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A novel C-terminal mutation resulting in constitutive activation of the *Listeria monocytogenes* central virulence regulatory factor PrfA

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The environmental bacterium *Listeria monocytogenes* survives and replicates in a variety of diverse ecological niches that range from the soil to the cytosol of infected mammalian cells. The ability of *L. monocytogenes* to replicate within an infected host requires the expression of a number of secreted bacterial gene products whose expression is regulated by the transcriptional activator PrfA. PrfA becomes activated following bacterial entry into host cells; however, the mechanism by which this activation occurs remains unknown. Here we describe a novel C-terminal mutation that results in the high-level constitutive activation of PrfA and yet, in contrast with other described *prfA** activation mutations, only modestly increases PrfA DNA binding affinity. *L. monocytogenes* strains containing the *prfA* P219S mutation exhibited high levels of PrfA-dependent virulence gene expression, were hyperinvasive in tissue culture models of infection, were fully motile and were hypervirulent in mice. In contrast with PrfA G145S and other mutationally activated PrfA proteins, the PrfA P219S protein readily formed homodimers and did not exhibit a dramatic increase in its DNA-binding affinity for target promoters. Interestingly, the *prfA* P219S mutation is located adjacent to the *prfA* K220 residue that has been previously reported to contribute to PrfA DNA binding activity. *prfA* P219S therefore appears to constitutively activate PrfA via a novel mechanism which minimally affects PrfA DNA binding *in vitro*.

INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterial pathogen that survives in a myriad of diverse environmental conditions that range from the soil and decaying plant matter to the cytosol of mammalian host cells (Czuprynski, 2005; Freitag *et al.*, 2009; Gandhi & Chikindas, 2007; Gray *et al.*, 2006; Lecuit, 2007; Toledo-Arana *et al.*, 2009). This resilient and highly adaptable bacterium can contaminate food sources and as a result has led to thousands of cases of food-borne illness as well as some of the most expensive food recalls in the USA (Centers for Disease Control and Prevention, 2004; Cossart, 2007; Gandhi & Chikindas, 2007; Lynch *et al.*, 2006; Mead *et al.*, 2006; Swaminathan *et al.*, 2006; Swaminathan & Gerner-Smidt, 2007). Once ingested by a susceptible individual, *L. monocytogenes* translocates across the gastrointestinal barrier and disseminates, resulting in severe infections that include meningitis and bacteraemia (Cossart & Toledo-Arana, 2008; Drevets & Bronze, 2008; Ramaswamy *et al.*, 2007; Swaminathan & Gerner-Smidt, 2007). In pregnant women, *L. monocytogenes* can cross the fetoplacental barrier and result in abortion, stillbirth or the premature birth of a severely infected infant (Delgado, 2008; Smith *et al.*, 2009; Smith *et al.*, 2008; Swaminathan & Gerner-Smidt, 2007).

The *L. monocytogenes* transition from life as a saprophyte to life as an intracellular pathogen requires bacterial recognition of its host environment and an associated increase in the expression of a number of genes whose products are required for cell invasion, phagosomal membrane disruption, cytosolic replication and bacterial spread to neighbouring host cells (Cossart & Toledo-Arana, 2008; Dussurget, 2008; Freitag *et al.*, 2009; Gray *et al.*, 2006; Kreft *et al.*, 2002; Scortti *et al.*, 2007; Toledo-Arana *et al.*, 2009; Vázquez-Boland *et al.*, 2001). The majority of *L. monocytogenes* gene products that are known to be required for host infection are regulated by a central virulence regulatory protein known as PrfA (Freitag, 2006; Scortti *et al.*, 2007). PrfA exists in a low-activity form in bacteria growing outside of host cells, but becomes highly activated via an unknown mechanism following host cell entry (Freitag *et al.*, 2009; Shetron-Rama *et al.*, 2002, 2003). Activation of PrfA is required for *L. monocytogenes* pathogenesis, and the transition of PrfA from

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Abbreviations: GUS, β-glucuronidase; CD, circular dichroism; EMS, ethylene methylsulphonate; EMSA, electrophoretic mobility shift assay; LLO, listeriolysin O; XG, 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

a low-activity to a high-activity form is thought to serve as a critical switch enabling *L. monocytogenes* to exploit its intracellular replication niche.

PrfA is a 27 kDa protein which belongs to the cAMP receptor protein (Crp)–Fnr family of transcriptional regulators (Eiting *et al.*, 2005; Lampidis *et al.*, 1994; Vega *et al.*, 1998). Members of this family appear to require the binding of a small molecule cofactor in order to induce an allosteric change that leads to protein activation (Harman, 2001). While the identity of the putative PrfA cofactor remains unknown, a number of mutations have been identified within PrfA that appear to result in protein activation in the absence of cofactor binding (*prfA** mutations) (Miner *et al.*, 2008a, b; Ripio *et al.*, 1997; Shetron-Rama *et al.*, 2003; Vega *et al.*, 2004; Wong & Freitag, 2004). These mutations map to different regions of *prfA*, but have generally clustered either near the putative PrfA cofactor binding site or near residues spanning amino acids 140–155 in the PrfA coding sequence. The most well characterized *prfA** mutation, *prfA* G145S, induces the repositioning of the PrfA helix–turn–helix DNA-binding domain and results in a substantial increase in PrfA DNA-binding affinity (Eiting *et al.*, 2005; Ripio *et al.*, 1997; Vega *et al.*, 1998). Substitutions at L140 also dramatically increase PrfA DNA binding (Miner *et al.*, 2008b; Wong & Freitag, 2004); however, the mechanisms leading to PrfA activation for other mutations (Y63C, E77K and G155S) are less clear (Miner *et al.*, 2008b; Mueller & Freitag, 2005). What has become apparent is that PrfA activation results in a variety of *L. monocytogenes* physiological changes that increase bacterial fitness within an infected host while decreasing fitness in broth culture (Bruno & Freitag, 2010; Wong & Freitag, 2004). Strains containing *prfA** mutations are hyperinvasive for tissue culture cells and hypervirulent in murine models of infection (Bruno & Freitag, 2010; Freitag & Portnoy, 1994; Mueller & Freitag, 2005); however, the mutants are defective in flagella-mediated swimming motility and are more susceptible to both acid and salt stress (Bruno & Freitag, 2010; Port & Freitag, 2007; Shetron-Rama *et al.*, 2003; Wong & Freitag, 2004). Environment-responsive regulation of PrfA activation thus appears essential for maximizing *L. monocytogenes* fitness both inside and outside of host cells.

While PrfA shares extensive structural homology with its well-studied family member *Escherichia coli* Crp, it differs from Crp in having an extended C-terminal domain consisting of three alpha helices (Eiting *et al.*, 2005; Lampidis *et al.*, 1994). This extended C-terminal domain has been postulated to stabilize PrfA DNA binding, and purified PrfA protein does indeed bind DNA with higher affinity than purified Crp lacking its cAMP cofactor (Eiting *et al.*, 2005; Herler *et al.*, 2001; Lampidis *et al.*, 1994; Scortti *et al.*, 2007; Vega *et al.*, 1998). A mutation within the C terminus of PrfA (K220T) has been reported to inactivate PrfA (Roche *et al.*, 2003; Velge *et al.*, 2007), as have deletions at the C terminus (Herler *et al.*, 2001). Here we present the characterization of a novel *prfA* P219S mutation within the extended C-terminal region of PrfA that serves to constitutively activate the protein, thus confirming a role for this region in the regulation of PrfA activity.

METHODS

Bacterial strains, plasmids and growth conditions. *L. monocytogenes* and *E. coli* strains used in this study are listed in Table 1. *E. coli* XL1-Blue (Agilent Technologies), One Shot TOP10 (Invitrogen), NEB $5\alpha F' I^{q}$ (New England Biolabs) and SM10 were used as host strains for maintenance and propagation of recombinant plasmids. *L. monocytogenes* and *E. coli* strains were grown at 37 °C in brain heart infusion (BHI) media (Difco) and Luria broth (LB) (Invitrogen). Bacteria containing the *L. monocytogenes* integration plasmid pPL2 (Lauer *et al.*, 2002) were maintained on media containing chloramphenicol (25 μg ml^{-1} for *E. coli* and 7.5 μg ml^{-1} for *L. monocytogenes*). Bacteria containing the 6 × histidine-tagged vector pQE30 (Qiagen) were maintained in *E. coli* with 100 μg ampicillin ml^{-1}. Streptomycin (200 μg ml^{-1}) was used in selection of *L. monocytogenes* following bacterial conjugation and for isolation of bacteria from infected mice.

Ethylene methylsulphonate (EMS) mutagenesis and *β*-glucuronidase assays. *L. monocytogenes* strain NF-L1124 containing an *actA–gus–neo* reporter fusion was chemically mutagenized using EMS (Sigma-Aldrich) as described previously (Shetron-Rama *et al.*, 2003). Bacteria expressing high levels of *actA* were selected on indicator plates containing 50 μg 5-bromo-4-chloro-3-indolyl *β*-D-glucuronide (XG) ml^{-1} and 10 μg neomycin ml^{-1}. Mutants that formed blue colonies on the XG indicator plates and were neomycin-resistant (in contrast with the wild-type which formed white colonies and were neomycin-sensitive) were selected for further analysis. Quantitative liquid culture enzymic assays for *β*-glucuronidase (GUS) activity were performed to confirm the increase in *actA* promoter expression levels. For GUS assays, overnight cultures of *L. monocytogenes* were diluted 1 : 50 in fresh BHI and grown with shaking at 37 °C. At various time points, the OD_{600} was determined for each culture and 1 ml of each sample was centrifuged to recover bacteria. Bacterial pellets were resuspended in 1 ml ABT buffer [1 M potassium phosphate (pH 7.0), 0.1 M NaCl, 1 % Triton] and GUS activity was measured as described by Youngman (1987) with the substitution of 4-methylumbelliferyl *β*-D-glucuronide (Sigma-Aldrich) in place of 4-methylumbelliferyl *β*-D-galactoside. The *prfA* promoter and coding regions of the strains that showed an increase in *actA–gus–neo* activity over wild-type levels were amplified by PCR and their DNA sequences were obtained.

Plasmid and bacterial mutant construction. The *prfA* P219S mutation was introduced into plasmid pNF1019, a pPL2 site-specific phage integration plasmid containing a wild-type copy of *prfA* with all promoters required for expression (Wong & Freitag, 2004), using the Quick Change site-directed mutagenesis kit (Stratagene) with primer pairs 5′-CTCAAAAGATATGCC**TCT**AAATTAGATGAATGGTTTTATTTAGCATGTCC-3′ and 5′-GGACATGCTAAATAAAACCATTCATCTAATTT**AGA**GGCATATCTTTTGAG-3′. Letters in bold type indicate the mutagenesis of proline (CCT) to serine (TCT) on both forward and reverse strands. The resulting plasmid, pNF1163, was conjugated into strain NF-L1003, which contains an in-frame deletion within *prfA* as well as the *actA–gus* reporter gene fusion, resulting in strain NF-L1452. For generation of purified PrfA proteins, the coding sequence of *prfA* was amplified by PCR from *L. monocytogenes* strains 10403S (wild-type), *prfA* P219S (NF-L1452) and *prfA* G145S (NF-L1226) using primer pairs 5′-AAA*GGTACC***AAC**GCTCAAGCAGAAG-3′ and 5′-GG*CTGCAG*T**TTA**ATTTAATTTTCCCCAAG-3′ and cloned into a pQE30 Expression vector (Qiagen), which contains an

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
NF-L1124	*L. monocytogenes* 10403S *actA–gus–neo–plcB*	Miner *et al.* (2008a)
NF-L1003	*L. monocytogenes* Δ*prfA* in 10403S *actA–gus–plcB*	Wong & Freitag (2004)
NF-L1041	*L. monocytogenes* NF-L1003 with pPL2–*prfA*	Wong & Freitag (2004)
NF-L1226	*L. monocytogenes* NF-L1003 with pPL2–*prfA* G145S	Port & Freitag (2007)
NF-L1452	*L. monocytogenes* NF-L1003 with pPL2–*prfA* P219S	This work
XL1-Blue	*E. coli* cloning strain	Agilent Technologies
NEB 5αF′I^q	*E. coli* protein expression strain	New England Biolabs
Plasmids		
pQE30	N-terminal His-tagged expression vector	Qiagen
pNF1019	pPL2 site-specific integration vector with full-length *prfA* and all promoters	Wong & Freitag (2004)
pNF1223	pNF-1019 *prfA* G145S	Port & Freitag (2007)
pNF1163	pNF-1019 *prfA* P219S	This work
pNF1797	pQE30 *prfA* P219S	This work
pNF2076	pQE30 *prfA* wild-type	This work
pNF3028	pQE30 *prfA* G145S	This work

N-terminal 6 × histidine tag and an IPTG-inducible promoter. Letters in bold type indicate the second codon and stop codon of the *prfA* coding sequence and italicized letters indicate the *Kpn*I and *Pst*I restriction endonuclease sites used for cloning the PCR fragment into pQE30. The resulting construct was initially propagated in *E. coli* TOP10 cells, isolated and then transformed into NEB 5αF′*I*^{q}. An overnight culture containing the expression construct was diluted 1 : 50 in fresh LB broth and the culture was incubated at 37 °C (with shaking) until an OD_{600} of 0.5 was reached. To induce expression of the PrfA protein, 1 mM IPTG (Inalco) was added to the culture and induction was allowed to proceed for 3–4 h. The bacterial cells were recovered by centrifugation followed by sonication with four repeated 10 s bursts and 1 min cooling on ice. The soluble fraction containing the *N*-His-PrfA protein was collected and purified using the His-Pur Purification kit (Thermo Scientific). Protein concentration was determined using a BCA Protein Assay kit (Thermo Scientific).

Assessment of growth in broth culture. Overnight cultures of strains grown in BHI were resuspended in fresh BHI media at a dilution of 1 : 20 and growth at 37 °C with shaking was assessed by measuring the OD_{600} at the indicated time points.

Measurement of haemolytic activity. Stationary-phase bacterial cultures were diluted 1 : 10 into LB medium and grown at 37 °C for 5 h with shaking. OD_{600} was determined, cultures were normalized to OD_{600} 0.5 and 1 ml of each culture was centrifuged at 13 000 ***g*** for 5 min. The supernatant was collected and was assayed for haemolytic activity with sheep erythrocytes washed with PBS (Gibco), as described previously (Camilli *et al.*, 1989). Haemolytic activity was determined as the reciprocal of the supernatant dilution at which 50 % lysis of erythrocytes was observed.

Assessment of phospholipase activity. *plcB*-dependent phospholipase production was assayed on egg yolk agar plates (Alonzo *et al.*, 2009; Mueller & Freitag, 2005). Antibiotic-free chicken egg yolk was added in a 1 : 1 (v/v) ratio to PBS and vortexed to form a suspension. A 5 ml aliquot of egg yolk suspension was added to 100 ml molten LB medium plus 0.2 % activated charcoal (Sigma-Aldrich) and 25 mM glucose-6-phosphate (Sigma-Aldrich) (Bitar *et al.*, 2008); 10 ml of this mixture was poured into Petri dishes. Bacterial strains were gently streaked onto the surface of the plate and incubated at 37 °C for 48 h. Phospholipase activity was visualized as a zone of opacity surrounding bacterial streaks.

Western blot analysis of PrfA protein. PrfA was detected within cytoplasmic fractions isolated from bacterial whole-cell extracts. A 25 ml culture of each *L. monocytogenes* strain was grown to mid-exponential phase in BHI at 37 °C with shaking. Cells were normalized to OD_{600} 0.8 and centrifuged and the bacterial pellets were resuspended in 1 ml PBS. Mutanolysin (100 U; Sigma-Aldrich) was added and the suspension was incubated at 37 °C for 2 h. A 10 μl volume of 100 × Protease Inhibitor Cocktail (Calbiochem) was added to the mutanolysin-treated cells, followed by sonication with three 40 s pulses with 2 min cooling on ice in between each pulse. Cellular debris was centrifuged at 24 000 ***g*** for 20 min at 4 °C and the supernatant containing the cytoplasmic components was collected and stored at −20 °C until further use. For detection of PrfA, 10 μl of the isolated cytoplasmic fraction was mixed with 10 μl 2 × Laemmli sample buffer (Bio-Rad), boiled for 5 min then separated by SDS-PAGE. Protein samples were transferred onto PVDF membranes. PrfA was detected using a 1 : 500 dilution of a monoclonal antibody directed against PrfA in 1 × PBST (PBS plus 0.05 % Tween-20) followed by incubation with a 1 : 2500 dilution of a polyclonal goat–anti-mouse secondary antibody conjugated to alkaline phosphatase (SouthernBiotech). Bands were visualized colorimetrically with the addition of 10 ml BCIP/NBT Plus solution (SouthernBiotech). Densitometry was determined using ImageJ software (http://rsbweb.nih.gov/ij/download.html).

Limited proteolysis. Limited proteolytic digestion of His-purified proteins with subtilisin (Sigma-Aldrich) was done as previously described by Miner *et al.* (2008b) with slight modifications. In brief, 2 μg purified protein was incubated with 500 ng subtilisin for 10 and 30 min at room temperature followed by the addition of 1 mM PMSF to terminate the reaction. Samples were boiled for 5 min in SDS-loading buffer and fragments were separated by SDS electrophoresis on a NuPAGE 4–12 % Bistris gel (Invitrogen). Bands were visualized by staining with Bio-Safe Coomassie G-250 (Bio-Rad).

Circular dichroism (CD). His-purified protein was purified and isolated as described above (Plasmid and bacterial mutant construction). Following purification, eluted protein samples were dialysed in 10 mM NaH_2PO_4 (pH 8) overnight at 4 °C and the concentration of the dialysed protein that remained in solution was determined using a BCA protein assay kit (Thermo Scientific). Samples (400 μl) containing 250 μg purified protein ml^{-1} were then analysed at room temperature on a Jasco J-710 CD spectrometer (UIC Center for

Structural Biology, Chicago, Illinois) in a 2 mm rectangular quartz cuvette. CD spectra parameters were set to read at wavelengths between 180 and 310 nm, continuous scanning mode, 2 nm data pitch, a scanning speed of 100 nm s^{-1}, response of 1 s, band width of 1.0 nm and an accumulation of 3. Data analysis was done using the SpectraManager software program.

Protein chemical cross-linking. Chemical cross-linking was done as previously described by Miner *et al.* (2008b) with minor modifications. In brief, after separation of samples by SDS-PAGE, proteins were transferred to PVDF membranes. Proteins were detected using a 1 : 500 dilution of a monoclonal antibody directed against PrfA in 1 × PBST followed by incubation with a 1 : 2500 dilution of a polyclonal goat–anti-mouse secondary antibody conjugated to alkaline phosphatase (SouthernBiotech). Bands were visualized colorimetrically with the addition of a BCIP/NBT Plus solution (Southern Biotech).

Electrophorectic mobility shift assays (EMSAs). Primer pairs used to amplify the *hly* promoter DNA fragment (~100 bp) were 5′-TCCTATCTTAAAGTGACTTTATGTT-3′ and 5′-GCTTCTAAAGA-TGAAACGCAATATTA-3′. The 3′ end primer was purchased with a biotin label (Sigma-Aldrich). EMSAs were done as described previously (Miner *et al.*, 2008b) with slight modifications. In brief, after DNA-binding reactions and electrophoresis, protein/DNA samples were transferred onto nylon membranes followed by detection using the Pierce chemiluminescent nucleic acid detection module (Thermo Scientific).

Intracellular growth. Bacterial intracellular growth assays in *Potorous tridactylis* kidney epithelial cells (PtK2) were performed as described previously (Marquis *et al.*, 1995; Mueller & Freitag, 2005; Wong & Freitag, 2004; Xayarath *et al.*, 2009). In brief, monolayers of cells were grown on glass coverslips to confluence and infected with bacterial strains with an m.o.i. of 100 : 1. One hour after infection of PtK2 cells, monolayers were washed three times in PBS and 5 μg gentamicin ml^{-1} was added to kill extracellular bacteria. At the indicated time points, coverslips were removed and tissue culture cells were lysed in 5 ml sterile H_2O to release intracellular bacteria for enumeration of intracellular growth, or the coverslips were processed for microscopy.

Plaque assays. Plaque assays were conducted as described previously (Sun *et al.*, 1990). Briefly, murine L2 fibroblasts were grown to confluence in six-well microtitre plates and infected with 20 μl of a normalized 1 : 20 dilution of overnight culture grown at 37 °C in BHI with shaking (m.o.i. 10 : 1). One hour post-infection, L2-infected monolayers were washed and 5 μg gentamicin ml^{-1} was added to kill extracellular bacteria. Three days post-infection, Neutral Red (Sigma-Aldrich) was added and plaques were visualized and measured using a micrometer (Finescale).

Mouse infections. All animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC) and performed in the Biological Resources Laboratory at the University of Illinois at Chicago. Overnight bacterial cultures were diluted 1 : 20 into fresh media and grown to OD_{600} ~0.6. A 1 ml volume of culture (corresponding to 6×10^8 c.f.u. ml^{-1}) was washed, diluted and resuspended in PBS to a final concentration of 1×10^5 c.f.u. ml^{-1}. Female 8–10-week-old ND4 Swiss Webster mice (Harlan Laboratories) received injections of 200 μl PBS containing 2×10^4 c.f.u. *L. monocytogenes* via the tail vein. Mice were killed and livers and spleens were harvested 72 h post-infection. Organs were homogenized with a Tissue Master 125 homogenizer (Omni International) and dilutions were plated onto BHI streptomycin (200 μg ml^{-1}) plates.

Swimming motility assays. Swimming motility was evaluated on semisolid [0.3 % (w/v) agar] BHI medium. The plates were inoculated with 5 μl mid-exponential phase (OD_{600} ~0.6) bacterial cultures grown in BHI at 37 °C. Inoculated plates were then incubated at 37 °C for 48 h. The diameter of the swimming colony in millimetres was measured at various time points, and swimming motility was expressed in millimetres travelled from the edge of the original drop diameter of inoculation. Each strain was assayed in triplicate.

RESULTS

Identification of a novel *prfA** mutation within the extended C-terminal region of PrfA

To identify unique and previously uncharacterized mutations resulting in the constitutive activation of PrfA, a *L. monocytogenes* strain containing an *actA–gus–neo–plcB* transcriptional reporter gene fusion was mutagenized with EMS, a chemical mutagen that most commonly induces G/C to A/T transitions. *L. monocytogenes* mutants exhibiting activation of PrfA activity were selected based on high-level *actA* promoter expression as indicated by increased levels of neomycin resistance and by enhanced blue colony colour on indicator plates containing the GUS substrate XG. Out of approximately 1×10^{10} total bacteria present in six independently EMS-treated pools, 282 mutants were isolated as dark blue neomycin-resistant colonies. The *prfA* promoter and coding regions of 60 of these isolates were sequenced. A total of 42 mutants contained mutations within *prfA*, with the largest group (46 %) containing the previously described *prfA** G145S mutation. Other previously described *prfA** mutations were also identified (*prfA* L140F, G155S and E77K). Only one of the 42 sequenced mutants possessed a novel *prfA* mutation not previously associated with PrfA* activity; this mutant contained a C to T transition resulting in a serine substitution for a proline at position 219 (P219S). This mutant was selected for further analysis.

Based on the PrfA crystal structure (Eiting *et al.*, 2005), the *prfA* P219S mutation is located in αH, one of the three C-terminal alpha helices present in PrfA but absent from Crp (Fig. 1a). These three alpha helices have been proposed to stabilize the C-terminal PrfA DNA-binding domain and to participate in homodimer interactions, such that residues within the three helices of one monomer form hydrogen bonds to *β* loops of the second monomer (Eiting *et al.*, 2005; Herler *et al.*, 2001). The proline residue at position 219 is the first amino acid of the αH helix, contributing to the transition from αG to αH (Fig. 1a).

The *prfA* P219S mutation results in constitutive activation of PrfA

The *prfA* P219S mutation was introduced into a *L. monocytogenes* Δ*prfA actA–gus–plcB* strain as a single copy on plasmid pPL2 to confirm that the mutation was sufficient to confer PrfA activation. In addition to *prfA* P219S, wild-type *prfA* and *prfA* G145S were also introduced into Δ*prfA actA–gus–plcB* strains as single copies using the plasmid integration vector pPL2 which contains all three promoters required for full *prfA* expression and

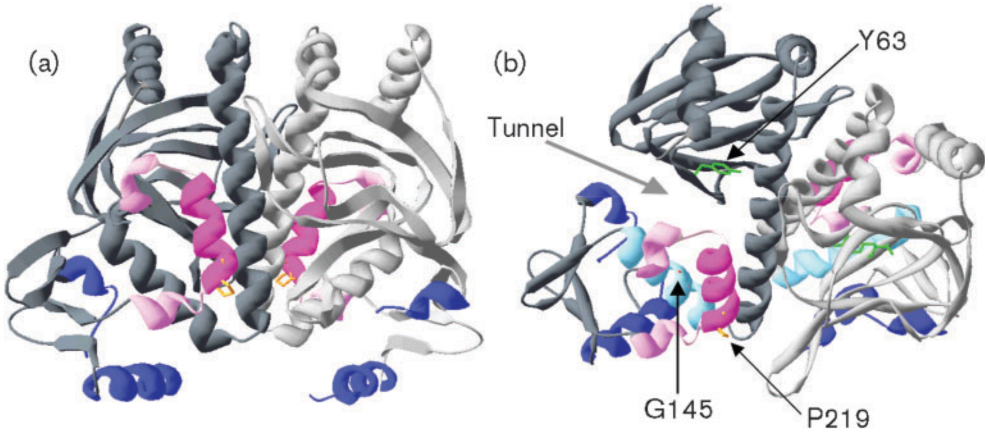

Fig. 1. Location of the PrfA P219S mutation. (a) Ribbon modelling of the PrfA dimer using DeepView-Swiss Pdb viewer v4.0 (http://spdbv.vital-it.ch/). The monomers that make up the dimer are coloured either light or dark grey. The DNA-binding helix–turn–helix helices are shown in blue and the three helices unique to PrfA that are located at the distal end of the C terminus are coloured pink (αG and αI) and magenta (αH). The *prfA* P219S mutation is located in αH and the proline residue at this specific location is shown in orange. (b) Ribbon modelling as done in (a) highlighting the tunnel suggested by Eiting *et al.* (2005) to be the putative ligand-binding pocket as indicated by the grey arrow. Colours for domains and secondary structures are the same as in (a), with the addition of αD shown in light blue. The specific residue Y63 (in β5) is shown in green and G145 (in αD) is in red. The G145 side chain is buried within helix D and is difficult to visualize but has been further highlighted by the black arrow pointing to its location.

complementation (Wong & Freitag, 2004). All comparisons of PrfA activity were thus carried out in isogenic Δ*prfA actA–gus–plcB* strains containing either wild-type or mutant alleles of *prfA*. The introduction of pPL2–*prfA* P219S resulted in a blue colony colour on plates containing the XG indicator for GUS activity, similar to that observed for strains containing pPL2-*prfA* G145S (data not shown). Strains containing the *prfA* P219S allele exhibited no obvious growth defect when grown in broth culture (Fig. 2a), but did display high levels of GUS activity in broth culture, similar to the levels observed for the highly activated *prfA** G145S mutant (Fig. 2b). In addition, overnight cultures of the *prfA* P219S strain grown in BHI at 37 °C without shaking settled to the bottom of culture tubes, exhibiting a phenotype similar to that reported for other high-activity *prfA** mutant cultures (Wong & Freitag, 2004) (data not shown).

The expression of listeriolysin O (LLO) and the broad-range phospholipase PC-PLC is increased following PrfA activation (Alonzo *et al.*, 2009; Portnoy *et al.*, 1988; Shetron-Rama *et al.*, 2003; Wong & Freitag, 2004). Consistent with the increase observed for *actA* promoter activity, the *prfA* P219S mutant exhibited increased LLO and PC-PLC activity with levels that were similar to those observed for the *prfA** G145S mutant (Fig. 2c, d). In addition, Western blot analysis using antibody directed against PrfA indicated that PrfA protein levels were approximately twofold higher in strains containing the *prfA* P219S and *prfA** G145S mutations in comparison with strains containing wild-type *prfA* (Fig. 3a), a result consistent with increased *prfA* expression resulting from PrfA-dependent activation of the upstream *plcA–prfA* promoter (Camilli *et al.*, 1993; Freitag & Portnoy, 1994). These data indicate that the *prfA* P219S mutation results in the constitutive activation of PrfA to a level that resembles that of the highly activated *prfA** G145S mutant strains.

The *prfA* P219S mutation alters PrfA conformation

Previously described *prfA** mutations have been reported to alter PrfA conformation in ways that can be detected by limited proteolytic digestion (Miner *et al.*, 2008b). Limited proteolysis enables a rapid assessment of protein conformational changes that can distinguish between active and inactive forms of Crp (Crp* mutants or Crp with or without cAMP) as well as PrfA and PrfA* mutants (Harman *et al.*, 1986; Miner *et al.*, 2008b; Tan *et al.*, 1991). Polypeptide fragments resulting from limited protease digestion were separated by SDS-PAGE and visualized by Coomassie staining (Fig. 3b). Heat-denatured PrfA samples were also subjected to subtilisin digestion to confirm that all protein samples were equally susceptible to proteolytic cleavage when denatured (Fig. 3b, position of subtilisin indicated by arrow). Limited proteolytic digestion of wild-type PrfA, PrfA P219S and PrfA* G145S proteins indicated that both PrfA G145S and PrfA P219S were more susceptible to proteolytic digestion in comparison to the wild-type protein (Fig. 3b). The PrfA P219S protein was also distinguishable from PrfA G145S, indicating that the P219S mutation resulted in PrfA conformational changes that are distinct from those imposed by G145S.

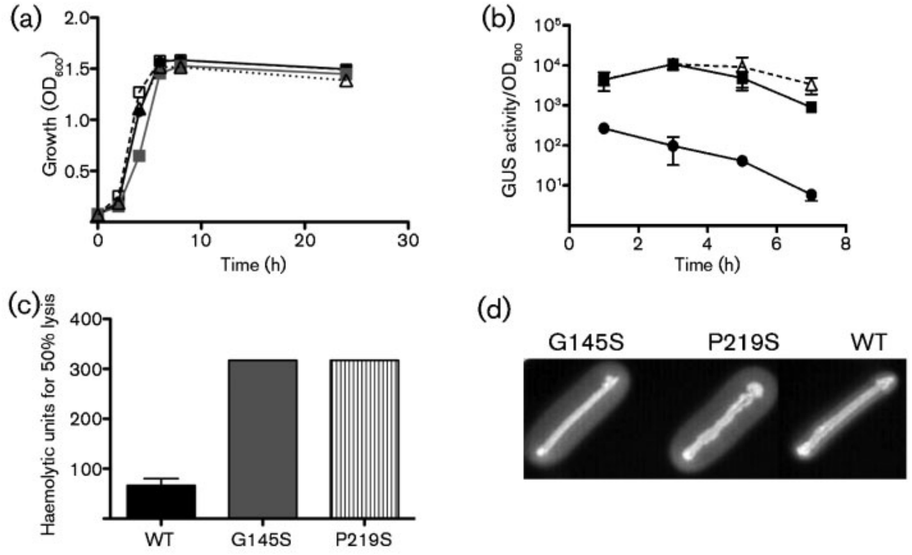

Fig. 2. The *prfA* P219S mutation dramatically increases PrfA-dependent virulence gene expression in broth culture. (a) Growth curve of the *prfA* P219S mutant (△) compared with wild-type (●), *prfA* G145S (■) and a Δ*prfA* strain (▨). Overnight cultures of strains grown in BHI were diluted 1 : 20 in fresh BHI and grown at 37 °C with shaking and the OD_{600} was measured at the indicated time points. The *prfA* P219S mutant grew similarly to all strains grown in rich media. (b) PrfA-dependent activation of *actA* expression as indicated by GUS activity. *L. monocytogenes* strains containing *actA–gus* transcriptional reporter fusions in the presence of *prfA* G145S (■), *prfA* P219S (△) or wild-type *prfA* (●) were grown in BHI at 37 °C with shaking. GUS activity indicative of *actA* expression was assessed at the indicated time points. Each data point is the mean ± 95 % confidence interval GUS activity measured in duplicate and the data shown are representative of at least two independent experiments. (c) Detection of PrfA-dependent LLO-associated haemolytic activity. Secreted LLO activity was assessed by measuring lysis of sheep erythrocytes from serial dilutions of bacterial culture supernatants. Haemolytic activity is represented as the reciprocal of the supernatant dilution at which 50 % lysis of erythrocytes was observed. Data shown represent the mean ± SEM activity measured in triplicate for two independent experiments. (d) Detection of PrfA-dependent PC-PLC activity. PC-PLC-associated phospholipase activity was assessed on egg yolk agar plates containing 0.2 % activated charcoal and 25 mM glucose 6-phosphate following incubation at 37 °C for 24 h. The white precipitate surrounding the bacterial streak is indicative of PC-PLC activity. Data are representative of at least three independent experiments.

To further assess conformational changes conferred by the P219S mutation, CD was used to compare protein secondary structure. Interestingly, both the PrfA P219S and PrfA G145S proteins exhibited similar CD profiles that were distinct from the spectrum observed for wild-type PrfA (Fig. 3c). PrfA P219S and PrfA G145S exhibited a more negative signal in the 208–222 nm range when compared with wild-type PrfA, suggestive of a higher alpha helical content. Thus, while PrfA P219S can be structurally distinguished from both wild-type PrfA and PrfA G145S via limited protease digestion, CD analysis suggests that overall PrfA P219S may adopt a confirmation more closely related to that of the constitutively activated PrfA G145S mutant than that of wild-type PrfA.

The *prfA* P219S mutation modestly reduces PrfA dimer formation

PrfA crystal structure analysis indicates that PrfA forms homodimers, and homodimers have also been observed via chemical cross-linking (Eiting *et al.*, 2005; Velge *et al.*, 2007). Mutationally activated forms of PrfA form homodimers; however, this homodimer formation is reduced in comparison with the wild-type protein (Miner *et al.*, 2008b). To assess the ability of purified PrfA P219S to form homodimers *in vitro*, purified protein was incubated with two distinct chemical cross-linking agents [sulpho-ethylene glycol bis[succinimidylsuccinate] and Bis suberate (BS^3)] and analysed by SDS-PAGE gel electrophoresis. Wild-type PrfA protein was observed to readily form dimers under these conditions, whereas the PrfA* G145S mutant exhibited reduced dimer formation as expected (Fig. 3d, the similar BS^3 data are not shown). Interestingly, despite its resemblance thus far in other assays to highly activated forms of PrfA, the PrfA P219S mutant formed dimers with an efficiency that was somewhat less than that of wild-type PrfA but greater than that of PrfA G145S.

PrfA P219S modestly enhances PrfA DNA-binding affinity

Highly activated PrfA G145S and PrfA L140F mutant proteins bind target DNA with an affinity that is

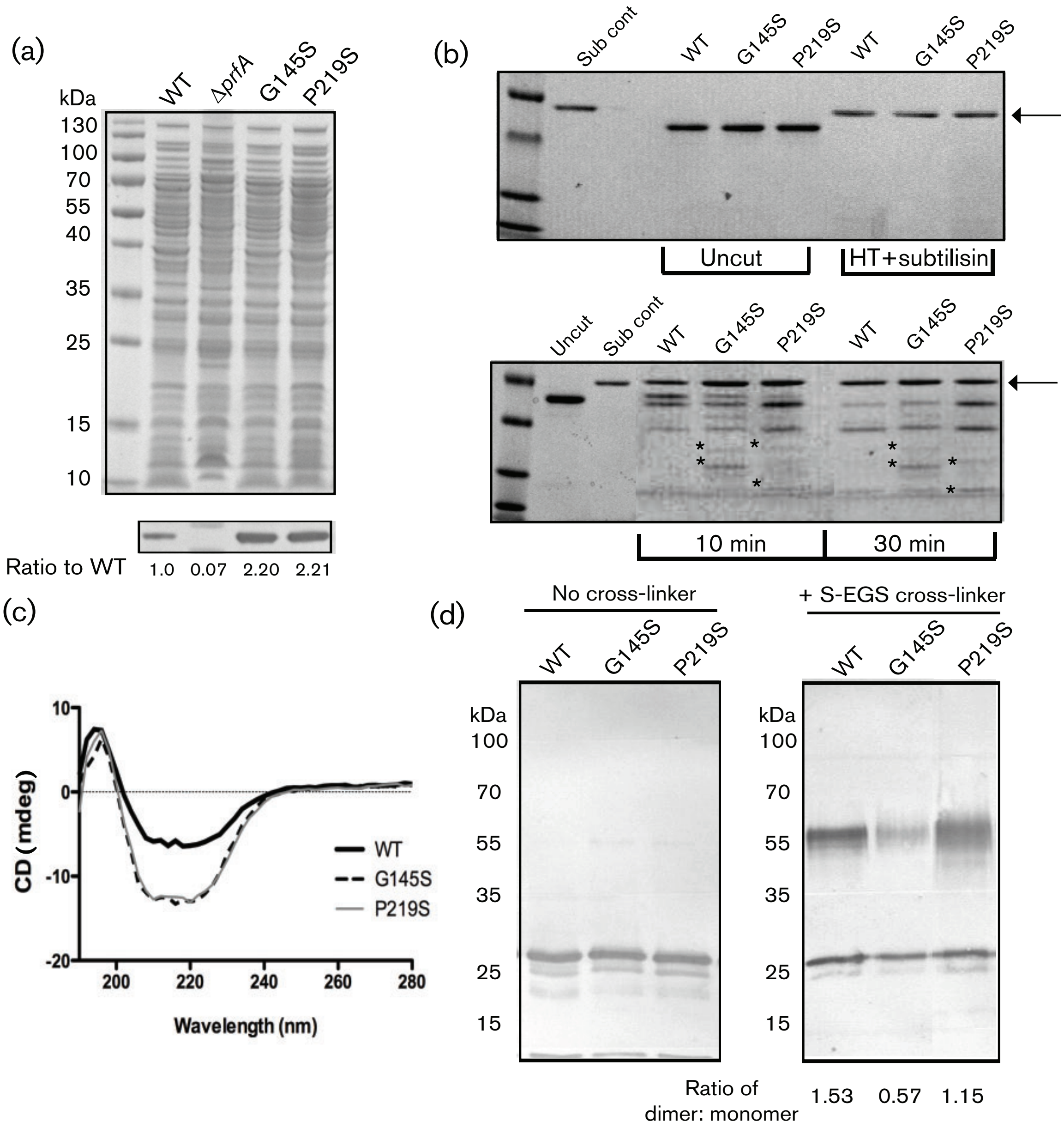

Fig. 3. The *prfA* P219S mutation influences PrfA protein conformation and dimer formation. (a) Western blot analysis of PrfA protein. Top panel: 20 μl isolated bacterial cytoplasmic fractions, normalized based on cell density, were loaded onto a 12 % Bis–Tris polyacrylamide gel and proteins were separated by gel electrophoresis. Polypeptides were visualized by staining with Coomassie blue. Bottom panel: PrfA protein was detected in the cytoplasmic fractions of the various *L. monocytogenes* strains using a monoclonal antibody directed against PrfA. Approximately twofold more PrfA protein was detected in both the *prfA* P219S and G145S mutant strains compared with the wild-type strain. Numbers below the Western panel indicate relative amounts of protein as determined by densitometry using ImageJ software (http://rsbweb.nih.gov/ij/download.html) in comparison to the levels of wild-type PrfA detected. (b) Comparison of PrfA protein conformation by limited proteolytic digestion. Samples (2 μg) of purified PrfA G145S, PrfA P219S or of the wild-type protein were digested with 500 ng subtilisin for 10 and 30 min at room temperature. Protein fragments were separated by SDS-PAGE and visualized by Coomassie staining. Untreated protein samples (uncut) and heat-denatured samples treated with subtilisin (Sub cont) were included as controls to demonstrate that denatured samples were equally susceptible to enzymic digestion. Arrows indicate the position of subtilisin and asterisks denote fragments that were not observed for the wild-type protein. PrfA P219S was more susceptible to proteolytic digestion than the wild-type protein but was also distinguishable from PrfA G145S. Gels are representative of three independent experiments. (c) CD analysis of the purified PrfA proteins. CD far-UV absorption signals between wavelengths of 180 and 310 nm were measured for 250 μg purified protein ml^{-1} in 400 μl 10 mM NaH_2PO_4 at room temperature. The graph is representative of two independent experiments run in triplicate. (d) PrfA homodimer formation as assessed by chemical cross-linking of purified PrfA. Samples (500 ng) of purified PrfA proteins were chemically cross-linked with 10 μM sulpho-ethylene glycol bis[succinimidylsuccinate]

(S-EGS) for 1 h at room temperature followed by SDS-PAGE and Western blotting for detection of PrfA dimers. The PrfA P219S mutant formed homodimers with an efficiency that was less than that observed for wild-type PrfA but greater than that observed for the PrfA* G145S mutant. The ratio of PrfA dimer to monomer formation was assessed by densitometry using ImageJ software (http://rsb.info.nih.gov/ij/download). Data are representative of at least three independent experiments.

significantly greater than that exhibited by the wild-type protein (Eiting *et al.*, 2005; Miner *et al.*, 2008b; Vega *et al.*, 1998). Given that the extended C-terminal region of PrfA has been proposed to enhance the DNA-binding stability of PrfA, we sought to compare DNA binding between the PrfA P219S mutant with wild-type and highly activated PrfA G145S. EMSAs confirmed that purified PrfA G145S bound a DNA fragment containing the *hly* promoter with significantly higher affinity than wild-type protein (Fig. 4). Unexpectedly, although the PrfA P219S mutant stimulated high levels of PrfA-dependent *hly*-encoded LLO activity, the mutant protein displayed only a modest enhancement in DNA-binding activity in comparison with wild-type PrfA (Fig. 4). The PrfA P219S mutation therefore affects PrfA function in a way that is distinct from that of the PrfA G145S mutation.

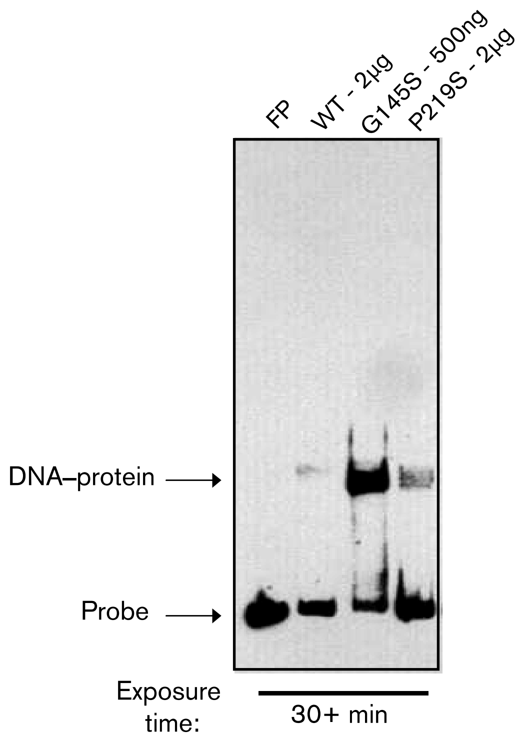

Fig. 4. PrfA P219S does not exhibit high-affinity *in vitro* DNA binding. The binding of purified PrfA wild-type and mutant protein to a biotin-labelled *hly* promoter DNA fragment was assessed by EMSA. FP, Free probe, no protein added. Data are representative of at least three independent experiments.

L. monocytogenes prfA P219S strains are hyperinvasive for tissue culture cells

PrfA activation leads to the increased expression of a number of gene products that contribute to bacterial invasion, including InlA, InlB, ActA and LLO (Freitag *et al.*, 2009; Ireton & Cossart, 1997; Ramaswamy *et al.*, 2007; Scortti *et al.*, 2007; Vázquez-Boland *et al.*, 2001). Consistent with the ability of the *prfA* P219S mutation to induce PrfA activation, *L. monocytogenes prfA* P219S mutants were hyperinvasive for both epithelial and fibroblast cell lines (Fig. 5). Cells infected with the *prfA* P219S mutants had significantly increased numbers of intracellular bacteria (up to 13-fold higher) at multiple time points post-infection of PtK2 epithelial cells, while the rate of intracellular bacterial replication was similar to that observed for wild-type bacteria (approximately 57 min doubling time for *prfA* P219S versus 61 min doubling time for wild-type) (Fig. 5a). Given the similar rate of intracellular bacterial replication, the increased numbers of *prfA* P219S bacteria at 3 h after infection (2 h after gentamicin treatment) are consistent with enhanced bacterial invasion of PtK2 epithelial cells. The *prfA* P219S mutant also appeared hyperinvasive for L2 fibroblast cells as indicated by the increased number of plaques formed by the mutant following the infection of monolayers in comparison with those infected with wild-type *L. monocytogenes* (Fig. 5b). Approximately 10-fold greater numbers of plaques were formed by the *prfA* P219S strain versus wild-type bacteria at a similar m.o.i. (Fig. 5b). The *prfA* P219S mutation therefore serves to enhance bacterial invasion of host cells in tissue culture.

The *prfA* P219S mutant strain is hypervirulent in mice

Highly activated *prfA** mutants have been shown to be hypervirulent in murine models of infection (Bruno & Freitag, 2010; Wong *et al.*, 2004). When mice were infected with 1×10^4 c.f.u. of either wild-type or the *prfA* P219S mutant via tail vein injection, approximately 5- and 10-fold more bacteria were recovered from the livers and spleens, respectively, of mice infected with the mutant strain (Fig. 6). Taken together, these results clearly indicate that the *prfA* P219S mutation results in high-level activation of PrfA as well as increased bacterial virulence.

Unlike *L. monocytogenes prfA* G145S mutants, *prfA* P219S mutants are not defective for swimming motility

Outside of host cells, it has been previously demonstrated that *prfA** mutants exhibit defects in swimming

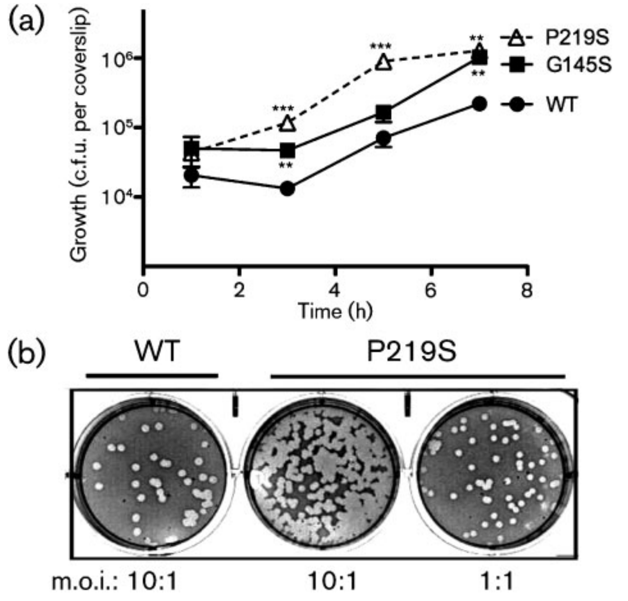

Fig. 5. Strains containing *prfA* P219S are hyperinvasive for tissue culture cells. (a) Bacterial invasion and intracellular growth in monolayers of PtK2 epithelial cells. PtK2 epithelial cells grown on glass coverslips were infected with the indicated strains at an m.o.i. of 100 : 1. Monolayers were washed and gentamicin was added 1 h post-infection (p.i.); coverslips were removed at the indicated time points for lysis of host cells and enumeration of intracellular bacteria. The number of bacteria recovered from the *prfA* P219S mutant was significantly different from the number recovered from the wild-type strain at 3, 5 and 7 h p.i., while the differences between the *prfA* G145S mutant and wild-type were significant at 3 and 7 h p.i. **$P\leqslant 0.005$, ***$P\leqslant 0.0005$ using an unpaired two-tailed Student's *t*-test (GraphPad Prism v.5.0A). In addition, the P219S mutant was significantly different from the G145S mutant at 3 and 5 h p.i. (**$P\leqslant 0.005$). Data shown represent the mean ± SEM of three independent experiments done in triplicate. (b) Bacterial infection of L2 fibroblast cells. The ability of the *prfA* P219S mutant and wild-type *L. monocytogenes* to invade, multiply and spread cell-to-cell within fibroblast tissue culture cell monolayers was determined by assessing plaque formation following infection with an m.o.i. of 10 : 1 or 1 : 1. At 1 h p.i. the cells were washed and gentamicin was added. Plaques were visualized 3 days p.i. Data shown are representative of three independent experiments done in duplicate.

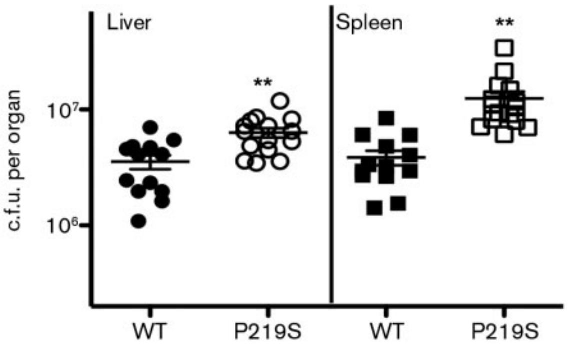

Fig. 6. The *L. monocytogenes prfA* P219S mutant is hypervirulent *in vivo*. Swiss Webster mice were intravenously infected with 2×10^4 c.f.u. through the tail vein. The livers and spleens were harvested 48 h post-infection, homogenized and enumerated for bacterial burdens. Each data point represents one mouse and the solid lines denote the median for each data group. Data were obtained from two independent experiments. Asterisks indicate statistical significance; **$P\leqslant 0.005$ using an unpaired two-tailed Student's *t*-test (GraphPad Prism v.5.0A).

motility that can be readily detected in soft agar media (Shetron-Rama *et al.*, 2003; Wong & Freitag, 2004). Swimming motility defects would be anticipated to compromise bacterial fitness in environments outside of host cells, as well as potentially reducing bacterial invasion of the intestinal epithelium (O'Neil & Marquis, 2006). It has been reported that gene products that contribute to flagella biosynthesis and chemotaxis, such as FlaA and MotA, are downregulated following PrfA activation, suggesting an inverse relationship between motility and *prfA* activity (Port & Freitag, 2007; Toledo-Arana *et al.*, 2009). To examine the motility of *prfA* P219S strains, *L. monocytogenes* swimming motility was assessed on semisolid BHI media at 37 °C over the course of 2 days. Defects in the initiation of bacterial swimming motility were readily detectable for *prfA* G145S but not for *prfA* P219S or wild-type strains during the first 24 h of growth (Fig. 7a). After approximately 24 h, *prfA* G145S strains exhibited rates of swimming motility that were similar to those observed for *prfA* P219S, wild-type and Δ*prfA* strains (Fig. 7b). These results indicate that PrfA activation does not prohibit the assembly of a functional flagellum nor interfere with bacterial chemotaxis. Interestingly, for strains containing some *prfA** mutations (such as G145S), PrfA activation delays the initiation of swimming motility, whereas other *prfA** strains (P219S) show no indication of motility defects.

DISCUSSION

PrfA activation appears to represent a critical switch that enables *L. monocytogenes* to transition from an environmental bacterium to a pathogen that can access and exploit the mammalian cytosol as a bacterial replication niche (Freitag *et al.*, 2009; Toledo-Arana *et al.*, 2009). While the signal that triggers PrfA activation remains unknown, the isolation of *prfA** mutant strains has provided insight into the effects of PrfA activation on bacterial fitness and physiology (Bruno & Freitag, 2010; Mueller & Freitag, 2005; Shetron-Rama *et al.*, 2003; Wong & Freitag, 2004). Phenotypic characterization of *prfA** mutants has established the critical importance of environmental regulation of PrfA to optimize bacterial survival in distinct habitats both outside and inside mammalian cells. Here we describe the isolation of a novel *prfA** mutation located within the distal C-terminal region of PrfA. This region has not been previously associated with PrfA activation and it is absent from the

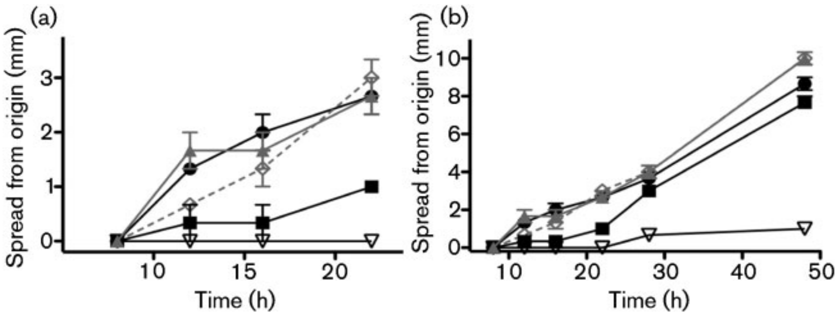

Fig. 7. *prfA* G145S but not P219S strains are delayed for initiation of swimming motility. Measurement of bacterial spread in soft agar. The diameter of bacterial spread (in mm) from the original inoculation site was measured at various time points up to 48 h for plates incubated at 37 °C. A Δ*flaA* mutant, which is defective for motility, was used as a negative control. The *prfA* G145S mutant strain is slow to initiate swimming motility, but then moves at a rate that is similar to that of wild-type, *prfA* P219S and *prfA* deletion strains. ●, wild-type; ▲, P219S; ◇, Δ*prfA*; ■, G145S; ▽, Δ*flaA*.

structurally related family member Crp (Eiting *et al.*, 2005; Lampidis *et al.*, 1994). While loss-of-function mutations have previously been associated with the PrfA C terminus (Herler *et al.*, 2001; Roche *et al.*, 2003; Velge *et al.*, 2007), our results are the first, to our knowledge, to show that mutations within this region are capable of conferring high-level constitutive activation to this key virulence regulator.

The *prfA* P219S mutation is located within the αH helix at the C-terminal end of PrfA. Based on the PrfA crystal structure, the three C-terminal alpha helices (αG, αH and αI) form an extended region that has been postulated to contribute to stabilization of PrfA DNA binding through both intra- and intermolecular homodimer interactions (Eiting *et al.*, 2005; Herler *et al.*, 2001). Within an individual PrfA monomer, hydrogen bonds are formed between helices αH and αI with helices αC and αD; PrfA mutants lacking the last 17 C-terminal residues (αH and αI) lose all ability to bind DNA (Eiting *et al.*, 2005; Herler *et al.*, 2001). In addition, the intermolecular contacts made between the helices of αG, αH and αI of one PrfA monomer and β6 and β7 in the N terminus of the second monomer reportedly stabilize the monomer–monomer interface (Eiting *et al.*, 2005). Residues participating in these contacts include K220 and Q223 in αH and T82 in β6–β7. The K220 residue has previously been implicated as playing an important role in PrfA activity in that a K220T mutant exhibited reduced PrfA DNA binding without affecting dimer formation (Roche *et al.*, 2003; Velge *et al.*, 2007). It was thus somewhat surprising that mutants containing the adjacent P219S substitution exhibited only modest alterations in both PrfA DNA binding and PrfA dimer formation, but nevertheless exhibited high level activation of PrfA and a corresponding dramatic increase in PrfA-dependent gene expression.

How does the *prfA* P219S substitution induce PrfA activation? In the absence of structural data for this mutant protein, the precise mechanism remains unclear. PrfA activation in the absence of a significant increase in PrfA *in vitro* DNA-binding affinity has been reported for one additional *prfA** mutant, *prfA* Y63C (Miner *et al.*, 2008b). The *prfA* Y63C mutation is located within a structural pocket that has been suggested to serve as the PrfA cofactor-binding site (Fig. 1b) and thus has been speculated to influence cofactor binding (Miner *et al.*, 2008b). The *prfA* P219S mutation is not located near the putative cofactor-binding region and is not therefore anticipated to enhance or influence cofactor binding unless it does so via a distal conformational effect. Such a distal effect is possible given that the P219 residue is located at the end of PrfA αH, near the transition of helix G to helix H. αH is in close proximity to αD, which contains G145 (Fig. 1b), for which the substitution of serine results in a repositioning of the PrfA helix–turn–helix DNA-binding domain and an increase in PrfA DNA-binding affinity (Eiting *et al.*, 2005). While it is possible that the P219S substitution alters the conformation of αH so as to mimic an activated state, the change imposed must be distinct from that conferred by G145S as there is no evident increase in PrfA P219S DNA-binding affinity (Fig. 4). Alternatively, it has been postulated that a component of the phosphoenol pyruvate phosphotransferase system may bind and sequester PrfA, thereby preventing PrfA binding at target promoters (Joseph & Goebel, 2007; Marr *et al.*, 2006; Mertins *et al.*, 2007). If this is the case, it is possible that the *prfA* P219S mutation disrupts this inhibitory interaction and prevents sequestration of PrfA, leaving it free to bind and activate its target promoters. Although the precise *in vivo* mechanism underlying activation of PrfA remains speculative, the isolation and characterization of *prfA** mutant proteins has made it readily apparent that there are multiple types of PrfA activation via amino acid substitution.

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