A novel growth based selection strategy identifies new constitutively active variants of the major virulence regulator PrfA in *Listeria monocytogenes*.
Listeria monocytogenes is a Gram-positive pathogen able to cause severe human infections. Its major virulence regulator is the transcriptional activator PrfA, a member of the Crp/Fnr family of transcriptional regulators. To establish a successful L. monocytogenes infection, the PrfA protein needs to be in an active conformation, either by binding the cognate inducer glutathione (GSH) or by possessing amino acid substitutions rendering the protein constitutively active (PrfA*). By a yet unknown mechanism, PTS-sugars repress the activity of PrfA. We therefore undertook a transposon-based approach to identify the mechanism by which PTS-sugars repress PrfA activity. For this, we screened a transposon mutant bank to identify clones able to grow in presence of glucose-6-phosphate as a sole carbon source. Surprisingly, most of the isolated transposon mutants also carried amino acid substitutions in PrfA. In transposon-free strains, the PrfA amino-acid substitution mutants displayed growth, virulence factor expression, infectivity and DNA binding, agreeing with previously identified PrfA* mutants. Hence, the initial growth phenotype observed in the isolated clone was due to the amino acid substitution in PrfA and unrelated to the loci inactivated by the transposon mutant. Finally, we provide structural evidence for the existence of an intermediate-activated PrfA state, which gives new insights into PrfA protein activation.

KEYWORDS Listeria monocytogenes, PrfA, PrfA*, Crystal structure, LLO, ActA
The Gram-positive bacterium *Listeria monocytogenes* is a human pathogen mainly affecting elderly, immunocompromised people and pregnant women. It can lead to meningoencephalitis, septicaemia and abortion. The major virulence regulator in *L. monocytogenes* is the PrfA protein, a transcriptional activator. Using a growth-based selection strategy, we identified mutations in the PrfA protein leading to constitutively active virulence factor expression. We provide structural evidence for the existence of an intermediate-activated PrfA state, which gives new insights into PrfA protein activation.

*INTRODUCTION*

*Listeria monocytogenes* is a Gram-positive bacterium that naturally resides in the soil. Occasionally, *L. monocytogenes* can become a human pathogen upon ingestion. The elderly, immunocompromised people, and pregnant women are at risk since the bacterium can cause meningoencephalitis, septicaemia and abortion (1-3). To cause an infection in the human host, *L. monocytogenes* concert the expression and action of an arsenal of virulence and host factors. Invasion of different cell types requires the expression of various surface proteins such as internalins (InlA and InlB) and actin assembly-inducing protein (ActA) (4). Once inside the cell, the bacterium is trapped in membrane-bound vacuoles that are lysed upon expression and secretion of bacterial proteins such as listeriolysin O (LLO) and two phospholipases (PlcA and PlcB) (1). After escape from the vacuole, the bacteria enter the cytosol where they start expressing the hexose-phosphate transporter Hpt. This enables the bacteria to make use of the sugar sources available inside the mammalian cell, thereby allowing bacterial replication (5). Inside the cytosol, the bacterium also starts expressing the
surface protein ActA, which allows them to move through the cell and into adjacent ones, using host cell actin polymerization for motility (6-8).

The major regulator of virulence factors in *L. monocytogenes* is the transcription activator PrfA, a member of the Crp/Fnr family of regulators. Outside the host, the expression of PrfA-regulated genes is low, but upon entering a host, PrfA becomes activated and turns on the expression of PrfA-regulated virulence genes. For activation, PrfA requires binding of the cofactor glutathione (9, 10). Glutathione binding stabilizes the DNA-binding helix-turn-helix (HTH) motif in a conformation compatible with DNA-binding, thereby allowing expression of PrfA-regulated virulence factors (10). The expression of virulence factors is tightly regulated to prevent their expression when they are not needed. How the bacterium controls this virulence factor expression is still not fully understood. It is known that PrfA-regulated gene products are repressed when the bacterium is grown in broth containing phosphoenolpyruvate phosphotransferase system (PTS) sugars such as cellobiose and glucose (reviewed in (1, 11-13)). However, when grown in LB media supplemented with non-PTS sugars carrying a phosphate group (i.e. sugar-phosphates such as glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), mannose-6-phosphate (M-6-P) and fructose-6-phosphate (F-6-P)), there is no repression of virulence gene expression (5, 14). Since sugar-phosphates, unlike glucose and cellobiose, are taken up by the Hpt transporter and not by the PTS, it has been suggested that an active PTS represses PrfA activity, although the mechanism remains unclear. This would repress PrfA activity when the bacterium lives in the soil, where its primary sugar sources are PTS sugars (5). In contrast, PrfA becomes active once inside the mammalian host where the sugar sources are available in the form of sugar-phosphate.
It has previously been shown that sugar-phosphate utilization is strictly dependent on PrfA activity; a *L. monocytogenes* strain carrying a glycine to serine substitution at position 145 (PrfA$_{G145S}$) renders the protein constitutively active. In contrast to a bacterial strain carrying wild-type PrfA (PrfA$_{WT}$), this PrfA$_{G145S}$ mutant is able to metabolize G-1-P (5, 14). Several other amino acid substitutions resulting in active PrfA proteins (called PrfA*) have also been identified (reviewed by (15)). These mutant strains are all characterized by elevated PrfA-dependent gene expression under non-virulence conditions, such as growth in the presence of PTS sugars. Different PrfA* mutants are able to activate virulence gene expression to varying extents.

Previous data show that *L. monocytogenes* is unable to grow in chemically defined media (DM) with sugar-phosphate as the sole carbon source (16). We found that *L. monocytogenes* PrfA* mutants can grow in DM supplemented with G-6-P and that this growth phenotype is strictly dependent on high expression of Hpt. Since growth of *L. monocytogenes* in G-6-P requires an active version of PrfA, we screened a transposon mutant library with the aim of identifying genes involved in sugar-mediated repression of PrfA activity. Surprisingly, for most of the isolated mutants, the ability of them to grow in the G-6-P media were due amino acid substitutions in PrfA, rendering them PrfA*. We identified three previously unidentified PrfA* variants of different classes that we characterized on the basis of their virulence factor expression, infectivity, and DNA-binding. Structure analyses show that the newly isolated PrfA* protein dimers fold into intermediate structures, i.e. without a collapsed central structure, with one HTH motif in an unstructured inactive form, and one HTH motif in an active folded form. We refer to these structures as intermediate-active forms of PrfA. Combined with previous work (17), there are now
structural evidence that PrfA can exist in at least three forms of activation: inactive, intermediate-active, and fully active.

RESULTS

Growth of *Listeria monocytogenes* in defined media supplemented with G-6-P requires a constitutively active PrfA protein. Growth of *L. monocytogenes* in the presence of sugar-phosphate requires the hexose-phosphate transporter Hpt (5). Hpt is expressed once the bacterium enters the host cytosol, where Hpt expression and sugar-phosphate uptake requires functional PrfA (5). In line with these findings, we tested if a constitutively active form of PrfA, PrfA*, could grow in defined media (DM) with sugar-phosphate as the sole carbon source. To test this hypothesis and evaluate it as a selection strategy for identifying genes involved in PTS-sugar mediated repression of PrfA activity, four strains were plated on DM supplemented with G-6-P. The strains tested were: a wildtype strain (WT); a strain lacking Hpt (Δhpt); a PrfA* strain (prfAG145S); and the same PrfA* strain lacking Hpt (prfAG145S, Δhpt) (Fig. 1A). The PrfAG145S mutant strain grew on the DM/G-6-P medium, in contrast to the WT and the Δhpt mutant strains. The growth of the PrfAG145S mutant was clearly dependent on Hpt, as there was no growth of the PrfAG145S Δhpt mutant (Fig. 1A).

Next, we tested the fosfomycin sensitivity of the WT; the prfAG145S, and the prfAG145S, Δhpt strain, as fosfomycin sensitivity correlates directly with levels of Hpt expression (18). As expected, the PrfAG145S mutant strain showed increased fosfomycin sensitivity as compared to the WT strain (Fig. 1B). Furthermore, the increased fosfomycin sensitivity was completely abolished in the PrfAG145S Δhpt strain. Since PrfA positively controls *hpt* expression (5, 19),
we examined hpt expression in the WT and the two PrfA_{G145S} mutant strains. All strains were grown in nutrient-rich brain heart infusion (BHI) broth prior to RNA-isolation and northern blot analysis (Fig. 1C). In agreement with the hypothesis that hpt expression requires active PrfA, only the PrfA_{G145S} mutant strain could express hpt. Taken together, these results suggest that growth of L. monocytogenes on G-6-P as a sole carbon source could be used as a selection strategy to better understand mechanisms of sugar-mediated repression of PrfA activity.

**Isolation of constitutively active PrfA while screening a transposon mutant library.** To identify genes involved in sugar-mediated repression of PrfA activity, we tested a previously generated Himar1-mariner transposon (Tn) mutant library for growth in DM medium supplemented with G-6-P as the sole carbon source (20). If the Tn mutants grew with G-6-P as the sole carbon source, we hypothesized that there were four possible explanations: i) the mutant strain had to carry an activated PrfA due to a Tn insertion in a gene encoding a protein involved in PrfA inhibition; ii) the Tn was inserted in a gene that acts as a repressor of Hpt expression in a PrfA-independent manner; iii) the mutant strain carried a transposon-independent mutation in the prfA gene that makes the PrfA protein constitutively active; or iv) the mutant strain carried a transposon-independent mutation in the hpt loci, making Hpt constitutively expressed.

The screen was conducted as described in Fig. S1 and materials and methods. Briefly, a library of individual transposon mutants (n=13,344) were inoculated in BHI overnight, before re-inoculation in DM supplemented with 0.2% (w/v) G-6-P, and tested for growth over several days. Of the 13,344 transposon mutants screened, 19 grew after three days in DM supplemented with G-6-P (corresponding to 0.14% of the tested colonies). As we were
interested in identifying mutants with an increased PrfA activity, the 19 mutants were plated on blood agar plates for haemolytic activity as a read-out for PrfA-activity. 12 of the 19 mutants had higher haemolytic activity than the WT control and were selected for further studies.

Before further characterization of transposon insertions, we sequenced the prfA gene of the 12 isolated mutants. We wanted to examine if any of them had acquired a transposon-independent mutation in the prfA gene rendering them constitutively active (PrfA*). Surprisingly, we found that most isolated transposon mutants (10 out of 12) also carried a point mutation in the prfA gene, leading to amino acid substitutions in the protein. The two remaining mutants did not have base-substitutions in the prfA gene and are currently undergoing further analysis in our laboratory. The prfA gene modifications included three previously characterized amino acid substitutions (1, 21, 22): four of the ten mutants carried the Gly to Ser substitution at residue 145 (PrfA_{G145S}), one carried the Gly to Cys substitution at residue 145 (PrfA_{G145C}), and two carried the Leu to Phe substitution at residue 140 (PrfA_{L140F}). In addition, we found three new PrfA mutants in the remaining three strains: Leu to His substitution at residue 140 (PrfA_{L140H}), Ala to Gly substitution at residue 218 (PrfA_{A218G}), and Ala to Val substitution at residue 94 (PrfA_{A94V}). Previously, Ala to Thr substitution at residue 94 (PrfA_{A94T}) has been shown to give rise to a PrfA* phenotype although this substitution has not been extensively characterized (22).

**Analysis of the identified PrfA mutants.** Our results indicated that the phenotypes of the mutants (growth in DM supplemented with G-6-P, and haemolytic activity) were due to mutations in the prfA gene. However, we needed to rule out the possibility that the transposons or other secondary site mutations were affecting the PrfA activity. L.
monocytogenes strains carrying the prfA mutations were therefore constructed in the integrative pPL2 plasmid in a genetic background devoid of transposons (23). To avoid variations in PrfA protein levels, the prfA\textsubscript{wt} together with the prfA mutants, were introduced in the pPL2 plasmid carrying the prfA\textsubscript{P1} and prfA\textsubscript{P2} promoters. At the same time, the PrfA-controlled plcA promoter was omitted to avoid unwanted indirect effects of the positive PrfA feedback loop. The WT and the mutant versions of prfA were introduced as a single copy into the strain EGDe (WT) containing an in-frame deletion of the prfA gene (ΔprfA). None of the PrfA mutant strains showed any growth defect at 37°C when grown in BHI, in agreement with previous observations (Fig. S2) (22). The mutant strains were further tested for growth in DM supplemented with G-6-P (Fig. 2A). All the previously identified PrfA* mutants, \textit{i.e.} PrfA\textsubscript{G145S}, PrfA\textsubscript{G145C} and PrfA\textsubscript{L140F} could grow in this medium with G-6-P, as could the PrfA mutants PrfA\textsubscript{L140H} and PrfA\textsubscript{A218G}, whereas the strain carrying the PrfA\textsubscript{A94V} mutation was unable to grow. As growth in G-6-P is completely dependent on Hpt expression, we tested the fosfomycin sensitivity of the mutants. All the mutants exhibited significantly increased fosfomycin sensitivity compared to the PrfA\textsubscript{WT}, with the A94V mutant showing the least sensitivity (Fig. 2B). We next tested hpt expression using northern blot. The results were consistent with the fosfomycin sensitivity test—all the prfA mutant strains showed significantly increased hpt expression compared to the wildtype strain, with the A94V substitution having a very minute increase in hpt expression (Fig. 2C). Based on these data, we hypothesize that the original A94V transposon mutant carried a secondary site mutation, allowing it to grow in medium having only G-6-P as the sole carbon source. In view of this, for this study we decided not to pursue this A94V strain further.

**Characterization of the PrfA* phenotype.** To further characterize the isolated prfA base-substitution mutants, we examined virulence factor expression using Western blots. ActA

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and LLO protein levels were upregulated in all the prfA mutant strains compared to wildtype levels (Fig. 3A and Fig. S3). The PrfA\textsubscript{G145S}, PrfA\textsubscript{G145C} and PrfA\textsubscript{L140F} mutants showed the highest expression levels, followed by PrfA\textsubscript{A218G} and PrfA\textsubscript{L140H}. Importantly, no significant differences in the amount of PrfA was observed among the strains. This indicates that the effect on the virulence gene expression was due to increased PrfA activity and not to increased PrfA expression (24). Based on the growth in DM supplemented with G-6-P, hpt expression, fosfomycin resistance, and virulence factor expression, all the PrfA mutants exhibited PrfA\textsuperscript{*} phenotypes, albeit at a varying degree.

\textit{L. monocytogenes} grown in BHI medium supplemented either with charcoal or with the non-polar adsorbent Amberlite XAD4 have increased virulence gene expression, presumably due to the removal (by the charcoal/Amberlite) of a hitherto unidentified inhibitory substance released by \textit{L. monocytogenes} during growth (24). Amberlite XAD4 is a polymeric adsorbent known to be especially effective against low-molecular-weight hydrophobic compounds (24). When grown in BHI supplemented with Amberlite XAD4 (BHIA), the wildtype and the PrfA\textsubscript{L140F} mutant strain showed a significantly increased expression of ActA compared to bacteria grown in only the BHI (Fig. 3B, Fig. S4). Also, the PrfA\textsubscript{L140H} and the PrfA\textsubscript{A218G} show an induced ActA expression despite not being statistically significant. Together, our data suggest that PrfA\textsubscript{WT}, PrfA\textsubscript{L140F} PrfA\textsubscript{L140H} and PrfA\textsubscript{A218G} but not PrfA\textsubscript{G145C} can be further activated.

\textbf{Intracellular phenotype of the PrfA\textsuperscript{*} mutants.} The phenotype of the identified mutants was investigated in more details. We started by investigating the intracellular growth of the mutants in the colon epithelial cell line Caco-2 (Fig. 4A). At 2 h post-infection, all tested mutants displayed a greater number of intracellular bacteria than the wildtype strain. However, the intracellular growth rates of the mutant strains were not markedly increased
compared to the wildtype strain, suggesting that PrfA\textsubscript{WT} is fully activated 2 h post-infection.

During infection, an important feature of \textit{L. monocytogenes} pathogenesis is its capacity to spread from cell to cell. This can be monitored by plaque formation in monolayers of tissue culture cells, which correlates well with the virulence seen in a mouse model (25). As a complementary strategy to further examine the ability of our strains to infect cells, we employed a modified plaque assay, which qualitatively assesses the ability of bacteria to adhere/invade and/or spread from cell to cell. We counted the number of plaques formed and correlated that with the wild-type (Fig. 4B). The mutants all showed an increased ability to form plaques in a TC7 cell line compared to the wildtype. In summary, the different PrfA* variants gave rise to diverse levels of PrfA-activity, with PrfA\textsubscript{G145S} being almost fully activated and the other mutants showing a lower activity.

**Differential DNA-binding capacity among the PrfA* proteins.** Previous studies with electrophoresis mobility shift assay or surface plasmon resonance (SPR) demonstrate that the PrfA\textsubscript{G145S} protein has a higher binding affinity than the PrfA\textsubscript{WT} protein for the \textit{hly} and \textit{actA} DNA promoters (17). Here we show that all the PrfA* proteins identified in our screen had higher binding affinities to \textit{hly}, \textit{actA} and \textit{hpt} promoter sequences than the PrfA\textsubscript{WT} protein (Table 1). The previously identified PrfA\textsubscript{G145S} protein showed the lowest equilibrium-binding constant, i.e., strongest binding of all the mutants, followed by the PrfA\textsubscript{A218G}, PrfA\textsubscript{L140H} and PrfA\textsubscript{L140F} proteins, respectively.

**Structural organisation of PrfA* homodimers.** To gain a deeper understanding of the PrfA* mutant proteins, the crystal structures of purified PrfA\textsubscript{L140H}, PrfA\textsubscript{L140F}, and PrfA\textsubscript{A218G} were determined as individual proteins and in complex with the PrfA \textit{hly} promoter DNA (Table S1). These structures were compared to the known structures of PrfA\textsubscript{WT} (PDB codes 2BEO (17) and 5F1R (19)), PrfA\textsubscript{G145S} (PDB code 2BGC (17)), the glutathione-activated PrfA
PrfA<sub>WT</sub> is a homodimer in which each monomer consists of an N-terminal domain (residues 1-108) and a C-terminal DNA-binding domain (residues 138-237), linked by a long α-helix (αC, residues 109-137) (17). Both the N- and C-terminal domains constitute an α/β-fold. Hydrophobic interactions between symmetry-related αC helices and loops β6-β7 stabilize the dimer interface. Two α-helices in the C-terminal domain, αE (residues 170-178) and αF (residues 183-197), constitute the two helices of the typical HTH-motif present in many prokaryotic transcription factors. In PrfA<sub>WT</sub>, parts of the first helix and the connecting turn of the HTH-motif were not defined by electron density, probably due to high flexibility (17)(Fig. 5A). A comparison of the structure of the constitutively active mutant PrfA<sub>G145S</sub> with that of PrfA<sub>WT</sub> revealed the first details of the structural differences between the inactive and active forms of PrfA (17). These changes have also been verified in the activated PrfA<sub>WT</sub>-GSH complex structure (PDB code 5LRR (10)). In activated PrfA, the HTH motif is folded; however, activation also leads to a more collapsed structure (17).

We expected that the crystal structures of PrfA<sub>L140H</sub>, PrfA<sub>L140F</sub>, and PrfA<sub>A218G</sub> would be similar to the PrfA<sub>G145S</sub> structure, since our in vivo characterization classified them as PrfA* mutations. However, rather their structures showed that each of the PrfA* variants had only one folded HTH motif, with the remaining structure residing in the PrfA<sub>WT</sub> conformation (Fig. 5B). Hence, the new mutant structures display features characteristic of both the inactive and active forms of PrfA. Noteworthy, we did not manage to get crystals of the PrfA<sub>WT</sub> under these crystallization conditions, however, crystals of PrfA<sub>A94V</sub>, which showed an almost PrfA<sub>WT</sub> activity levels (Fig. 2), were obtained. The PrfA<sub>A94V</sub> protein also had one folded HTH and one unfolded (WT) motif identical to the PrfA* mutants (Fig. 5B). Thus, the new
structures of PrfA* variants and the wild type-like PrfA_{A94V} protein, presented here, represent an intermediate-activated form of the protein. The most striking structural difference between the inactive, intermediate-active, and fully active structures is the position of their recognition helices. When superimposed on monomer B, the recognition helix in monomer A is shifted with up to 6 Å (Fig. 5C). However, when PrfA is bound to promoter DNA, both monomers are almost identical with each other in all of our PrfA mutants (Fig. 5D).

**DISCUSSION**

The rationale for this work was to further understand the mechanism by which PTS sugars repress PrfA activity (Ref 1, 11-13). We therefore undertook a transposon-based strategy to isolate mutants with a PrfA-activated phenotype. To do this, we selected for growth of *L. monocytogenes* in medium containing glucose-6-phosphate as the only carbon source. Most transposon mutants that were able to grow under these conditions also carried base-substitution mutations in the *prfA* gene, resulting in amino-acid substitutions and constitutively activated forms of PrfA (PrfA*). Interestingly, they showed a varying degree of PrfA activation, as judged by “classical” PrfA* phenotypes such as fosfomycin sensitivity, virulence factor expression, bacterial uptake into eukaryotic cells, and intracellular growth.

Also, the PrfA* proteins that we isolated also showed a diverse ability to bind the promoter regions of difference PrfA-regulated genes. Conclusively, our isolated PrfA* mutants resembles phenotypes of other previously characterized PrfA* mutants (15).

We determined the crystal structures of the medium- to high-activated PrfA* mutants to see if their varying activation levels were reflected structurally. PrfA_{WT} has flexible HTH-domains allowing for only weak DNA-binding, whereas the fully-activated PrfA*_{G145S} mutant...
and the glutathione-activated PrfA WT have structured HTH-domains that allow maximal DNA-binding (10, 17). In addition to folded HTH motifs, the activated PrfA proteins also have a collapsed central core structure (17). We found that the structures of the PrfA* proteins in this study had mixtures of structural features characteristic of both inactive and active PrfA. The PrfA L140H, PrfA L140F, and PrfA A218G PrfA* mutants all had a folded HTH motif in one monomer of the homodimer, but an uncollapsed central core structure. Consequently, we suggest these mutant structures represent an intermediate-activated form of the protein with features characteristic of both inactive and activated PrfA. We hypothesise that this form is also present during activation of PrfA WT or PrfA G145S, but too short-lived to be captured in crystal structures. Taken together, these experiments give a structural explanation of why there exist different activity levels of the PrfA* mutants.

By adopting an ensemble of interconverting conformational states under physiological conditions, transcriptional activators like PrfA are primed to quickly switch their function in response to changes in their environments.

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MATERIAL AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table S3 and S4. Listeria monocytogenes EGDe (serotype 1/2a) strains were subcultured in BHI (Thermofisher Scientific), BHI supplemented with 1% (w/v) Amberlite XAD4 (#06444, Fluka)
Escherichia coli strains were subcultured in Luria-Bertani (LB) media. Antibiotics were added as appropriate.

**Genetic manipulation.** All oligonucleotides are listed in Table S5. The pMAD vector (Eurofins) was used to delete the hpt allele as described previously (27). For Construction of the G145C, L140F, L140H, A94V and A218G prfA mutants were performed as follows: 10 ng of plasmid pLis35 (28) or pET-His1a-prfA was amplified using either primer pair PrfAG145CFwd and PrfAG145CRev (for G145C), PrfAL140FFwd and PrfAL140FRev (for L140F), PrfAL140HFwd and PrfAL140HRev (for L140H), PrfAA94VFwd and PrfAA94VRev (for A94V) or PrfAA218GFwd and PrfAA218GRev (for A218G). Afterwards, the PCR products were digested using 10 U of Dnpl (Thermo Scientific) at 37°C for 1 hour. The reaction mixture was transformed into E. coli strain DH5α. The point mutations were verified by sequencing the prfA gene.

**Glucose-6-phosphate screen** A previously constructed Himar1-marinerm transposon (Tn) mutant library (20) was inoculated into 96-well plates containing BHI and subsequently grown at 37°C with shaking o/n. The following day, the cultures were spun down and washed three times in 1 x PBS before they were replica-plated into 96-well plates containing DM supplemented with 0.2 % glucose-6-phosphate (G-6-P). The bacteria were grown at 37°C with shaking for 2 days. The cultures were examined daily for growth and the Tn mutants that showed growth were recovered by re-streaking onto BHI plates. To verify their ability to grow in the screening conditions, the colonies were re-inoculated into DM supplemented with 0.2 % G-6-P in 15 ml falcon tubes and grown at 37°C with shaking. In addition, the colonies were streaked onto blood agar plates to examine their haemolytic ability.
**Fosfomycin sensitivity test.** Fosfomycin sensitivity was determined by using 100 µg fosfomycin antibiotic discs (Liofilchem). BHI plates with or without 7 µg/ml chloramphenicol were inoculated using swabs soaked with a bacterial suspension in sterile saline. The suspension was adjusted to a turbidimetry of OD$_{600}$ = 0.5 on the MacFarland’s scale. A fosfomycin antibiotic disc was placed on the plates and they were incubated overnight at 37°C. The next day the inhibition zone was measured.

**Intracellular growth.** Human colon epithelial Caco-2 cells were seeded into 24-well dishes (Corning BioCoat Cellware, Collagen Type I, VWR) at a density of 8·10$^4$ cells per well and infected with o/n with cultures of bacterial strains at a multiplicity of infection of 10. The cells and bacteria were centrifuged at 130 x g for 5 min to synchronize the infection. At one hour post-infection, the Caco-2 cells were washed twice with PBS. Cell growth media supplemented with 50 µg/ml gentamycin was added to kill off extracellular bacteria. At 2, 4 and 6 hours postinoculation, the cells were lysed in water and the bacteria plated on LA plates.

**Plaque assay.** Plaque assay was performed as described previously with some modifications (29) giving a more qualitative number of infectivity. Briefly, TC7 cells (a kind gift from Andrea Puhar, Umeå University, Sweden) were seeded into six-well dishes (Corning BioCoat Cellware, Collagen Type I, VWR) at a density of 10$^6$ cells/well and infected with a multiplicity of infection of 1:500 with PBS-washed overnight cultures grown in BHI at 37°C with shaking. Two hours post infection, the TC7 monolayer was washed and an agarose overlay was added consisting of DMEM, 0.7% agarose, 20% fetal calf serum and 50 µg/ml gentamycin. Two days post infection, the agarose overlay was removed and the cells were fixed with absolute ethanol for 5 min and stained with Giemsa before counting of the plaques.
Isolation of RNA. Isolation of RNA was performed essentially as previously described in (30). Overnight cultures in BHI (37°C) were diluted 50-fold in BHI and incubated at 37°C with shaking and grown until OD_{600} = 1.0. The bacteria were collected by centrifugation in at 4°C, 6000 x g for 10 min and frozen at -80°C. Pelleted bacteria were resuspended in resuspension solution (10% glucose, 12.5 mM Tris-HCl pH 7.6, 5 mM EDTA). After transferring the samples to a bead beater tube containing 0.4 g glass beads and 0.5 ml phenol (pH 4.5) the cells were homogenized in a mini-bead beater (Biospect products) for 75 sec. The mix was then centrifuged for 5 min at 16800 x g, 4°C before the addition of 1 ml Trizol (Ambion) and 100 μl of a 24:1 ratio of chloroform/isoamylalcohol added to the aqueous phase. The samples were centrifuged for 5 minutes at 16800 x g, 4°C. After centrifugation, two more chloroform/isoamylalcohol extractions were performed before precipitation of the RNA by addition of 0.7 volumes of isopropanol, placed in the freezer for 30 min. The sample was centrifuged for 20 minutes at 4°C, 16800 x g. The dried RNA pellet was dissolved in 200 μl DEPC treated water. Samples were subjected to DNaseI treatment, 20 U and incubated for 30 min at 37°C. The reaction was stopped by addition of phenol/chloroform/isoamylalcohol (Ambion) and centrifuged for 5 minutes, 16800 x g, 4°C. The aqueous phase was extracted with of chloroform/isoamylalcohol as above before centrifugation. The purified RNA was pelleted by addition of 1/10 volume of DEPC-treated 3M NaOH, (pH 4.5) and 2.5 volumes of 99.5% ethanol, incubated -20°C for 30 min and pelleted by centrifugation, 16800 x g (4°C for 20 min). The RNA was dissolved in 200 μl of DEPC-treated water. The extracted RNA was analyzed on a 1.2% agarose gel to verify transcript integrity. The concentration of the RNA was measured on a Nanodrop ND-1000 spectrophotometer.

Northern blot. Northern blotting was performed as described previously (19). In brief, 25 μg of RNA was separated on an agarose gel (1.2% agarose, 1 x HEPES buffer (20 mM
HEPES, 5 mM NaAc, 1 mM EDTA, adjusted to pH 7), 7.3% formaldehyde). The gel was run in 1× HEPES buffer at 100 V for 4 h and the RNA was transferred to a Hybond-N membrane (GE Healthcare) by capillary transfer in 20 × SSC buffer. The membranes were cross-linked by UV-light, pre-hybridized at 50°C (for hpt) or 60°C (for tmRNA) in Rapid hyb buffer (GE Healthcare) for about 2 h and then hybridized with DNA probes at 50°C or 60°C overnight, respectively. Membranes were washed (0.1% sodium dodecyl sulfate, 2 × SSC), at room temperature for 15 min, followed by a second wash (0.1% SDS, 0.1 × SSC) at 50°C or 60°C for 15 min. Thereafter, the membranes were exposed in a phosphorimager cassette and developed using the Typhoon FLA9500 Scanner (GE Healthcare). The probes were created by amplifying genomic L. monocytogenes EGDe DNA with PCR and primers uhpT-U/uhpT-D for uhpT and tmRNA-U/tmRNA-D for tmRNA. The primer sequences are in Table S5. Probes were subsequently labeled with α-32P dATP (PerkinElmer) using Megaprime DNA labelling system (GE Healthcare), according to the manufacturer’s instructions.

Western blots. Western blots were performed as described previously, with minor changes (19). Bacterial cultures were grown in BHI supplemented with the appropriate antibiotics at 37°C with shaking. At OD$_{600}$ = 1.0, the cultures were processed either as a whole cell fraction or a secreted fraction as follows.

Supernatant fraction: 1 ml of the culture supernatant was precipitated trichloroacetic acid: A one-fourth volume of ice-cold 50% trichloroacetic acid was added to the samples, which were then incubated on ice for 1 h. The samples were spun down (10 min, 16800 x g) and the precipitate washed in 80% ice-cold acetone. The dried protein pellets were suspended in 1 x Laemmli buffer (31) and run on SDS-PAGE and Western blotting. The Western blot was developed with rabbit anti-LLO (#ab43018, Abcam), HRP-conjugated goat-
anti rabbit secondary antibodies (#as09602, Agrisera), and HRP-conjugated rabbit- anti
mouse secondary antibodies (Dako P0260).

**Whole cell fraction:** The cultures were added to an equal volume of 1:1 ethanol:acetate and frozen at -20°C o/n. Subsequently the samples were centrifuged and the bacterial pellet lysed in lysis buffer (20 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 20% sucrose) with added lysozyme and DNase. The samples were heated at 37°C for 1 h and run on SDS-PAGE and Western blotting. The Western blot was developed using anti-ActA (19), anti-PrfA R79IS4b (kindly provided by Pascale Cossart, Institute Pasteur, Paris, France), anti-RNA polymerase beta (RpoB) (#ab202891, Abcam) and HRP-conjugated secondary antibodies (#as09602, Agrisera) or anti-RpoB (BioSite check) and HRP conjugated rabbit- anti mouse secondary antibodies (Dako P0260).

**Amberlite induction.** Overnight *L. monocytogenes* cultures grown in BHI supplemented with the appropriate antibiotic were diluted into BHI supplemented with 1% (w/v) Amberlite XAD4 (#06444, Fluka) and grown at 37°C until OD₆₀₀ = 1.0. Samples were processed as described under *Western blots.*
Surface plasmon resonance (SPR). The interaction study was performed using a ProteOn™ XPR36 biosensor (Bio-Rad, USA) equipped with an NLC sensor chip (Bio-Rad, USA).

Biotinylated dsDNA strands 5´-TTTTGTTTCTGCATGATAACAAGTGTTAATGACGGAAAG-3´ (hpt-promoter), 5´-AGTTGGGTTAATGAGAAATGGACTAAAAAATA-3´ (act-promoter) and 5´-CTTTTATGGGCATTACAGTTAATGACGATAAA-3´ (hly-promoter) were immobilized to a density of 50-90 response units (RU). All SPR experiments were performed at 25 °C in 30 mM Tris-HCl pH 7.4 containing 200 mM NaCl and 0.05% Tween 20. A blank surface or interspots was used as a reference and subtracted from the data. Graded concentrations of PrfA and its derivatives were injected over the different promoters. The rate and dissociation constants were derived by global fitting of at least four different PrfA concentrations with ProteOn software (Bio-Rad, USA).

Protein expression and purification. The PrfA_{L140F}, PrfA_{L140H}, PrfA_{A94V} and PrfA_{A218G} constructs, cloned as described above, encode the full length PrfA protein (M1-N237) as well as a 6-His tag and a Tobacco etch virus (TEV) protease cleavage site. This results in the addition of two non-native N-terminal residues (GA) upon TEV cleavage. Proteins were overexpressed in E. coli BL21 (DE3) plysS cells (Novagen) grown at 37°C in LB medium, supplemented with 50 μg/ml kanamycin and 34 μg/ml chloramphenicol, then induced with isopropyl β-D-1-thiogalactopyranoside (final concentration of 0.4 mM) at OD_{600}=0.6. Growth was continued o/n at 20°C and cells were then harvested by centrifugation and lysed by sonication on ice.

Purification of PrfA proteins for assays and crystallization was performed using Ni-NTA Superflow FF (Qiagen) in a lysis buffer containing 50 mM sodium phosphate pH 8.0, 20 mM imidazole, 500 mM NaCl. The columns were washed with ten column volumes of lysis buffer followed by ten column volumes of 50 mM sodium phosphate pH 8.0, 1000 mM NaCl before
elution of PrfA proteins with 50 mM sodium phosphate pH 8.0, 300 mM imidazole, 500 mM NaCl. The poly-histidine tag was removed by overnight cleavage with TEV protease at 4°C in 50 mM sodium phosphate pH 7.1, 200 mM NaCl. Cleaved target proteins were separated from the 6-His-tagged TEV protease, 6-His-tag fragments, and un-cleaved target proteins by nickel affinity chromatography as described above. The eluted target protein was dialysed into a final buffer consisting of 20 mM Tris-HCl pH 7.1, 100 mM NaCl.

**Crystallization of PrfA* mutants, and the mutants in complexes with the hly promoter DNA.** For crystallization screening, proteins were additionally purified by ion-exchange (GE Healthcare) and size-exclusion chromatography. Prior to ion-exchange, the sample pH was adjusted to 6.5 and the MonoS 5/5 column (GE Healthcare) was eluted with a linear gradient of 200-650 mM NaCl in 20 mM sodium phosphate pH 6.5.. Purified proteins eluted at ~250 mM NaCl. The peak fractions of PrfA were pooled and applied to a HiLoad Superdex 75 16/60 column (GE Healthcare) equilibrated with 50 mM sodium phosphate pH 6.5, 200 mM NaCl. Protein used for co-crystallization with hly DNA was further buffer-exchanged to 20 mM Tris-HCl pH 8.0, 150 mM NaCl. Each protein was concentrated using Amicon Ultra centrifugal filter devices (Millipore) before being flash-frozen in liquid N₂ and stored at -80°C.

Two complementary 30 bp DNA oligonucleotides, representing the hly PrfA box motif, obtained from Eurofins Genomics (5'-TTGAGGCATTACATTTGTAACGACGATA-3', reverse complement: 5'-TATCGTCGTTAAATGTTAATGCCTCAA-3'), were annealed by cooling from 95°C to room temperature over 3 h in 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA. This formed a blunt-ended DNA duplex.

The PrfA*variants were crystallized by the hanging-drop vapor-diffusion method in VDX plates (Hampton Research) at 18°C. Droplets of 2-4 μl protein solution at 3 mg/ml were
mixed with 2 μl reservoir solution consisting of 24% PEG 4000, 100 mM sodium citrate pH 5.5, and 17% isopropanol. Crystals used for data collection were obtained after 2-5 days. For crystallization of PrfA-DNA complexes, the protein and hly PrfA box motif duplex DNA were incubated together at a ratio of 1:1.3 (PrfA dimer:hly DNA) at final concentrations of 50 μM and 70 μM respectively in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, for 30 min at room temperature, before crystallization screening. Crystals were obtained after 24 h by mixing 4 μl protein-DNA solution with 2 μl reservoir solution consisting of 8% PEG 8000, 100 mM sodium acetate pH 4.6, 100 mM magnesium acetate, 20% glycerol. Crystals of PrfA-DNA complexes were cryoprotected in reservoir solution supplemented with 30% glycerol before vitrification in liquid nitrogen. PrfA crystals were vitrified directly from their drop solutions.

**Data collection and structure determinations.** Diffraction data were collected at -173°C at the European Synchrotron Radiation Facility (ESRF) beamline ID30-B. Images were processed with XDS (32, 33) and subsequently scaled and merged using AIMLESS, a component of the CCP4 software suite (34). Structures were solved by molecular replacement using the previously determined structures of PrfA<sub>WT</sub> (PDB code 5F1R (19)), PrfA<sub>G145S</sub> (PDB code 2BGC (17)), and the PrfA<sub>WT</sub>-hly DNA complex (PDB code 5LEJ (10)) as search models with the program PHASER from the PHENIX suite (35). Atomic models were iteratively rebuilt manually and refined using the programs COOT (36) and phenix.refine (35). Bases of the two chains of the palindromic hly PrfA box motif DNA are numbered from -15 to +15. Data collection, refinement and validation statistics are presented in Table S1. Superimpositions are based on all main chain atoms of residues 2-237 using the program SSM (37). All structural figures were prepared with CCP4mg (38).

**Accession number(s).** The atomic coordinates and the structure factors have been deposited with the Protein Data Bank (PDB codes 6QVY for PrfA<sub>A94V</sub>, 6QVZ for PrfA<sub>L140H</sub>.
6QW1 for PrfA<sub>L140F</sub>, 6QW2 for PrfA<sub>A218G</sub>, 6QWF for PrfA<sub>V94V</sub>-DNA, 6QWH for PrfA<sub>L140H</sub>-DNA, 6QWK for PrfA<sub>L140F</sub>-DNA, and 6QWM for PrfA<sub>A218G</sub>-DNA

SUPPLEMENTARY MATERIAL

Supplemental material for this article may be found at https:

### TABLE 1

SPR analysis of kinetic rate constants and equilibrium affinities for PrfA-promotor interactions

| Promotor | hly          | actA         | hpt          |
|----------|--------------|--------------|--------------|
|          | k<sub>a</sub> (M<sup>-1</sup>s<sup>-1</sup>) | k<sub>d</sub> (s<sup>-1</sup>) | K<sub>D</sub> (nM) | k<sub>a</sub> (M<sup>-1</sup>s<sup>-1</sup>) | k<sub>d</sub> (s<sup>-1</sup>) | K<sub>D</sub> (nM) | k<sub>a</sub> (M<sup>-1</sup>s<sup>-1</sup>) | k<sub>d</sub> (s<sup>-1</sup>) | K<sub>D</sub> (nM) |
| Mutant   |             |              |              |             |              |              |             |              |              |
| PrfA<sub>WT</sub> | 1.7*10<sup>6</sup> | 1.9*10<sup>-3</sup> | 110          | 6.2*10<sup>5</sup> | 4.2*10<sup>-2</sup> | 690          | 4.2*10<sup>5</sup> | 5.9*10<sup>-2</sup> | 1040          |
| PrfA<sub>L140F</sub> | 1.7*10<sup>6</sup> | 2.5*10<sup>-3</sup> | 15           | 9.2*10<sup>4</sup> | 8.5*10<sup>-2</sup> | 95           | 7.0*10<sup>4</sup> | 1.9*10<sup>-2</sup> | 270           |
| PrfA<sub>L140H</sub> | 6.6*10<sup>5</sup> | 6.8*10<sup>-3</sup> | 10           | 3.5*10<sup>3</sup> | 3.0*10<sup>-2</sup> | 85           | 2.6*10<sup>5</sup> | 5.4*10<sup>-2</sup> | 210           |
| PrfA<sub>A218G</sub> | 1.6*10<sup>6</sup> | 6.4*10<sup>-3</sup> | 4            | 8.6*10<sup>5</sup> | 2.3*10<sup>-2</sup> | 27           | 7.1*10<sup>5</sup> | 3.9*10<sup>-2</sup> | 55            |
| PrfA<sub>G145S</sub> | 1.9*10<sup>6</sup> | 3.8*10<sup>-3</sup> | 2            | 4.0*10<sup>5</sup> | 5.0*10<sup>-3</sup> | 12           | 2.5*10<sup>5</sup> | 7.7*10<sup>-2</sup> | 31            |

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FIG 1  Growth of *Listeria monocytogenes* in defined medium (DM) supplemented with glucose-6-phosphate (G-6-P). (A) Four strains were tested for growth in DM with 0.2% G-6-P as the sole carbon source. These were: 1) wildtype EGDe strain (WT); 2) the strain carrying an in-frame deletion of the *hpt* gene (Δ*hpt*); 3) the strain carrying the Gly to Ser substitution of codon 145 in the PrfA protein (PrfA<sub>G145S</sub>); and 4) the PrfA<sub>G145S</sub> strain carrying an in-frame deletion of the *hpt* gene (PrfA<sub>G145S</sub> Δ*hpt*). Growth was followed by viable count for three days and shown as mean values with standard deviations (n=3). Statistical analysis compared growth of the mutant strains with the wildtype strain at each time-point (Student T-test (two-tailed, p<0.05,*; p<0.01,**)). (B) Fosfomycin resistance of the indicated strains. Fosfomycin discs were used and the clear zone measured. The radius of the clearing zone is indicated relative to the wildtype as an average of three independent experiments. Statistical analysis compared fosfomycin sensitivity of the PrfA* strain with the WT and the PrfA*, Δ*hpt* strains (student's T-test (two-tailed, p<0.001,***)). (C) Upper panel. Expression of *hpt* in the indicated strains grown in BHI until OD<sub>600</sub>=1. RNA was isolated and *hpt* expression was examined by Northern blot using radiolabelled probes against *hpt* and tmRNA (control). A representative of three independent experiments is shown. Lower panel. Measurement of *hpt* expression from top panel. Expression is relative to WT (set to 1). Student T-test (two-tailed, p<0.001,***).

FIG 2  Effect of the amino acid substitutions on PrfA activity. (A) Transposon-free strains expressing PrfA proteins with the wildtype sequence or indicated amino-acid substitutions were tested for growth in DM supplemented with 0.2% G-6-P for 72 hours. A representative of five independent experiments is shown. (B) Fosfomycin resistance of the indicated strains. Fosfomycin discs were used and the clearing zone measured. The radius of the clearing zone is indicated relative to the wildtype as an average of three independent experiments. Statistical analysis compared fosfomycin sensitivity of the PrfA* strain with the wildtype and the PrfA*, Δ*hpt* strains (student's T-test (two-tailed, p<0.05,*; p<0.01,**; p<0.001,***)) (C) Upper panel. Expression of *hpt* in the indicated strains were grown in BHI until OD<sub>600</sub>=1. RNA was isolated and *hpt* expression was examined by Northern blot using radiolabelled probes against *hpt* and tmRNA (control). A representative of three independent experiments is shown. Lower panel. Quantification of *hpt* expression from top panel. Expression is relative to WT (set to 1). Student T-test (two-tailed, p<0.05,*; p<0.01,**; p<0.001,***).

FIG 3  The identified point mutations in PrfA give rise to PrfA* phenotypes. (A) ActA, PrfA and LLO virulence factor expression of the indicated strains examined by Western blots. The
strains were grown in BHI until \( \text{OD}_{600} = 1 \). RNA polymerase beta (RpoB) was used as a loading control for whole cell fraction samples (ActA and PrfA) and P60 was used as a loading control for the secreted fraction (LLO). A representative of four independent experiments is shown. (B) Expression of ActA and PrfA virulence factors in presence of 1% Amberlite. The strains were grown in BHI with or without 1% Amberlite XAD4 until \( \text{OD}_{600} = 1 \) before sample preparation and western blotting. RNA polymerase beta (RpoB) was used as a loading control. A representative of four independent experiments is shown. See also Fig. S3 for quantification of ActA, PrfA and LLO expression levels from Fig. 3A, and Fig. S4 for quantification of ActA expression levels from Fig. 3B.

**FIG 4** The PrfA* mutant strains show a larger uptake and cell-to-cell spread compared to a wildtype strain. (A) Intracellular growth of the indicated strains was tested by viable count in the colon epithelial cell line Caco-2. An average with standard deviation of three independent experiments is shown. Statistical analysis (inset) compared number of CFUs for the wildtype strain with the mutant strains at 2, 4, and 6 h post-infection (student’s T-test, two-tailed, \( p < 0.05, *, p < 0.001, ***, \text{ns} = \text{no significant difference} \)). (B) The indicated strains were tested for infection (adhesion/invasion and/or cell-to-cell spread) of the Caco-2 derivative TC7 cell line using a multiplicity of infection of 1:500. The number of plaques formed were counted and represented as amount of plaques relative to WT (set to 1). An average with standard deviation of three independent experiments is shown. Statistical analysis compared infection of the wild type strain with the mutant strains (student’s T-test (two-tailed \( p < 0.01, **, p < 0.001, *** \))).

**FIG 5** Superimposed structures of PrfA. All superimpositions are based on residues 2-237 in monomer B. For root-mean-square (rms) deviations see Table S2 (A) The PrfA\(_{WT}\) homodimer (PDB code 2BEO (17)). The HTH motifs are shown in dark blue of which only the recognition helix (\( \alpha \)F) is folded. (B) Superimposed structures of the four intermediate-activated structures of PrfA studies: PrfA\(_{A94V}\), PrfA\(_{L140H}\), PrfA\(_{L140F}\), and PrfA\(_{A218G}\). Only the HTH motifs in monomer B are folded. (C) Superimposed structures of PrfA\(_{WT}\), PrfA\(_{G145S}\), and one representative of the intermediate-activated fold (PrfA\(_{A218G}\)). Noteworthy is the shift with up to 6 Å in the position of the recognition helix in monomer A in PrfA\(_{A218G}\) compared to monomer A in PrfA\(_{G145S}\). The Calpha trace of PrfA\(_{G145S}\) is shown in sea green with the folded HTH motifs coloured in orange. (D) Seven structures of PrfA in complex with DNA: PrfA\(_{WT}\) (PDB code 5LEJ (10)), PrfA\(_{WT}\)-GSH (PDB code 5LRS (10)), PrfA\(_{G145S}\) (PDB code 5LEK (10)), PrfA\(_{A94V}\), PrfA\(_{L140H}\), PrfA\(_{A140F}\), and PrfA\(_{A218G}\). Note the close to identical structures of all PrfA when in complex with DNA (rms deviations < 0.5 Å).

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Figure 1

A  Growth in DM + 0.2% G-6-P

B  Fosfomycin resistance relative to WT

C  hpt expression, relative to WT

WT  PrfA*  PrfA*, Δhpt

Δhpt  PrfA*WT

PrfA* expression, relative to WT

hpt expression, relative to WT

Δhpt

WT  PrfA*  PrfA*, Δhpt

hpt

tmRNA

***  **  *  **  ***
Figure 2

A Growth in DM + 0.2% G-6-P

Growth, CFU x ml$^{-1}$

- Inoculum
- 24 hour
- 48 hour
- 72 hour

B Fosfomycin resistance relative to WT

C hpt expression, relative to WT

hpt

pPL2:prfA

*** *** ** *

pPL2:prfA

hpt

tmRNA

*** ** ** *

pPL2:prfA
Figure 3

A

Whole cell fraction:

|         | WT | G145S | G145C | L140F | L140H | A218G | ΔprfA | ΔactA |
|---------|----|-------|-------|-------|-------|-------|-------|-------|
| ActA    |    |       |       |       |       |       |       |       |
| PrfA    |    |       |       |       |       |       |       |       |
| RpoB    |    |       |       |       |       |       |       |       |

Secreted fraction:

|         | WT | G145S | G145C | L140F | L140H | A218G | ΔprfA | Δlly  |
|---------|----|-------|-------|-------|-------|-------|-------|-------|
| LLO     |    |       |       |       |       |       |       |       |
| P60     |    |       |       |       |       |       |       |       |

B

Whole cell fraction:

|         | WT | G145S | L140F | L140H | A218G | ΔprfA | ΔactA |
|---------|----|-------|-------|-------|-------|-------|-------|
| ActA    |    |       |       |       |       |       |       |
| RpoB    |    |       |       |       |       |       |       |
Figure 4

A: Intracellular growth

- Intracellular growth over time (h)
- CFU x ml$^{-1}$

B: Infection, relative to WT

- Comparison of infection rates for different mutants
- Significance levels indicated

Significance

| Hours p.i. | 2 | 4 | 6 |
|-----------|---|---|---|
| WT        |   |   |   |
| G145S     | * | * | * |
| L140F     | *** | * | ns |
| L140H     | ns | ns | * |
| A218G     | * | * | ns |

pPL2:prfA

Infection, relative to WT
