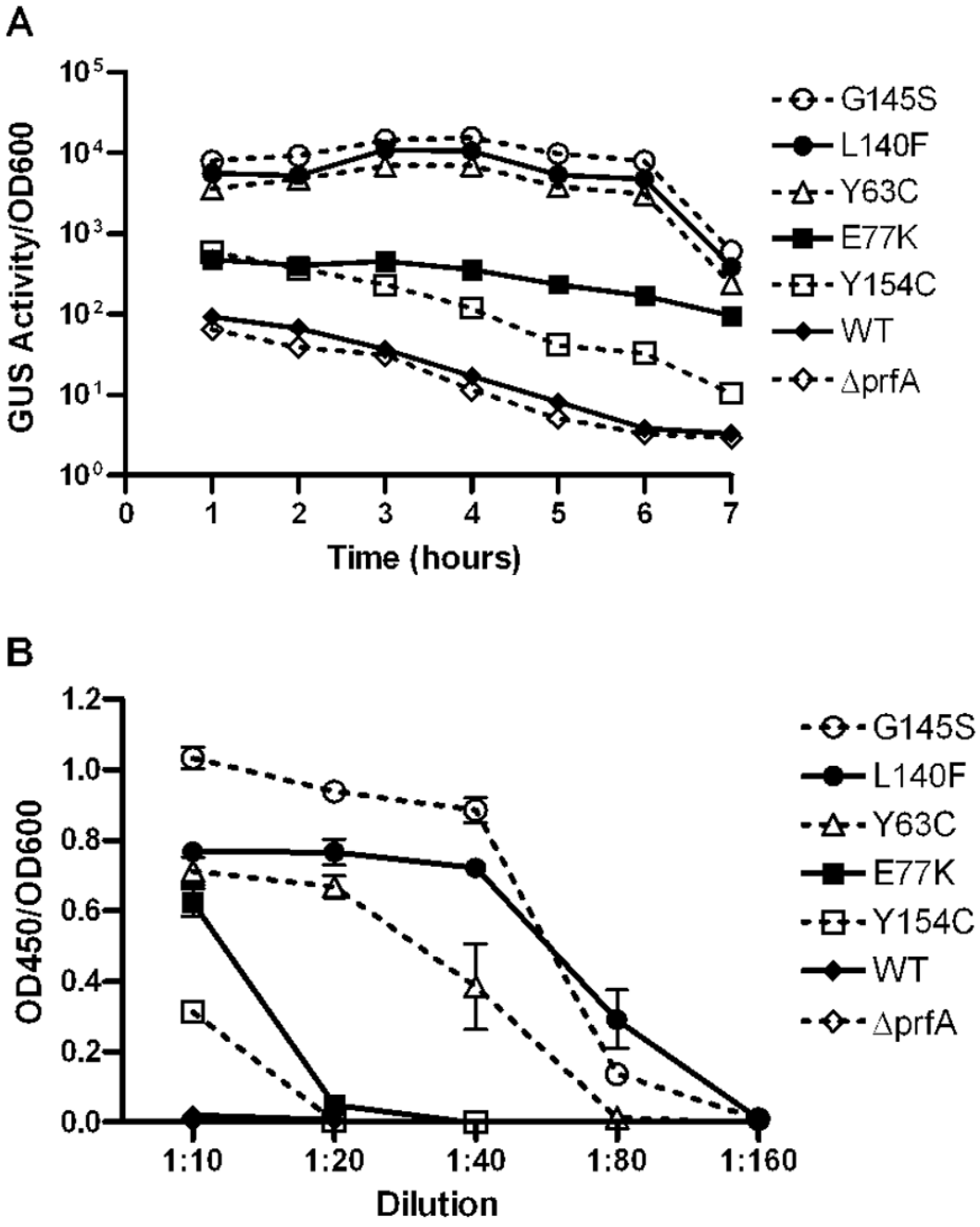

Figure 2. *prfA strains exhibit increased expression of PrfA-dependent gene products in broth culture**

A. Levels of *actA* expression measured by monitoring GUS activity. Each time point represents the mean +/− the SEM of duplicate samples, and the data is representative of three independent experiments. *B.* Secreted hemolytic activity as measured by erythrocyte (RBC) lysis. Serial dilutions of bacterial supernatants were incubated with RBCs and cell lysis was determined by measuring absorbance at 450 nm (OD450). Each point represents the mean +/− SD, and the data is derived from three independent experiments.

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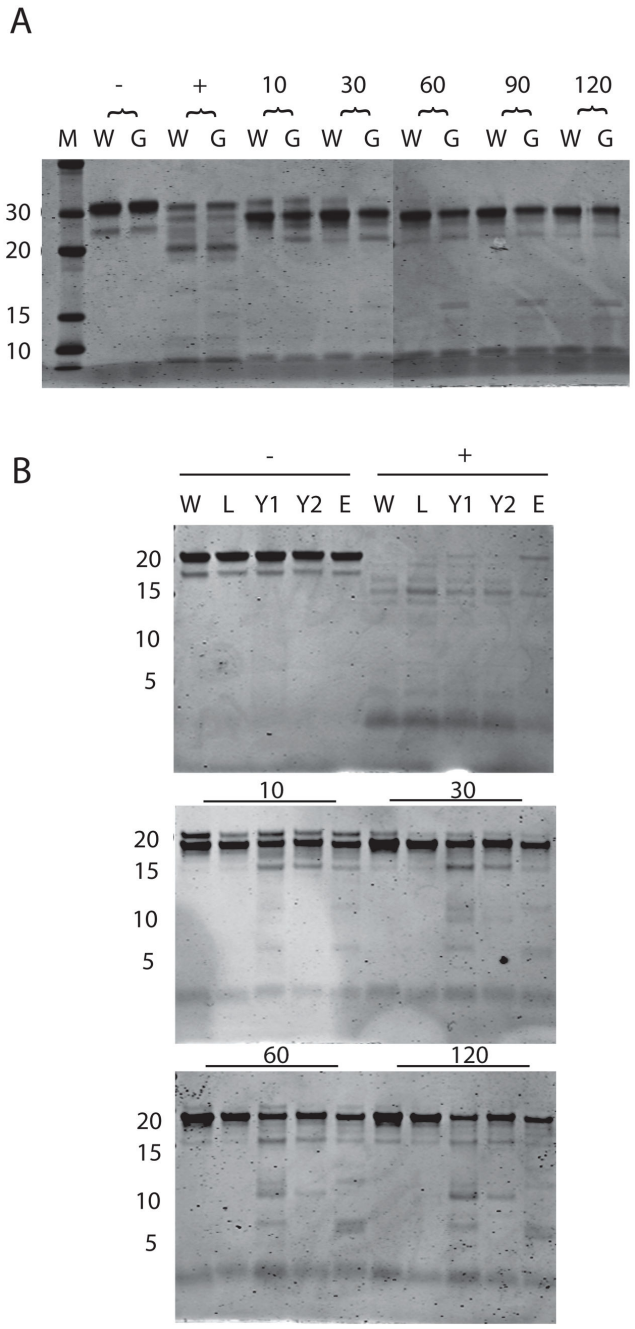

Figure 3. Protease sensitivity of WT PrfA and PrfA* mutants

A. Wild type (W) and PrfA G145S (G) recombinant proteins were digested for 10, 30, 60, 90, and 120 minutes with trypsin, subjected to SDS-PAGE and visualized by Coomassie stain. Minus symbol denotes protein without trypsin, plus symbol denotes trypsin digestion of denatured protein. Lane M contains molecular weight markers. *B.* Wild type and PrfA* proteins were treated with trypsin for 10, 30, 60, and 120 minutes. W, wild type; L, L140F; Y1, Y63C, Y2, Y154C; E, E77K. Minus symbol indicates samples without trypsin, plus

symbol indicates trypsin treated denatured protein. Numbers on left represent molecular weight in kD. Gel is representative of three similar experiments.

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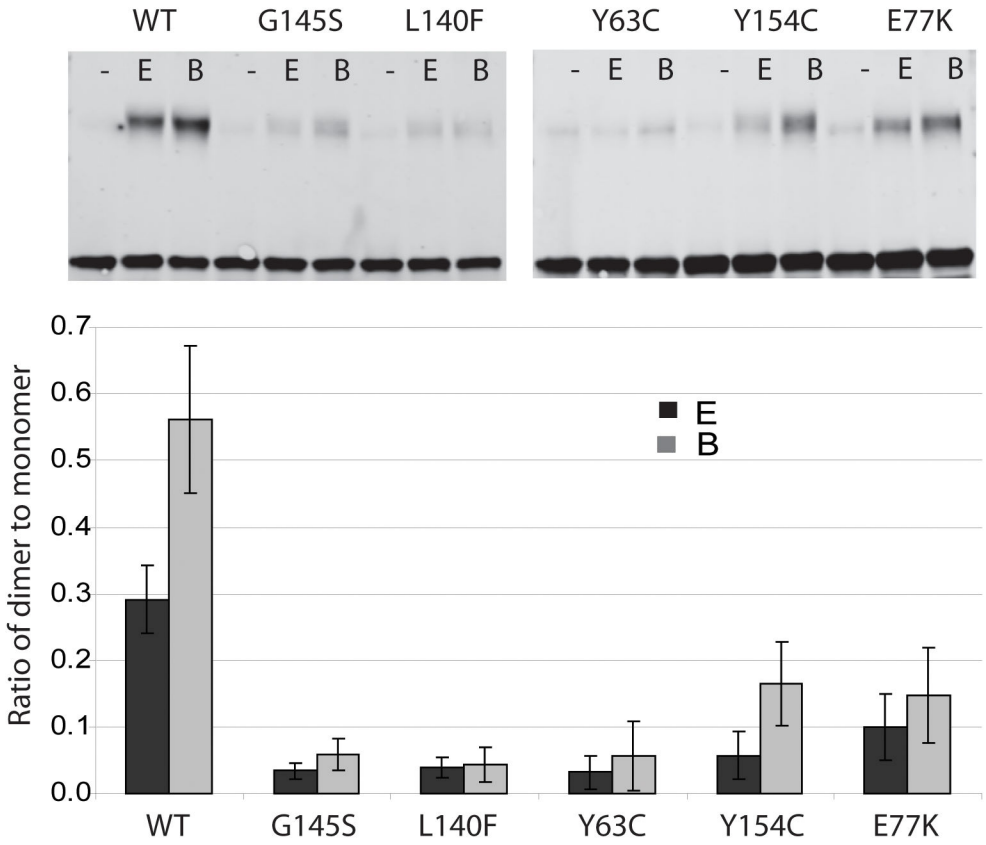

Figure 4. Dimer formation of PrfA and PrfA* proteins

A. Purified wild type and PrfA* protein (500 ng) was incubated with 10uM EGS (E) or BS^3 (B) or without crosslinking agent (-) for one hour at room temperature and subjected to SDS-PAGE and Western analysis with polyclonal antibody directed against PrfA (Greene & Freitag, 2003). Arrows indicate molecular weight in kD. *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 standard deviations.

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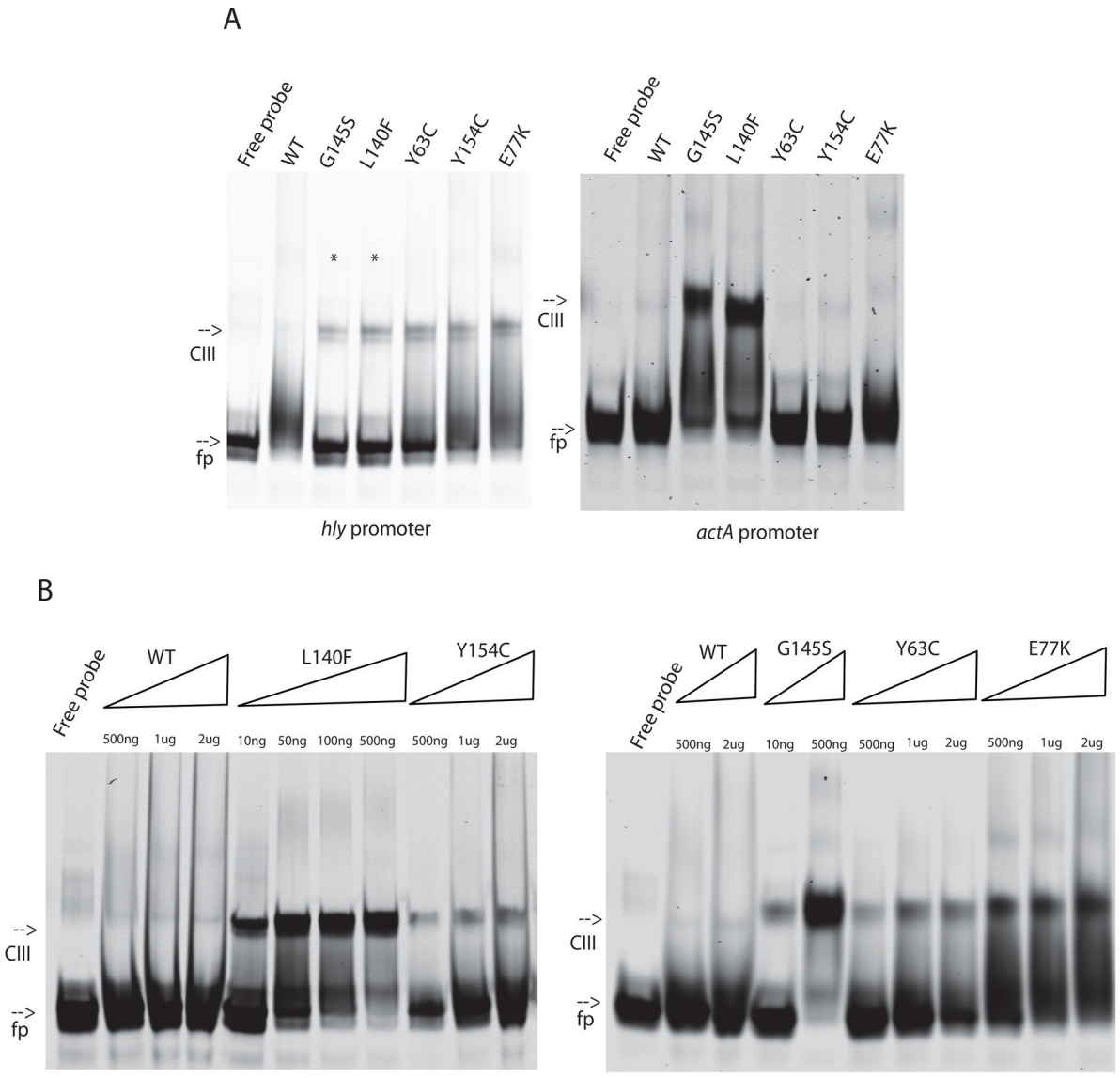

Figure 5. *In vitro* DNA binding activity of WT PrfA and PrfA* proteins

A. Purified protein was incubated with labeled *hly* or *actA* DNA fragments. All reactions contained 500 ng protein except those denoted with * which contained 10 ng. *B.* Increasing amounts of purified protein were incubated with *hly* DNA fragments. Amounts of protein are denoted above the lane. Gels are representative of three experiments. fp, free probe; CIII, PrfA-bound DNA [CIII designation derived from (Bockmann *et al.*, 2000)].

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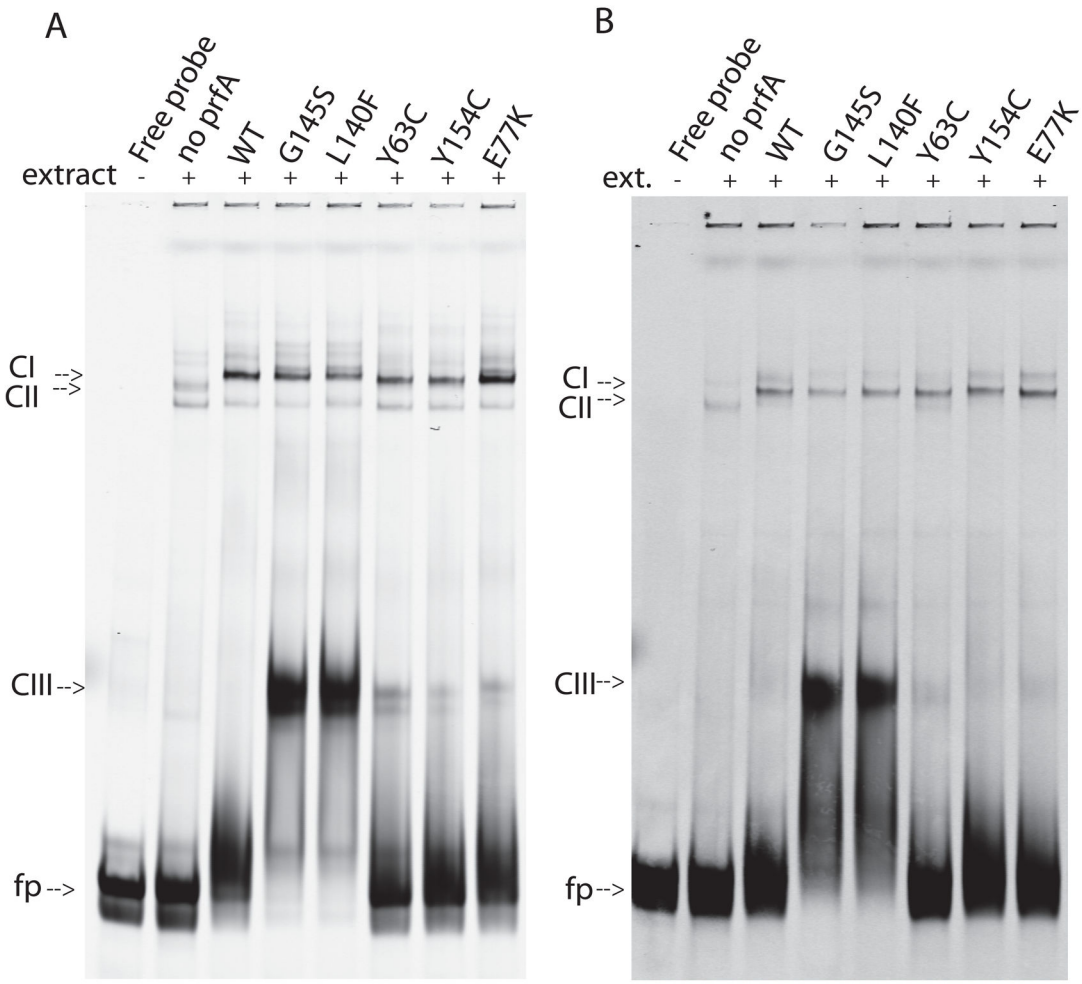

Figure 6. PrfA and PrfA* formation of DNA-RNAP complexes

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 Δ*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 (Bockmann *et al.*, 2000)].

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Table 1

Bacterial strains used in this work

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

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Table 2

Oligonucleotides used in this study

Primer Name	Sequence	Characteristics
5'hly	TCCTATCTTAAAGTGACTTTTATGTT	5' *hly* promoter
3'hly	GCTTCTAAAGATGAAACGCAATATTA	3' *hly* promoter
5'actA	GATGCTTCTAAAAAAGTTGCTGAAGC	5' *actA* promoter
3'actA	TATTCATGAATTATTTTAAGAATATCA	3' *actA* promoter
+374 prfA	GCGCTGCAGGAAACTTGTTTTTGTAGGGTTTGG	3' truncated *prfA* ORF
5' prfA orf	CACCATGAACGCTCAAGCAGAA	5' *prfA* ORF for protein expression
3' prfA orf	TCCTCATTGAGGAATACTGTT	3' *prfA* ORF for protein expression
prfA-trunc- nt3-F[b]	GCGCTGCAGGAACGCTCAAGCAGAAG AATTC	5' truncated *prfA*
prfA-trunc- nt841-R[b]	GCGCTGCAGGGAACAACTATCTGTTGC AGCTC	3' truncated *prfA* ORF
prfA-L140F- QkCh-F[b]	GATTTTTCGATTAACGGGAAGTTTGGC TCTATTTGCGGTCAAC	5' L140F Quikchange
prfA-L140F- QkCh-R[b]	GTTGACCGCAAATAGAGCCAAACTTCC CGTTAATCGAAAAATC	3' L140F Quikchange
prfA-G145S- QkCh-F[b]	GGAAGCTTGGCTCTATTTGCAGTCAAC TTTTAATCCTGACC	5' G145S Quikchange
prfA-G145S- QkCh-R[b]	GGTCAGGATTAAAAGTTGACTGCAAAT AGAGCCAAGCTTCC	3' G145S Quikchange