A naturally occurring mutation K220T in the pleiotropic activator PrfA of *Listeria monocytogenes* results in a loss of virulence due to decreasing DNA-binding affinity

P. Velge,¹ M. Herler,² J. Johansson,³ S. M. Roche,¹ S. Témoïn,¹
A. A. Fedorov,⁴ P. Gracieux,¹ S. C. Almo,⁴ W. Goebel² and P. Cossart³

Correspondence

P. Velge

Philippe.velge@tours.inra.fr

¹Institut National de la Recherche Agronomique, UR1282 Infectiologie animale et santé publique, 37380 Nouzilly, France

²Lehrstuhl für Mikrobiologie, Theodor-Boveri-Institut für Biowissenschaften der Universität Würzburg, Am Hubland, 97074 Würzburg, Germany

³Institut Pasteur, Unité des Interactions Bactéries-Cellules, 28 rue du Docteur Roux, 75015 Paris, France

⁴Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, 1300 Morris Park Avenue, Bronx, NY 10461, USA

The sequencing of *prfA*, encoding the transcriptional regulator of virulence genes, in 26 low-virulence field *Listeria monocytogenes* strains showed that eight strains exhibited the same single amino-acid substitution: PrfAK220T. These strains exhibited no expression of PrfA-regulated proteins and thus no virulence. This substitution inactivated PrfA, since expression of the PrfAK220T mutant gene in an EGDΔ*prfA* strain did not restore the haemolytic and phosphatidylcholine phospholipase C activities, in contrast to the wild-type *prfA* gene. The substitution of the lysine at position 220 occurred in the helix αH. However, the data showed that the PrfAK220T protein is dimerized just as well as its wild-type counterpart, but does not bind to PrfA-boxes. PrfAK220T did not form a PrfA–DNA complex in electrophoretic mobility shift assays, but low concentrations of CI complexes (PrfAK220T–RNA polymerase–DNA complex) were formed by adding RNA polymerase, suggesting that PrfA interacted with RNA polymerase in solution in the absence of DNA. Formation of some transcriptionally active complexes was confirmed by *in vitro* runoff transcription assays and quantitative RT-PCR. Crystallographic analyses described the structure of native PrfA and highlighted the key role of allosteric changes in the activity of PrfA and especially the role of the Lys220 in the conformation of the helix–turn–helix (HTH) motif.

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INTRODUCTION

Listeria monocytogenes, a facultative intracellular pathogen, is a major cause of food-borne infection in humans (Tompkin, 2002). Although rare, invasive listeriosis is an infection of public health concern because of its potential to cause epidemics, the possible after-effects and its severity, with a fatality rate evaluated at 20–30 % (Rocourt & Cossart, 1997). The main virulence determinants of *L. monocytogenes* have been extensively identified. Entry involves at least two proteins of the internalin family, InlA (or internalin) and InlB (Dramsi *et al.*, 1995; Gaillard *et al.*, 1991). Intracellular *L. monocytogenes* lies in a phagocytic vacuole which is quickly lysed by the pore-forming toxin listeriolysin O

(LLO) encoded by the *hly* gene and the two phospholipases C [phosphatidylinositol phospholipase C (PI-PLC) and phosphatidylcholine phospholipase C (PC-PLC)] encoded by the *plcA* and *plcB* genes respectively (Goebel *et al.*, 1988; Smith & Portnoy, 1993). Free *L. monocytogenes* in the cytosol can replicate, and its ActA protein induces polymerization of actin filaments, promoting intracellular bacterial movement (Domann *et al.*, 1992). This protrusion may contact a neighbouring cell, allowing entry and giving rise to a two-membrane vacuole lysed by PC-PLC and LLO (Vazquez Boland *et al.*, 1992). Thus, the bacterium is disseminated by direct cell-to-cell contact. These bacterial traits allow plaque formation in the cell monolayer, which can be used to estimate the virulence of *L. monocytogenes* strains (Roche *et al.*, 2001). Most of these genes are located within a 10 kb cluster, which also encodes the main virulence regulator PrfA.

Abbreviations: EMSA, electrophoretic mobility shift assay; HTH, helix–turn–helix; PC-PLC, phosphatidylcholine phospholipase C; r.m.s., root mean square; RNAP, RNA polymerase; SeMet, selenomethionyl.

The protein encoded by the *prfA* gene is a key factor for *L. monocytogenes* pathogenesis, and strains lacking this gene are avirulent in a mouse model of infection (Mengaud *et al.*, 1991). PrfA, a 237 amino-acid protein, belongs to the cAMP receptor protein (Crp)-Fnr family of pleiotropic transcription regulators. The C-terminal region of PrfA contains a helix–turn–helix (HTH) motif (Lampidis *et al.*, 1994), which allows PrfA to bind to a 14 bp DNA palindromic sequence called the 'PrfA-box', present in the –40 region of target gene promoters (Williams *et al.*, 2000). PrfA binding is less efficient on imperfect promoter palindromes (such as those upstream of *mpl* and *actA*) than on perfect ones (*hly* and *plcA*) (Sheehan *et al.*, 1995). The symmetrical structure of PrfA-boxes and the similarity in the amino-acid sequences of Crp and PrfA suggest that PrfA, like Crp, binds to its target DNA as a dimer (Lampidis *et al.*, 1994). However, PrfA is longer than Crp and the relevance of the C-terminal region, containing helices α G, α H and α I, has not been completely determined.

We previously identified 26 low-virulence *L. monocytogenes* strains using a method that combines a plaque-forming (PF) assay with the subcutaneous inoculation of mice (Roche *et al.*, 2001). These strains exhibited a low lethality in mice and their full virulence could not be restored after 10 successive *in vivo* inoculations (Roche *et al.*, 2003). Using the results of cell infection assays and phospholipase activities, the low-virulence strains were assigned to one of four groups by cluster analysis. All strains belonging to Group I exhibited a mutated PrfA. Out of the 11 strains of this group, which were unrelated in origin and isolation date, eight strains had a single amino-acid substitution, PrfAK220T (Roche *et al.*, 2005). This mutation was

sufficient to explain the low virulence of these field strains, as introduction *in trans* of a wild-type *prfA* gene from the EGDe strain restored their virulence, as demonstrated by a plaque forming assay and lethality in mice (Roche *et al.*, 2005).

The aim of this study was to analyse the effects of the modifications induced by the K220T substitution on the activity of PrfA and on the protein conformation. The results obtained highlight the crucial role of Lys220 in PrfA activity.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains used in this study are described in Table 1 and the plasmids in Table 2. The *Escherichia coli* strain MC1061 was used for constructing the plasmids. *L. monocytogenes* strains were grown in brain heart infusion (BHI; Difco) broth and *E. coli* strains in trypticase soy broth (Difco). Strains harbouring plasmid pHT1618 (Lereclus & Arantes, 1992) were grown in the presence of the following antibiotics: ampicillin (100 μ g ml⁻¹) for *E. coli*, or tetracycline (10 μ g ml⁻¹) for *Listeria*. Strains harbouring plasmid pP1 (Drams *et al.*, 1995) were grown in the presence of erythromycin (8 μ g ml⁻¹).

Cloning and *Listeria* electrotransformation. The EGDA Δ *prfA* strain was transformed with the wild-type *prfA* gene from the EGDe strain, or with the mutated *prfA* genes from the low-virulence strains (CNL895806 and SO49). The pP1 vector, carrying a strong constitutive promoter from *Streptococcus cremoris* protease gene, was used to clone and express the *prfA* ORFs of each strain. All ORFs with the transcriptional terminator of the *prfA* gene were amplified by PCR with *Pfu* DNA polymerase (Promega) using primers O25 and O26 for 35 cycles of 30 s at 95 °C, 45 s at 54 °C and 110 s at 72 °C (Table 3). The PCR fragments were ligated with a DNA ligation kit (Takara, BioWhittaker) into *Sall*/*Xba*I-digested pP1 vector and

Table 1. Characteristics of *Listeria* strains used

<i>Listeria</i> strain*	Source	Serotype	Virulence <i>in vitro</i>		Virulence <i>in vivo</i>		
			Cell entry (%)	PF assay† (mean ± SD)	LD ₅₀ ‡	s.c. test (spleens)§ (mean ± SD)	I/T
<i>L. monocytogenes</i>							
LO28	Human isolate	1/2c	0.5	5.21 ± 0.16	2.9 × 10 ⁶	4.66 ± 0.29	5/5
CDI5	LO28 mutant	1/2c	ND	ND	ND	ND	ND
EGDe BUG1600	Animal clinical case	1/2a	1.0	6.35 ± 0.12	4.2 × 10 ⁴	5.54 ± 0.46	5/5
EGDe Δ <i>prfA</i>	EGDe BUG1600 mutant	1/2a	<0.001	0	> 1 × 10 ⁹	0	0/5
CNL895806	Food product	1/2a	<0.001	0	> 1 × 10 ⁹	0	0/5
SO49	Food product	1/2a	<0.001	0	> 1 × 10 ⁹	0	0/5
<i>L. innocua</i>							
BUG499		6a	<0.001	0	> 1 × 10 ⁹	0	0/5

ND, Not determined.

*The strains have previously been described (Renzoni *et al.*, 1997; Roche *et al.*, 2003, 2005).

†Plaque-forming assay. Log numbers of plaques per 10⁷ c.f.u. deposited. Values are from two independent experiments performed in duplicate.

‡LD₅₀ was calculated by a probit dose–response model after subcutaneous (s.c.) injection into the left hind footpad (Roche *et al.*, 2003)

§Log numbers of *Listeria* recovered in spleens 3 days after s.c. injection into the left hind footpad of immunocompetent Swiss mice with 10⁴ c.f.u. in 50 μ l (Roche *et al.*, 2003).

||Ratio of infected mice to inoculated mice in s.c. test.

Table 2. Plasmids used in this study

Plasmids	Characteristics	Source or reference
pHT1618	Derivative from pBC16 vector of <i>Bacillus cereus</i> and pUC18 vector; Tet ^r Amp ^r	Lereclus & Arantes (1992)
pP1	pAT18 derivative carrying the promoter of the protease of <i>Streptococcus cremoris</i> ; Ery ^r	Dramsi <i>et al.</i> (1995)
<i>prfA</i> _{EGDe}	pP1 carrying wild-type <i>prfA</i> gene of EGDe strain	This study
<i>prfA</i> _{CNL895806}	pP1 carrying mutated <i>prfA</i> gene of CNL895806 strain encoding mutated PrfA (PrfAK220T)	This study
<i>prfA</i> _{SO49}	pP1 carrying mutated <i>prfA</i> gene of SO49 strain encoding mutated PrfA (PrfAK220T)	This study
<i>hly</i> _{EGDe}	pHT1618 carrying wild-type <i>hly</i> gene of EGDe strain and its promoter region	This study

introduced into *E. coli* MC1061. Transformant strains were selected by antibiotic resistance, and the integrity of the inserts for each construct was checked by double-strand sequencing. EGDΔ*prfA* was then transformed with the plasmids by electroporation as described previously (Sheehan *et al.*, 1995).

The *Listeria innocua* BUG 499 strain was co-transformed with the wild-type *hly* gene from the EGDe strain and with either the wild-type *prfA* gene from the EGDe strain or the mutated *prfA* gene from the low-virulence strain CNL895806. The *prfA* genes were introduced into *L. innocua* as previously described for *L. monocytogenes*. The *hly* gene was PCR amplified using the O29–O30 primer pairs (Table 3) and *Pfu* DNA polymerase for 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 150 s at 72 °C. PCR fragments of *hly* were cloned into pHT1618 expression vector after digestion with *SalI* and *XbaI*, using T4 DNA ligase (Promega). Integrity of the inserts for each construct was checked by sequencing. Plasmids containing the *hly* and *prfA* genes from EGDe or CNL895806 strains were then introduced into *L. innocua* BUG 499 by electroporation.

RNA extraction and real-time RT-PCR. Overnight cultures in BHI were harvested by centrifugation. Total bacterial RNA was obtained as previously described (Glatron & Rapoport, 1972) with some modifications. In brief, after resuspension, bacteria were subjected to mechanical disruption with glass beads (425–600 µm, Sigma) for 45 s at maximum speed (Fast Prep FP120, Bio 101

Savant). After centrifugation at 4 °C at 13 000 *g* for 20 min, two acid phenol/chloroform (5/1, v/v) and two chloroform extractions were performed. The amount and quality of the RNA were determined spectrophotometrically and by agarose gel electrophoresis. Two DNase treatments were then performed according to the manufacturer's instructions (Invitrogen) in the presence of 20 U RNase inhibitor (RNasin, Promega) per µg RNA. One microgram of DNase-treated total RNA from three separate cultures was reverse transcribed to cDNA with AMV reverse transcriptase using random hexamer primers (Promega) according to the manufacturer's protocol. The reaction was performed at 42 °C for 60 min and stopped at 95 °C for 5 min. Real-time RT-PCR was performed to measure the transcriptional levels of *hly* and housekeeping *rpoB* genes (Stritzker *et al.*, 2005) using the LightCycler system (Roche) and SYBR Green I. Optimal PCR conditions were determined for each primer pair (Table 3) and hybridization was performed at 65 °C using 2 mM MgCl₂ and 0.5 µM of each primer. Quantification of cDNA samples was achieved by reference to a standard curve generated from a series of dilutions (10^{−1}–10^{−6}) of positive control pCR4-TOPO (Invitrogen) plasmid containing the amplicon of interest. Following amplification, melting curves were generated to verify PCR product identity. Expression of the *hly* gene was normalized with 10² copies of the housekeeping *rpoB* gene.

Enzyme assays. Haemolytic activity was assayed as described by Roche *et al.* (2001). One haemolytic unit (HU) is defined as the reciprocal of the dilution at which 50 % haemolysis is detected. The PC-PLC (lecithinase) activity was titrated as described by Geoffroy *et al.* (1991). PC-PLC units are defined as the amount of enzyme increasing the A₅₁₀ by 0.1 units in 1 h.

Protein preparation and Western blot analysis. Proteins were prepared from total extracts of bacteria. Aliquots (1 ml) of bacterial cultures with an OD₆₀₀ of 0.6 were centrifuged. The resulting pellets were sonicated three times for 5 min and the bacterial extracts were collected by centrifugation and separated by SDS-PAGE in 10 % polyacrylamide gels. Proteins were stained with Coomassie brilliant blue. Western blots were prepared and probed with rabbit anti-PrfA antibody (Renzoni *et al.*, 1997) and anti-rabbit horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using chemiluminescence (ECL+, Amersham) according to the manufacturer's protocol.

Protein–protein cross-linking. The LO28, CDI5 (an LO28 mutant strain; Renzoni *et al.*, 1997) and SO49 strains were cultured overnight, harvested, washed twice in 10 mM Tris/HCl (pH 8.0) and suspended in cross-linking buffer [1 M triethanolamine-HCl (pH 8.5), 0.25 M NaCl and 5 mM dithiothreitol]. They were then disrupted by sonication (10 × 10 s at 6 % amplitude) using a Vibriocell 72408 (Bioblock Scientific). Cell debris was removed by centrifugation at 15 000 r.p.m. for 30 min and the supernatant was incubated with or without 0.1 mg ml^{−1} dimethyl-suberimidate (DMS, Sigma) for 60 min. The SDS sample buffer was then added, the proteins were separated by SDS-PAGE and PrfA was revealed by Western blotting with rabbit anti-PrfA.

Table 3. Primers used for constructions or for real-time RT-PCR

Restriction sites are underlined.

Name	Primer sequence (5'–3')	Gene
Cloning		
O29	GTGTCGACTAGAGCGGACATCC	hly
O30	<u>TCTAGAAAAAA</u> AATTAAAAATAAGC-CTGTTTCTACATTCTTCAC	
RB1	GCGCATGCGATGACGATGACAAAAT-GAACGCTCAAGCAGAAGAA	prfA
RB2	GGT <u>GTCGAG</u> TCGTACGCGTTCATGA	
O25	<u>GGGTCGACC</u> AGCTCTTCTTGGTGAAG	
O26	GGT <u>CTAGAC</u> CGATTGGGGGATGAGAC	
Real-time RT-PCR		
rpoBF	ATCTGGGTTTCGTCTGTATCTC	rpoB
rpoBR	GGACCGTTACCAAGGATTCTC	
hlyF	AGTATACCACGGAGATGCAGTG	hly
hlyR	TTTACGAGAGCACCTGGATAGG	

Electrophoretic mobility shift assays (EMSAs). The mutant *prfA* gene was amplified using the RB1 and RB2 primers (Table 3) and cloned into the pQE-30 expression vector (Qiagen) digested with *SphI* and *PstI*. The wild-type *prfA* gene was cloned into the same vector as previously described (Bockmann *et al.*, 1996). The His₆-tagged PrfA protein was purified using 1 ml HiTrap chelating columns (Amersham). Protein purity was analysed by staining SDS-polyacrylamide gels with Coomassie brilliant blue. EMSAs with the purified PrfA proteins were performed with the 109 bp double-stranded DNA probe of the *hly* promoter region as previously described (Dickneite *et al.*, 1998; Herler *et al.*, 2001). Several concentrations of PrfA were tested (0–800 ng). CI complex formation was assayed by adding partially purified RNA polymerase (RNAP), from *L. monocytogenes* cultivated in minimal medium, to the binding assay mixtures containing PrfA bound to the DNA. This mixture was then incubated for 5 min at 37 °C and 10 min on ice. The DNA–protein complexes were separated on native 5% polyacrylamide gels in low ionic strength buffer and visualized by autoradiography (Bockmann *et al.*, 2000).

In vitro runoff transcription assays. *In vitro* transcription was performed in runoff experiments (Bockmann *et al.*, 2000) using the probe of the strictly PrfA-dependent *uhpT* promoter region (Glaser *et al.*, 2001), previously named *hpt* (Chico-Calero *et al.*, 2002), [α -³²P]UTP (Amersham) and 0.45 nM RNAP.

Crystallization of PrfA and X-ray data collection. PrfA was cloned in pET28a vector and expressed in *E. coli* BL21(DE3) pLysS cells (Invitrogen). Transformed *E. coli* strains were grown at 37 °C in LB medium supplemented with kanamycin (30 µg ml⁻¹) and chloramphenicol (30 µg ml⁻¹). PrfA expression was induced for 4 h at 37 °C by 1 mM IPTG. Bacteria were pelleted, resuspended in binding buffer (500 mM NaCl, 10 mM imidazole, 20 mM Tris/HCl pH 7.4) and sonicated four times for 15 s. After 30 min incubation on ice with benzonase nuclease (Novagen; 15 µl to 1 ml of buffer) and centrifugation at 16 000 r.p.m. in an SS-34 rotor for 45 min, PrfA protein was recovered with Ni-NTA agarose beads (Qiagen). The hexahistidine tag was removed by thrombin cleavage. PrfA was then dialysed against 20 mM HEPES, 150 mM NaCl, 1 mM DTT and purified by cation-exchange chromatography (MonoS5/5 column, Amersham) equilibrated with the same buffer. The column was eluted with a linear gradient of 150–1000 mM NaCl and the protein came out as a single peak at 250 mM NaCl. PrfA was concentrated using a Biomax-10 centrifugal filter device (Millipore) to 5–6 mg ml⁻¹. Selenomethionyl (SeMet)-substituted protein was expressed using the *E. coli* Met auxotroph B 834 system. Purification was identical to that of the native protein.

The best diffraction-quality crystals were obtained using a protein concentration of 5 mg ml⁻¹. Crystals were grown at room temperature by the hanging-drop vapour-diffusion method. The well solution contained 20% ethanol, 0.1 M Tris/HCl buffer (pH 7.4). After 1 day, the crystals grew to 0.4 × 0.3 × 0.15 mm.

The native and SeMet MAD data were collected at beamline X9A (NSLS, Brookhaven National Laboratory). All diffraction measurements were done at cryogenic temperature (100 K). The native crystals diffract to 2.8 Å (0.28 nm) resolution and 250 frames were collected using a MARCCD-165 detector at a distance of 210 mm with 1.0° oscillations. MAD data to 3.2 Å (0.32 nm) resolution were collected for wavelengths of 0.098019, 0.098000 and 0.095737 nm. For each wavelength 400 frames were collected on a MARCCD-165 detector at a distance of 250 mm with 0.5° oscillations. The native and SeMet crystals exhibited the diffraction pattern consistent with the orthorhombic space group P2₁2₁2₁ [*a* = 69.43 Å (6.943 nm), *b* = 72.09 Å (7.209 nm), *c* = 114.33 Å (11.433 nm)] with two molecules per asymmetric unit. The value of Matthews coefficient *V*_M (Matthews, 1968) is 2.62 Å³ Da⁻¹ (0.262 nm³ Da⁻¹), which

corresponds to a solvent volume fraction 51.3%. Data were integrated and scaled using DENZO and SCALEPACK, and the data statistics are reported in Table 4. Figures showing the crystal structure of PrfA were generated using PyMOL from the sequence published on the web site (Thirumuruhan *et al.*, 2003).

RESULTS

PrfAK220T substitution prevents the expression of virulence genes

Out of 26 naturally occurring low-virulence *L. monocytogenes* strains previously identified, unrelated in origin, isolation date and rDNA gene restriction patterns, we detected an A-to-C transition at the first position of *prfA* codon 220 in eight strains (Roche *et al.*, 2005). This nucleotide replacement led to a Lys220Thr substitution in PrfA (PrfAK220T). Two strains (CNL895806 and SO49) with this mutation were analysed in this study. They were avirulent according to their inability to enter cells, form plaques, or kill or colonize the spleen of subcutaneously inoculated mice (Table 1).

The direct influence of the mutation on the activity of PrfA was demonstrated by *trans* complementation of the mutated *prfA* gene into an EGD strain lacking the *prfA* gene (EGDΔ*prfA*). This transformed strain had the same low haemolytic activity as the EGDΔ*prfA* parent strain and the EGDΔ*prfA* strain carrying the pP1 vector without an insert, although PrfA production was observed by immunoblotting (data not shown). The low haemolytic activity recorded was probably due to *hly* regulation, which is in part PrfA independent (Ripio *et al.*, 1996). In contrast, the haemolytic titre of the EGDΔ*prfA* strain carrying the wild-type *prfA* gene in *trans* was eight times higher than that of the parent strain (Fig. 1). Similar results were obtained for PC-PLC activity. No activity was detected for the EGDΔ*prfA* strain or for the derivative strains carrying only the pP1 vector or the recombinant plasmid with the mutated *prfA* (Fig. 1). PC-PLC activity was only recorded with the EGDΔ*prfA* strain carrying the wild-type *prfA* gene in *trans*. Thus, our data show that introducing the gene encoding PrfAK220T into the EGDΔ*prfA* strain did not restore PC-PLC or haemolytic activities, unlike complementation with the wild-type *prfA* gene, suggesting that this substitution inactivates PrfA.

PrfAK220T is unable to induce normal *hly* transcription

To determine whether the decrease in haemolysin activity and the lack of PC-PLC activity was related to a decrease in the transcript number, we used quantitative RT-PCR to assess *hly* gene expression. To ensure detection at the putative low transcription level, we analysed the influence of mutated *prfA* on the *hly* transcription in a *L. innocua* genetic background after co-transformation with two plasmids containing a strong constitutive promoter. A *L. innocua* strain was co-transformed with the plasmid pP1 bearing the wild-type or mutated *prfA* gene of the CNL895806 strain

Table 4. X-ray data collection and refinement statistics for *prfA*

Data collection	$\lambda 0$ (native)	$\lambda 1$ (edge)	$\lambda 2$ (peak)	$\lambda 3$ (remote)
Space group	P212121	P212121	P212121	P212121
Unit cell				
a (Å)	69.430	69.978	69.836	69.908
b (Å)	72.091	71.926	72.815	71.879
c (Å)	114.335	114.769	114.627	114.676
Wavelength (Å)	0.9795	0.9795	0.9791	0.9574
Resolution range (Å)*	30–2.8 (3–2.8)	25–3.2 (3.3–3.2)	25–3.2 (3.3–3.2)	25–3.2 (3.3–3.2)
Number of reflections				
Total reflections	183 188	76 399	71 652	70 960
Unique reflections	13 304	18 367	18 337	18 188
Completeness	96.9 (100)	99.6 (99.7)	99.1 (99.7)	99.3 (98.1)
I/ σ *	32.1 (8.7)	51.3 (4.0)	46.0 (4.2)	44.4 (4.2)
R _{merge} (%)	6.3 (33.8)	2.4 (30.9)	2.4 (26.3)	2.1 (26.1)
Overall figure of merit (FOM) for MAD phasing of PrfA				
Acentric	0.36 (0.55)			
Centric	0.34 (0.55)			
Refinement				
Resolution range (Å)	20–2.8			
Reflections	14309			
R _{cryst} (%)	24.4			
R _{free} (%)†	27.8			
Wilson B-factor (Å ²)	68.0			
Overall B-factor (Å ²)	76.10			
R.m.s deviation from ideal values				
Bond lengths (Å ²)	0.007			
Bond angles (°)	1.30			

*Values in parentheses correspond to highest resolution shell.

†The R_{free} was calculated with 5 % of the data omitted from the structure refinement.

and the plasmid pHT1618 bearing the *hly* gene and its own promoter. Production of PrfA was checked by immunoblotting and similar amounts of proteins were detected when we deposited the same number of bacteria transformed with wild-type PrfA or PrfA K220T (data not shown). The K220T substitution resulted in a 10-fold reduction in *hly* gene transcripts compared to the wild-type PrfA. However, transcription of the *hly* gene was five times higher when PrfAK220T was synthesized by *L. innocua* than

when *L. innocua* harboured only the pP1 vector without *prfA*. This result shows that PrfAK220T continued to induce a low level of *hly* transcription.

Formation of homodimers by PrfAK220T protein

The low-virulence phenotype of the PrfAK220T strains could be due to the amino-acid substitution preventing dimerization of the PrfA protein. This is in line with

Fig. 1. Haemolysin and lecithinase activities of wild-type and *trans*-complemented strains. Haemolytic titres are shown in grey and lecithinase activity in black. See Methods for details of units. Means of two independent experiments are shown. The EGDΔ*prfA* strain was *trans*-complemented without plasmid (none), or with pP1 plasmid without insert (pP1), pP1 plasmid carrying the wild-type *prfA* gene (*prfA*_{EGDΔ}), or pP1 plasmid carrying the mutated *prfA* gene that encodes PrfAK220T (*prfA*_{CN1895806} and *prfA*_{SO49}).

Fig. 2. Protein-protein cross-linking. Homodimerization of PrfA was assessed without (–) or with (+) dimethylsuberimidate (DMS) for the LO28 and CDI5 strains, which express a wild-type PrfA, and for the SO49 strain, which expresses PrfAK220T. Proteins were separated by electrophoresis, and monomer and dimers of PrfA were revealed by Western blotting with an anti-PrfA serum. The positions of the two molecular-mass markers closest to PrfA are shown.

previous findings suggesting that the C-terminal region is involved in homodimerization of PrfA (Herler *et al.*, 2001). We investigated this by measuring the capacity of PrfA to form dimers with the help of dimethylsuberimidate (DMS) cross-linking. The CDI5 strain is *L. monocytogenes* LO28 with a transposon insertion in *plcA*, in which *prfA* is only expressed as monocistronic transcripts (Renzoni *et al.*, 1997). We observed (Fig. 2) that in the presence of DMS the wild-type PrfA, expressed both from the PrfA-dependent bicistronic transcript and the PrfA-independent monocistronic transcript (LO28), was able to dimerize equally well as PrfA expressed from only the PrfA-independent monocistronic transcript (CDI5) as previously described (Mengaud *et al.*, 1991). Under the same conditions, the PrfAK220T formed dimers just as well as the wild-type PrfA from the LO28 and CDI5 strains (Fig. 2).

Therefore, the K220T substitution does not alter the dimerization of PrfA.

Lack of PrfAK220T binding to the PrfA-box

Since the K220T substitution does not impair dimerization of PrfA, the low-virulence phenotype could be the result of PrfAK220T not binding to target DNA and thus not activating transcription. We measured the binding of the recombinant PrfAK220T protein to the *hly* promoter region by EMSAs, and its ability to promote transcription by *in vitro* transcription assays (Bockmann *et al.*, 2000).

The binding of the recombinant wild-type PrfA protein to the PrfA-box resulted in the formation of the so-called CIII complex (PrfA–DNA complex; Dickneite *et al.*, 1998). No PrfAK220T was detected bound to the *hly* promoter region, while wild-type PrfA bound excellently at the same concentration, showing that both PrfA had different affinity for DNA (Fig. 3a). The CIII complex shifted to the CI complex (PrfA–RNAP–DNA complex) when partially purified *L. monocytogenes* RNAP was added (Dickneite *et al.*, 1998). This shift was strong with wild-type PrfA, whereas PrfAK220T predominantly formed the CII complex (RNAP bound to the promoter) and less CI complex, the latter being approximately 15 % of the wild-type PrfA CI complex (Fig. 3b). The finding that PrfAK220T plus RNAP was able to shift to CI was somewhat unexpected, since there was no detectable CIII complex formed with PrfAK220T alone. This observation has, however, already been described (Vega *et al.*, 2004). The presence of RNAP seemed to increase and/or stabilize the binding of PrfAK220T to the *hly* promoter and led to the formation of a certain amount of a transcriptionally active complex. The *in vitro* transcription experiments with the strictly PrfA-dependent *uhpT* promoter showed that PrfAK220T continued to weakly activate transcription (Fig. 4a), to a level of about 14 % of that of wild-type PrfA after subtraction of the

Fig. 3. EMSA with ^{32}P -labelled *hly* promoter DNA fragment and purified wild-type (WT) and mutant PrfA protein K220T. (a) Formation of CIII complexes with 0, 50, 100, 200, 400 or 800 ng PrfA and target DNA. The arrowhead indicates the position of the CIII complex. (b) Formation of CI complex with partially purified RNAP (0.125 nM) and 200 ng PrfA per lane. CI, CII: positions of the various complexes formed (see text for details). Control (C) contains the ^{32}P -labelled promoter probe but no PrfA protein.

Fig. 4. *In vitro* runoff transcription assay. (a) *In vitro* transcription starting from the *uhpT* promoter with RNAP (0.45 nM) and the purified wild-type PrfA (WT) or mutated PrfA protein (K220T). Purified PrfA proteins (0, 5, 10 and 20 ng) were applied. (b) Densitometric analysis of the autoradiogram shown in (a). Transcription efficiency with 20 ng wild-type PrfA was defined as 100%. Runoff assays were carried out with wild-type PrfA (black bars) or mutated PrfA (PrfAK220T; grey bars). The experiments were repeated three times with similar results. The bars in (b) show the standard deviation of the three independent experiments.

background transcription i.e. transcription without PrfA (Fig. 4b).

Structure determination

Out of 12 selenium sites, eight were found by the SnB software. A total of eight selenium atoms were input into the MLPHRE program (Collaborative Computational Project, 1994) to refine the heavy atoms. The initial phases were calculated to a 3.2 Å (0.32 nm) resolution. The electron density map was improved by solvent flattening, two-fold non-crystallographic symmetry (NCS) averaging and phase extension to 2.8 Å (0.28 nm) using the program DM (Cowtan, 1994), allowing most α -helices and some β -strands to be identified. The PrfA protein exists as a dimer and the monomers are related by a non-crystallographic two-fold axis. The model was built by using program O and CNS (Brunger *et al.*, 1998; Jones *et al.*, 1991) including simulated annealing refinement, individual B-factor refinement and a bulk solvent correction. The model building was facilitated by using SeMet residue positions from an anomalous map as markers. Since the unit cell dimensions of the native crystal were slightly different from those of the SeMet crystal, a rigid body refinement was carried out after switching to the native dataset. Structure refinement was supported by manual model building and simulated annealing. The final model, which shows good

stereochemistry, consists of 224 residues in monomer A, 227 residues in monomer B and one ligand (glycerol) molecule in each monomer, giving an R-factor of 24.4 % with an R-free of 27.8 % at 2.8 Å (0.28 nm) resolution. The root mean square (r.m.s.) deviations from ideality are 0.007 Å (0.0007 nm) for bond lengths and 1.3 Å (0.13 nm) for bond angles. The high mean B-factor for the model [76.10 Å² (7.610 nm²)] is reflected in the high Wilson B-factor [68.0 Å² (6.8 nm²)]. Residues 1–4 and 175–183 of monomer A and 1–4 and 177–182 of monomer B are not seen in the electron density due to conformational disorder. Ramachandran analysis using the program PROCHECK (Laskowski *et al.*, 1996) indicated that 85.8 % of the residues were in the most favoured region, 1 % within the generously allowed region, and no residues were in the disallowed region.

Crystal structure of PrfA

As can be seen from Figs. 5 and 6(a), the PrfA crystal structure obtained here is closely similar to that presented by Eiting *et al.* (2004, 2005). The C-terminal domain of PrfA (residues 138–237) consists of four anti-parallel β -strands and six α -helices. It is linked to the N-terminal domain by a long helix α C (residues 109–137). The N-terminal domain (residues 1–108) consists of an eight-stranded anti-parallel β -barrel and two α -helices. Two monomers of the current PrfA dimer are connected by a local twofold axis with an angle of 178.9° between monomers. Two monomers of the dimer are very similar and can be superimposed with a r.m.s. deviation of 0.47 Å (0.047 nm) over 169 α -carbon pairs. Maximal deviations were found for the tips of two β -hairpins β 4– β 5 and β 9– β 10 and for helices E and F. The loop between helices E and F is disordered in both monomers and is not included in the final model. The glycerol molecule from the cryoprotectant solution is bound in the cavity between loop β 2– β 3 and β -strand β 7 in both PrfA monomers. The overall structure of PrfA is similar to that of catabolite activator protein (CAP; Schultz *et al.*, 1991) also named cAMP receptor protein (Crp; Harman, 2001), but Crp does not have the three C-terminal α -helices α G, α H and α I that are present in the PrfA structure. Despite the general similarity between the PrfA and Crp structures they have essential differences in mutual orientation of monomers inside the dimers and mutual orientation of domains inside the monomers. The superpositions of PrfA dimer and Crp dimer from the complex with cAMP and DNA (PDB ID 1CGP) show r.m.s. deviations of 2.7 Å (0.27 nm) over 374 equivalent α -carbon pairs. The superposition of corresponding monomers gives a r.m.s. deviation of 2.6 Å (0.26 nm) over 186 α -carbon pairs. The superpositions of individual N- and C-terminal domains of PrfA and Crp structures lead to r.m.s. deviations of 2.0 Å (0.2 nm) and 2.5 Å (0.25 nm) over 99 and 59 α -carbon pairs respectively. So the N-terminal domains inside the monomers are the most similar parts of the PrfA and Crp structures. The relative orientation of α -helices in the C-terminal domains of PrfA and Crp is slightly different, including helices E and

10MI

2BEO

Fig. 5. Secondary structure comparison between two PrfA files published in the RCSB protein database: 10MI (Thirumuruhan *et al.*, 2003) and 2BEO (Eiting *et al.*, 2004). extended strand; turn; disulfide bond; alpha helix; 310 helix; pi helix. Greyed-out residues have no structural information.

F, which form the DNA-binding HTH motif in both transcriptional factors. The formation of the dimer in PrfA is supported by multiple monomer–monomer interactions. The side chains of Lys220 and Glu223 located in helix α H of the C-terminal domain form hydrogen bonds with main chain atoms of Tyr82 from the N-terminal domain of another monomer (Fig. 6b). The side chain of Lys220 also forms hydrogen bonds with the side chain of Ser50 from the N-terminal domain of the neighbouring monomer. The residues Phe128 and Phe131 from helix α C of one monomer form hydrophobic contacts with the N-terminal domain residues of another monomer Tyr83 (loop β 6– β 7) and Leu60 (β 5) respectively. The main contribution to PrfA dimer stabilization is represented by hydrophobic interactions between residues of long helix α C of one monomer and identical residues of the same helix of another monomer. These helices are connected by a local two-fold axis and have

a lot of interacting hydrophobic residues at the dimer interface: Leu110, Phe113, Phe114, Phe117, Leu120, Val124, Phe131 and Phe134.

DISCUSSION

Evidence that PrfA can exist in two functional states, weakly and highly active, was provided by the identification of a spontaneous mutant exhibiting the constitutively over expressed PrfA regulon (Ripio *et al.*, 1997). Other induced mutations have furthered our understanding of the amino acids involved in the highly active conformation and the domains involved in PrfA activity (Vega *et al.*, 2004). We have described a new spontaneous mutation that inactivates PrfA and which is observed in *L. monocytogenes* field strains. This mutation seems to be important for *L. monocytogenes* survival, because eight out of 26 low-virulence strains

Fig. 6. Schematic representation of PrfA. All representations were performed with PYMOL v0.99. (a) Structure of the wild-type PrfA dimer. Each monomer is colour-coded differently in blue or green: N-terminal domain (Nter) in pale colour, dimerization-helix (DH) in medium colour and C-terminal domain (Cter) in dark colour. Lysine 220 is in red and DNA helices are orange. The probable DNA position was determined after superposition of the current PrfA structure (PDB ID 1OMI) and the structure of the CAP complex with cAMP and DNA (PDB ID 1CGP). (b) Schematic representation of interactions between lysine 220 and serine 50 and tyrosine 82. Dotted lines denote hydrogen bonds.

analysed exhibited this lysine substitution by a threonine at position 220 (Roche *et al.*, 2005). This widespread mutation could reflect an evolution of *L. monocytogenes* strains as observed with *L. innocua*, which has lost the virulence gene cluster (Vazquez-Boland *et al.*, 2001).

The role of this substitution in the inactivation of PrfA has previously been demonstrated by complementation of the low-virulence strains having this mutation with PrfA from the virulent EGDe strain; this trans-complementation restored the ability of the low-virulence strains to form plaques and to infect mice (Roche *et al.*, 2005). Moreover, we have shown that PrfAK220T was inactive because it was unable to restore haemolysin and PC-PLC activities in the EGDe strain lacking the *prfA* gene, unlike insertion of the wild-type *prfA*. The lack of virulence protein expression was related to a decrease in gene transcripts, as observed by quantitative RT-PCR. However, this substitution still allowed expression of a low level of virulence gene transcripts, as observed in the *in vitro* runoff transcription assay and quantitative RT-PCR in a *L. innocua* background.

Our data support the hypothesis that Lys220 does not affect PrfA dimerization, as the PrfAK220T protein is dimerized just as well as its wild-type counterpart, but it could affect DNA binding. Indeed, by EMSA no PrfAK220T–DNA complex (CIII complex) could be detected. However, although we detected no interaction between PrfAK220T and PrfA-boxes, some CI complexes (PrfAK220T–RNAP–DNA complexes) were formed by adding RNAP. The low number of CI complexes continued to be able to induce transcription as observed by RT-PCR and *in vitro* runoff

transcription assays, but was unable to induce protein expression, as observed by Western blotting or lack of phospholipase activity (Roche *et al.*, 2005). The low transcription level could be due to an initial interaction between PrfAK220T and the RNAP, followed by the binding to DNA of the complex formed. This result suggested that RNAP can increase the binding of at least the mutant PrfA to the promoter. This hypothesis is strengthened by the fact that Crp, a transcription regulator of *E. coli* whose sequence is homologous to that of PrfA, interacted with RNAP in solution in the absence of promoter DNA (Harman, 2001). The domain involved in binding PrfAK220T to RNAP should be preserved, since the PrfA RNAP binding domains are present in the N-terminal region of PrfA (Herler *et al.*, 2001).

Overall, the PrfAK220T substitution did not modify the homodimerization of PrfA, but prevented PrfA from binding to DNA. However, the crystal structure of PrfA did not support direct interaction between DNA and Lys220. When we superposed the wild-type PrfA structure and the structure of the Crp–DNA complex, we concluded that Lys220 is not in contact with DNA. It is the HTH motif present at positions 168–196 which is involved in DNA binding through two α -helices (residues 169–174 and 184–195), and the connecting loop (174–184) (Eiting *et al.*, 2005; Sheehan *et al.*, 1996). The three α -helices of the C-terminal region which are not conserved in Crp protein were involved in stabilization of the DNA binding site (Fig 6a). Substituting Lys220 by Thr leads to the breaking of important bonds between the C-terminal domain of one monomer and the N-terminal domain of

the second monomer. This break could affect the relative orientation of the C-terminal domain, causing the negative repositioning of the DNA-binding α -helices.

As can be seen from Fig. 6(b), Lys220 has a very important position in helix α H of the C-terminal domain. Its side chain forms hydrogen bonds to Ser50 OG (β 4) and Tyr82 CO (β 7) of the N-terminal domain of the adjacent monomer. Lys220 therefore plays a key role in stabilizing the monomer–monomer interface. When Lys220 was replaced by Thr, the hydrogen bonds mentioned above were broken. Model building showed that new inter-monomer hydrogen bonds, including Thr-OG1, were impossible. This weakening of monomer–monomer interaction could lead to some changes in relative orientation of C- and N-terminal domains, and to possible inactivation of the HTH motif. The N-terminal domain is indeed susceptible to conformational changes that modify the positioning of the HTH motif (Vega *et al.*, 2004). The ability to form dimers remains after the breaking of hydrogen bonds between residue 220 and residues from the N-terminal domain of the neighbouring monomer because the remaining interactions between monomers are still in place.

Overall, our results strengthen the minimal model previously described by Vega *et al.* (2004) in which PrfA-dependent expression is controlled at three hierarchical levels: (1) PrfA activity, via allosteric changes in the protein, as observed with the positive mutations in α D (G145S and L140F) and the negative mutations in α H (K220T); (2) PrfA concentration, which depends on the autoregulation of the *prfA* gene; (3) *cis*-acting control of the interaction of PrfA and RNAP with DNA, where RNAP could stabilize a weakly active conformation.

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