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A Gly145Ser Substitution in the Transcriptional Activator PrfA Causes Constitutive Overexpression of Virulence Factors in *Listeria monocytogenes*

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Virulence genes in *Listeria monocytogenes* are coordinately expressed under the control of the transcriptional activator PrfA, encoded by *prfA*, a member of the cyclic AMP (cAMP) receptor protein (CRP)/FNR family of bacterial regulators. Strain P14-A is a spontaneous mutant of *L. monocytogenes* serovar 4b which produces elevated levels of virulence factors (M. T. Ripio, G. Domínguez-Bernal, M. Suárez, K. Brehm, P. Berche, and J. A. Vázquez-Boland, Res. Microbiol. 147:371–384, 1996). Here we report that P14-A and other variant strains with the same phenotype carry a point mutation in codon 145 of *prfA*, leading to a Gly→Ser substitution. *trans*-complementation experiments with PrfA-deficient mutants demonstrated that the Gly145Ser *prfA* allele causes overexpression of virulence factors in *L. monocytogenes*, to the levels found in the virulence factor-overexpressing variants. In strain P14-A with a chromosomal Gly145Ser *prfA* background, transcription of *prfA* and of PrfA-dependent virulence genes remained constitutively high under culture conditions in which virulence factor expression is downregulated in wild-type *L. monocytogenes*. The Gly145Ser substitution is located in a PrfA stretch (residues 141 to 151) showing high sequence similarity to the D α-helix of CRP. Interestingly, well-characterized *crp mutations, which make CRP functionally active in the absence of cAMP, map in this region (i.e., Gly141Ser and Ala144Thr substitutions). By analogy with the CRP model, the phenotype conferred to *L. monocytogenes* by the Gly145Ser substitution in PrfA could be due to the mutant regulatory protein being locked in a transcriptionally active, cofactor-independent conformational state. Our observations allow the construction of a model for PrfA-dependent virulence gene regulation in which the levels of virulence factor expression depend primarily on the conformational state of the PrfA protein, which alternates between active and inactive forms according to its interaction with an environmentally regulated signal molecule or cofactor.**

The gram-positive, facultative intracellular pathogen *Listeria monocytogenes* is the causal agent of listeriosis, a severe foodborne, opportunistic infection of humans and animals characterized by meningoencephalitis, abortion, and septicemia (11). Known listerial virulence factors are chromosomally determined, and most of the corresponding genes form a cluster encompassing the *hly* locus (34). *hly* is monocistronically transcribed and encodes listeriolysin O (LLO), a cholesterol-binding, SH-activated toxin which confers a hemolytic phenotype to *Listeria* colonies (9, 29). Downstream from *hly* is the so-called lecithinase operon, which comprises the genes *mpl*, *actA*, and *plcB*, in that order. Their products are, respectively, Mpl, a metalloprotease which cleaves the *plcB* product to its active form; ActA, a surface protein which mediates actin-based bacterial motility within infected host cells; and PlcB, a wide-range phospholipase C (PLC), or lecithinase. These proteins are produced from a single mRNA driven by the *mpl* promoter or from shorter transcripts, one of which starts at a promoter in front of *actA* and covers *actA* and *plcB* (30, 46). Upstream, and transcribed divergently from *hly*, is the *plcA-prfA* operon, which encodes a phosphatidylinositol-specific PLC, and PrfA, a transcriptional activator. The PrfA protein exerts tight control over all of the above-described transcriptional units, and its presence is absolutely necessary for virulence expression (8, 26, 30). Elsewhere on the chromosome is the *inlA-inlB* operon, coding for the surface proteins InlA (or internalin) and InlB, which are involved in invasion and cell tropism (15). This operon is partially regulated by PrfA (3, 28, 41; see references 24, 34, and 39 for reviews on molecular determinants and cell biology of *Listeria* pathogenesis).

PrfA is the only virulence regulator identified so far in *L. monocytogenes*. It has overall sequence similarity with the cyclic AMP (cAMP) receptor protein (CRP) and other members of the CRP family of transcriptional regulators (22, 25). The structural similarities between the listerial regulator and CRP appear to be functionally significant, since, as shown recently (40), single substitutions in the C-terminal DNA-binding helix-turn-helix (HTH) motif of PrfA, affecting residues predicted to be involved in DNA contact, alter DNA binding and virulence gene activation. Like CRP-regulated elements, all PrfA-controlled transcriptional units are preceded by conserved DNA sequences with dyad symmetry. These palindromic structures, called PrfA boxes, are 14 bp long and are centered near position −41. There is experimental evidence that PrfA binds to them. PrfA-regulated genes are differentially regulated, possibly due to sequence variability within PrfA boxes: promoters with perfectly symmetrical palindromes (i.e., P*hly* and P*plcA*) are more efficiently transcribed than those having nucleotide mismatches (i.e., P*mpl*, P*actA*, and P*inlA*) (3, 12–14, 28, 41). PrfA-dependent virulence gene expression is thermoregulated (10, 27). Transcription is maximal at mammalian body temperatures, suggesting that *L. monocytogenes* may use this environmental variable to recognize its transition from free to parasitic life. The PrfA-dependent promoter of the *plcA-prfA* operon, which is responsible for the PrfA transcriptional amplification

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source and/or reference(s)
L. monocytogenes		
PrfA-proficient strains		
P14	Type 1, genetic group I (sv 4b), human clinical isolate	36, 45
P14-A	Type 2, spontaneous derivative from strain P14	36
L028[b]	Type 3, genetic group II (sv 1/2c), laboratory strain, originally a human isolate	P. Cossart (36, 47)
EGD	Type 1, genetic group II (sv 1/2a), laboratory strain, presumably one of the first isolates of *L. monocytogenes* (E.G.D. Murray, 1924)	T. Chakraborty (36)
EGD-A	Type 2, genetic group II (sv 1/2a), laboratory strain, presumably an EGD derivative (identical isoenzyme types [37]); also known as EGD-Mackaness	P. Berche (36)
NCTC 7973	Type 2, genetic group II (sv 1/2a), laboratory strain	Collection (36)
CLIP 545	Type 2, genetic group I (sv 4b)	Collection (36)
P7	Type 1, genetic group I (sv 4b), animal clinical isolate	44
CO244	Type 2, genetic group II (sv 3a), food isolate	This work
PrfA-deficient mutants		
MS5	P14-A derivative, Tn*917* insertion 4 bp upstream from P2*prfA*	This work (42)
OD43	L028 derivative, Tn*917-lac* insertion 4 bp upstream from P1*prfA*	P. Cossart (30)
Δ*prfA* mutant	*prfA* deletion mutant from L028	P. Cossart (41)
E. coli		
DH5α	Cloning host strain	18
MOS*Blue*	Cloning host strain	Amersham
Plasmids		
pKK3535	*rrnB* ribosomal RNA operon of *E. coli*	6
pMOS*Blue*	T-vector for cloning of PCR products	Amersham
pMK4	*E. coli-Bacillus*/*Listeria* shuttle vector	43
pTV1	Thermosensitive delivery vector for Tn*917*	49
pRWT	pMK4 plus wild-type *prfA* from P14, monocistronic construct	This work
pRWT-b	pMK4 plus wild-type *prfA* from P14, *plcA-prfA* bicistronic construct	This work
pRG145S	pMK4 plus Gly145Ser mutant *prfA* allele from P14-A, monocistronic construct	This work
pRG145S-b	pMK4 plus Gly145Ser mutant *prfA* from P14-A, *plcA-prfA* bicistronic construct	This work

[a] Types: 1, wild type; 2, hyperhemolytic/hyperlecithinase variant; 3, moderately hyperhemolytic/hyperlecithinase variant. Genetic groups I and II, two serovar-related groups into which *L. monocytogenes* strains can be divided according to *prfA* nucleotide sequence (see text and Table 2). sv, serovar.
[b] Although displaying a variant hemolysin/lecithinase phenotype (type 3), strain L028 has a wild-type *prfA* sequence.

loop, appears to play a key role in this thermoregulation (27; see references 5 and 23 for recent reviews on virulence gene regulation in *Listeria*).

By examining a wide panel of *L. monocytogenes* strains for LLO and PLC production as markers for virulence factor expression, we recently established that wild-type strains, when growing at 37°C in a rich medium such as brain heart infusion (BHI), express virulence factors very weakly. This phenotype was designated type 1 (36). We have also shown that these type 1 strains can substantially (10- to 100-fold) increase *prfA* and PrfA-dependent virulence gene expression in charcoal-treated BHI (36). This demonstrated that in addition to temperature, there is at least one other environmental factor, in this case in the medium composition, which is critical for full expression of PrfA-controlled genes in *L. monocytogenes*. In the same study, we identified several strains with a variant phenotype, or type 2, characterized by constitutively high, charcoal-unresponsive levels of LLO and PLC activities in BHI. Interestingly, all of these hyperhemolytic and hyperlecithinase variants were strains that had been kept under laboratory conditions for a long time, with some of them being well-known collection widely used in research on listerial virulence (e.g., NCTC 7973 and EGD Mackaness [or EGD-A, as it has been redesignated by us to avoid confusion with wild-type EGD]) (36) (Table 1). In this work we characterized the genetic basis of the type 2 hyperhemolytic/hyperlecithinase variant phenotype of *L. monocytogenes*. We report that a single amino acid substitution in PrfA, in a position which in the homologous protein CRP leads to a cofactor-independent, transcriptionally active conformation, is sufficient to cause constitutive overexpression of virulence factors in *L. monocytogenes*. Our data strongly support the hypothesis that PrfA, like CRP, may reversibly shift from transcriptionally inactive to active forms upon interaction with a cofactor or signal molecule, in response to environmental signals.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacteria and plasmids used in this work are shown in Table 1. *L. monocytogenes* and *Escherichia coli* strains were cultured in BHI and Luria-Bertani media, respectively, with antibiotics whenever appropriate.

DNA methods. Restriction and modification enzymes were purchased from Pharmacia and used as prescribed by the manufacturer. The Expand High Fidelity PCR system (Boehringer Mannheim) was used to amplify specific DNA segments. PCR products were purified from gels with the Qiaquick (Qiagen) Gel Extraction kit. Plasmid DNA was extracted from *E. coli* with the Plasmid Purification kit from Qiagen. Chromosomal and plasmid DNAs from *L. monocytogenes* were extracted by using the cetyltrimethylammonium bromide and alkaline lysis miniprep protocols (1) after pretreatment of bacterial cells with lysozyme (10 to 20 mg/ml) for 1 h at 37°C in glucose-Tris-EDTA or Tris-EDTA-sucrose, respectively. DNA sequencing was performed on both strands of plasmid DNA with an Applied Biosystems 377 apparatus. DNA probes consisted of PCR-amplified fragments internal to the genes of interest; they were radiolabeled by nick translation (Boehringer Mannheim kit) with [α-^{32}P]dCTP (Amersham).

***prfA* constructs.** *prfA* was amplified from *L. monocytogenes* chromosomal DNA by PCR, either alone or together with *plcA*, using the oligonucleotide pairs MR2 (5′-CAACTAACATATATTATTCCT-3′)-MR10 (5′-CTTGGTGAAGCAATC GTACGC-3′) or MR9 (5′-TTCGCTTCTAAAGATGAAACG-3′) -MR10, respectively. The *hly-plcA* intergenic region was PCR amplified with primers MR3 (5′TCTTATACAAAATGGCCC-3′) and MR4 (5′-TTTCATGGGTTTCACTC

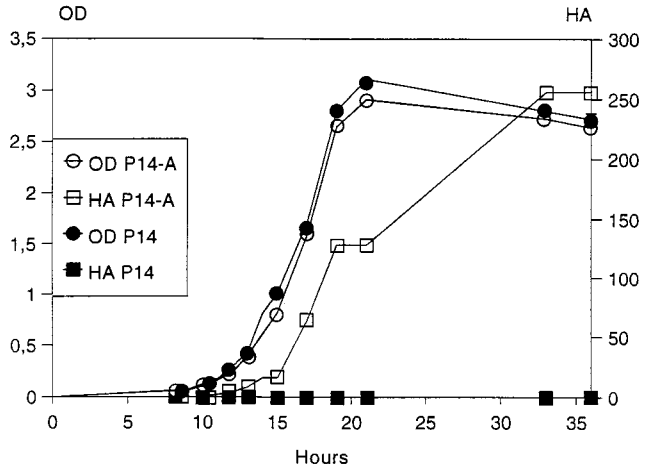

FIG. 1. Growth curves (optical density at 578 nm [OD]) of and production of hemolytic activity (HA) (in complete hemolytic units [36]) by the wild-type strain P14 and the type 2 hyperhemolytic mutant P14-A at 20°C in BHI. Note that P14-A produces high levels of hemolytic activity at 20°C (the same as at 37°C [36]), whereas hemolysin production is abolished in wild-type strain P14 at the same temperature.

T-3′), corresponding to the 5′ ends of *plcA* and *hly*, respectively. PCR products were cloned in *E. coli* by using the pMOS*Blue* T-vector kit (Amersham). For complementation experiments with *L. monocytogenes*, *prfA*-containing inserts in pMOS*Blue* were transferred to the *E. coli-Bacillus*/*Listeria* shuttle vector pMK4 (43), using *Sal*I and *Sma*I restriction sites, giving rise to the plasmids described in Table 1. *prfA* constructs in pMK4 were introduced into *L. monocytogenes* by electroporation as described by Park and Stewart (32). Transformants were isolated in BHI with 7 μg of chloramphenicol per ml and then routinely subcultured in the same medium with a higher antibiotic concentration (10 μg/ml).

Hemolytic and lecithinase activity determinations. Hemolytic and lecithinase activities of *L. monocytogenes* were determined in supernatants of 15-h cultures as previously described (36).

RNA extraction and transcription analysis. Total RNA from *L. monocytogenes* was extracted from 100 ml of late-log-phase cultures, and semiquantitative transcription analysis was performed by a slot blot technique, as described elsewhere (36).

RESULTS AND DISCUSSION

The *L. monocytogenes* hyperhemolytic variant strain P14-A carries a constitutive mutation in the virulence regulon. The *L. monocytogenes* type 2 variant strain P14-A spontaneously diverged from P14, a wild-type clinical isolate of serovar 4b (36, 45). The mutant P14-A secretes significantly higher levels of LLO and PLC into the culture supernatant than its isogenic parent strain. This is accompanied by increased levels of the *hly*, *plcB*, and *prfA* mRNAs (36). Unlike wild-type P14, P14-A does not respond, or at most responds very poorly, to charcoal in the medium, with its already high virulence gene expression levels remaining virtually unchanged in charcoal-treated BHI (36). In addition, culture in the presence of the β-glucoside cellobiose, a condition that has been reported to repress *hly* and *plcA* expression in *L. monocytogenes* (31), does not substantially affect hemolysin expression in P14-A (36). These observations suggest that *L. monocytogenes* P14-A is a spontaneous mutant expressing constitutively high levels of virulence factors.

A major environmental factor that controls virulence gene expression in *L. monocytogenes* is temperature: PrfA-mediated transcription is activated at temperatures of >30°C and is shut off at around 20°C or below (27). We therefore studied hemolysin expression in P14 and in P14-A at 20 and 37°C. In P14, hemolysin production was repressed at 20°C. P14-A, in contrast, secreted equally high levels of hemolytic activity at both temperatures (Fig. 1), indicating that *hly* expression is not thermoregulated in this mutant strain.

To characterize further the regulatory defect in P14-A, we comparatively analyzed transcription of *prfA* and PrfA-dependent virulence genes in P14 and P14-A at 20 and 37°C (Fig. 2). L028, a laboratory strain widely used in research on *L. monocytogenes* virulence (see, e.g., references 9, 30, 38, 40, and 46) (Table 1), was also included in the experiments. As previously reported (36), L028 represents a third class of *L. monocytogenes* variant (type 3), which expresses intermediate levels of hemolysin and lecithinase activities in BHI (Table 1). Unlike type 2 variants, L028 is able to respond to charcoal by enhanced *hly* and, particularly, *plcB* expression (36).

In P14-A, the same high levels of transcription were observed for the genes of the virulence cluster at each growth temperature, confirming that its virulence determinants are not subject to thermoregulation (Fig. 2). *prfA* mRNA levels were identically high at both temperatures, and transcription of *plcA* was similarly abundant, suggesting that the P*plcA*-mediated PrfA amplification loop is fully functional in P14-A independently of the temperature. In contrast, transcription of

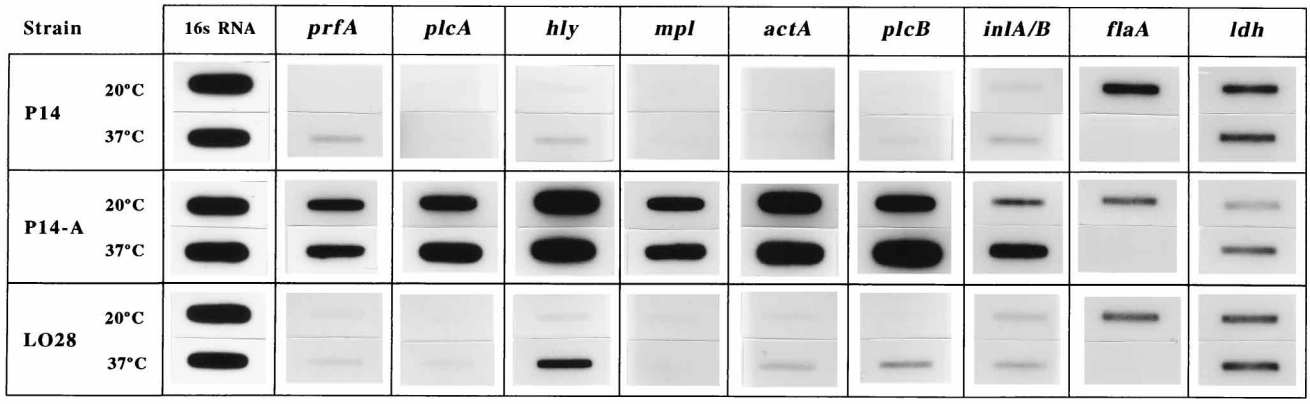

FIG. 2. Transcriptional analysis of the genes of the virulence cluster (*prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*) and the *inlAB* locus in the Gly145Ser mutant P14-A, in its wild-type parent strain P14, and in L028 grown at 20 and 37°C. For controls, transcription levels of the housekeeping gene *ldh* (46) and the thermoregulated flagellin subunit gene *flaA* (which is preferentially expressed at 20°C and repressed at 37°C) (38) were also determined. The plasmid pKK3535, containing the *rrnB* ribosomal RNA operon of *E. coli* (6), was used as a reference for the amount of total RNA present in the blots. The same results as with P14 and P14-A were obtained with other type 1 (wild-type) and type 2 (hyperhemolytic mutant) *L. monocytogenes* strains.

prfA and the other genes of the virulence cluster was almost completely abolished in both P14 and L028 at 20°C. At 37°C, these two strains gave different patterns, consistent with their different phenotypes in BHI (36): in P14, mRNA levels for *prfA* and *hly* were slightly increased, whereas transcripts for the other genes of the virulence cluster could not be detected; in L028, significantly higher levels of transcription were observed for *actA* and, particularly, *plcB* and *hly*, but *prfA* transcription was as weak as at 20°C, clearly below that in P14. In P14 and L028 cultured at 37°C in charcoal-treated BHI, transcription of virulence genes was comparable to that in P14-A (not shown).

Transcription of the *inlAB* operon was also investigated (Fig. 2). In strains P14 and L028, *inlAB* transcription was detected at 20°C and was only slightly higher at 37°C. In P14-A, transcription levels were significantly higher than in P14 and L028 at both temperatures. However, and in contrast to the case for genes of the virulence cluster, transcription of *inlAB* in P14-A was thermoregulated, with markedly higher levels at 37°C (Fig. 2). These results are consistent with previous reports indicating that expression of the *inlAB* locus is only partially dependent on PrfA (3, 28, 41).

In conclusion, the virulence cluster appears to be constantly overexpressed in P14-A, presumably because this mutant strain has lost the capacity to downregulate PrfA-dependent virulence genes in response to repressor signals from the environment.

A point mutation in codon 145 of *prfA* is present in all type 2 hyperhemolytic variants of *L. monocytogenes*. The phenotype of P14-A could be due to an alteration in PrfA which increases its activity and/or renders it (or its expression) insensitive to environmental signaling. To test this hypothesis, we PCR amplified, cloned, and sequenced *prfA* and its promoter regions (i.e., the *plcA-prfA* and *plcA-hly* intergenic regions) from P14 and P14-A. DNA sequences were identical in both strains except for a G-to-A transition at the first position of triplet 145 of P14-A (GGT in P14 versus AGT in P14-A). This nucleotide replacement leads to a Gly145Ser substitution in PrfA. The deduced peptide sequence for P14 was identical to that previously reported (30) for strain L028.

To assess the significance of the mutation, we also determined the *prfA* sequences in several other type 2 hyperhemolytic/hyperlecithinase variants of *L. monocytogenes* (strains EGD-A, NCTC 7973, CO244, and CLIP 545) and in two additional wild-type (type 1) strains (EGD, which appeared to be isogenic with the variant strain EGD-A as determined by isoenzyme typing [37] , and P7) (Table 1). The deduced PrfA sequences of wild-type strains were identical to that of P14, and all type 2 variant strains had the Gly145Ser substitution.

In strains EGD-A, NCTC 7973, and CO244, an additional Cys229Tyr replacement (triplet TGT changed to TAT) was present. Interestingly, these strains belong to one of the two groups into which the *L. monocytogenes* strains studied could be divided according to their *prfA* sequences. Group I contained strains P14, P14-A, CLIP 545, and P7, all of serovar 4b. Group II contained strains EGD, EGD-A, and NCTC 7973, of serovar 1/2a; CO244, of serovar 3a; and L028, of serovar 1/2c. The two groups differed from each other by conservative changes at the third positions of 16 codons (Table 2). This strain grouping and the corresponding serovar distribution are in perfect agreement with previous findings, based on multilocus enzyme electrophoresis (2, 33) and on variability of *hly* sequences (35), which demonstrated that the bacterial populations within the species *L. monocytogenes* belong to two genetically distinct, serovar-correlated evolutionary branches. Our *prfA* sequence-based groups I and II correspond, respectively, to the phylogenetic divisions I and II of *L. monocytogenes* described by Piffaretti et al. (2, 33).

TABLE 2. Differences (conservative changes) in *prfA* sequence between genetic groups I (serovar 4b) and II (serovars 1/2a, 1/2c, and 3a) of *L. monocytogenes*[a]

Codon no.	Amino acid	Codon in group:	
		I	II
40	Phe	TTC	TTT
45	Ile	ATT	ATC
48	Leu	CTT[b]	CTC
49	Thr	ACA	ACG
63	Tyr	TAT	TAC
65	Gly	GGT	GGG
74	Ile	ATC	ATT
90	Glu	GAA	GAG
108	Lys	AAG	AAA
127	Ser	AGC	AGT
129	Ala	GCC	GCT
140	Leu	CTC	CTT[b]
175	Gly	GGC	GGA
178	Ser	AGC	AGT
191	Ser	TCT	TCC
218	Ala	GCT	GCC

[a] The *prfA* sequence-based genetic groups I and II correspond, respectively, to the serovar-related phylogenetic divisions I and II previously described by Piffaretti et al. (33) on the basis of cluster analysis of isoenzyme types of *L. monocytogenes*.

[b] Nucleotide change that creates a *Hin*dIII site. There is a single *Hin*dIII site in *prfA*, at positions 412 to 417 and 688 to 693 in group I and group II strains, respectively (position numbering corresponds to that of the nucleotide sequence of the *prfA* gene region in reference 30).

In conclusion, (i) all of the type 2 hyperhemolytic/hyperlecithinase variants examined had a point mutation at codon 145 of *prfA*, leading to a Gly→Ser replacement in PrfA, and (ii) the Cys229Tyr substitution was present only in a subgroup of variant strains. Therefore, it might be that the Gly145Ser substitution alone is sufficient to determine virulence factor overexpression in *L. monocytogenes*.

The Gly145Ser substitution in PrfA causes virulence factor overexpression in *L. monocytogenes*. *prfA* in *trans* on a multicopy plasmid can complement an *L. monocytogenes* chromosomal *prfA* mutant (13, 26, 30). To determine whether the Gly145Ser substitution affected PrfA-mediated virulence gene expression in *L. monocytogenes*, we inserted the wild-type and mutant *prfA* alleles from strains P14 and P14-A, respectively, into the *E. coli-Bacillus*/*Listeria* shuttle vector pMK4 (43) and introduced the resulting plasmids into a PrfA-deficient background. *prfA* can be transcribed either monocistronically, from at least two promoters in the *plcA-prfA* intergenic region, or as a bicistronic transcript comprising *plcA* and *prfA*, driven by the *plcA* promoter. P*plcA* is PrfA dependent so that, by means of the *plcA-prfA* bicistronic mRNA, PrfA autoinduces its own synthesis (Fig. 3). Full activation of virulence genes is dependent on the presumably high levels of PrfA generated through this transcriptional amplification loop (7, 13, 14, 30). Therefore, constructs which contained *prfA* alone or as the *prfA-plcA* bicistron were prepared. These constructs contained all of the transcription signals for *prfA*, including the PrfA box in front of P*plcA* (Fig. 3).

Two PrfA-deficient transposon mutants, MS5 from P14-A (*L. monocytogenes* genetic group I) and OD43 from L028 (genetic group II), were transformed with the above-described constructs. MS5 was obtained by Tn*917* mutagenesis with the thermosensitive plasmid pTV1 (49) after selection for virulence-related phenotype deficiency (42). The transposon in-

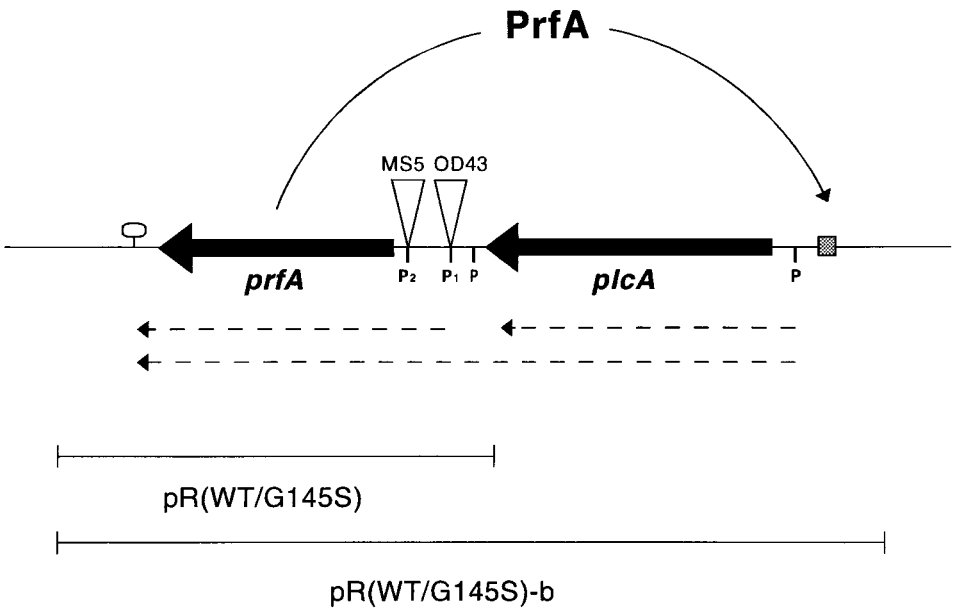

FIG. 3. Physical and transcriptional organization of the *plcA-prfA* operon of *L. monocytogenes* and locations of transposon insertions (triangles) in the PrfA mutants MS5 and OD43. Known promoters are indicated (P); their designations correspond to those previously published (7, 13, 14, 30), except for the P promoter in the *plcA-prfA* intergenic region, which was designated P2 by Mengaud et al. (30). The PrfA box in front of *plcA*, responsible for the PrfA transcriptional autoamplification loop, is indicated by a gray square. Mono- and bicistronic transcripts are shown as dotted arrows; the segments below represent the DNA fragments inserted into pMK4 to give rise to the monocistronic (plasmid pRWT or pRG145S) or bicistronic (pRWT-b or pRG145S-b) *prfA* constructs (Table 1). The small loop downstream from *prfA* represents the terminator which is present at the 3′ end of the gene.

serted in this mutant 4 bp upstream from the P2*prfA* transcription start point and consequently abolished transcription from any of the known monocistronic *prfA* promoters (Fig. 3). OD43 has been described elsewhere (21, 30) and has a Tn*917-lac* insertion 4 bp upstream from the P1*prfA* start point (Fig. 3). A Δ*prfA* mutant from L028 (41) was also used (Table 1), as a control. Hemolytic and lecithinase activities, whose respective genetic determinants *hly* and *plcB* belong to two independent transcriptional units of the virulence regulon, were used as markers to monitor PrfA-dependent virulence gene expression (36) in the complementation experiments.

Complementation of PrfA-deficient mutants with the wild-type monocistronic *prfA* construct (plasmid pRWT) in multicopy had no effect on (MS5) or only caused a small increase in (OD43 and the Δ*prfA* mutant) hemolysin and lecithinase activities. In contrast, complementation with the monocistronic Gly145Ser mutant *prfA* allele (plasmid pRG145S) resulted in a pronounced enhancement of hemolysin (4- to 100-fold increase) and, particularly, lecithinase (170- to 500-fold) activities (Table 3). The expression levels in the PrfA mutants complemented with pRG145S were similar to those in P14-A (Table 3) and in wild-type strains (e.g., P14) grown in charcoal-treated BHI (reference 36 and results not shown).

The bicistronic construct of wild-type *prfA* (plasmid pRWT-b), with a functional PrfA autoamplification loop, was, as expected, significantly more efficient than the corresponding monocistronic construct at inducing *hly* and *plcB* expression (Table 3). Presumably, therefore, higher levels of PrfA are indeed produced by the bicistronic constructs. Complementation with pRG145S-b and pRWT-b had a similar effect on hemolysin production, whatever the *prfA* allele introduced: with both plasmids, almost full activation of the PrfA-dependent *hly* promoter was attained, since the levels of expression reached were comparable to those in P14-A and in PrfA mutants complemented with pRG145S (Table 3). Similar results were obtained for *plcB* expression, although in this case pRWT-b was significantly (10 times) less efficient in activating *plcB* expression in MS5 and OD43 compared to pRG145S-b (Table 3).

Overall, these results indicate (i) that the Gly145Ser substitution in PrfA causes overexpression of PrfA-dependent virulence factors in *L. monocytogenes*, to levels that are comparable to the constitutive levels in the type 2 hyperhemolytic/hyperlecithinase mutants; (ii) that high levels of expression of the PrfA-dependent virulence genes are attained either with elevated levels of wild-type PrfA or with low levels of the Gly145Ser mutant PrfA; and (iii) that the PrfA-dependent promoters from which *hly* and *plcB* are expressed are differently activated.

Indeed, the differences were more clear-cut when PrfA-dependent virulence gene activation was monitored by assaying lecithinase activity (Table 3). Thus, promoters directing *plcB* expression (i.e., P*mpl* and P*actA*) appear to be more stringent and are fully transcribed only above a certain, relatively elevated PrfA-mediated activation threshold. This is in agreement with the notion that PrfA-dependent promoters are differentially activated (3, 13, 15, 41), with high-affinity promoters (i.e., P*hly* and P*plcA*) being fully stimulated at PrfA concentrations lower than those required by low-affinity promoters (i.e., P*mpl* and P*actA*). Our findings suggest that the Gly145Ser substitution lowers the PrfA-dependent activation threshold of *hly* and, especially, *plcB*, rendering in addition the PrfA regulatory system insensitive to environmental signals that modulate virulence gene expression.

Evidence for competition between two PrfA forms with different promoter-activating capacities. Complementation experiments were also performed with the PrfA-proficient strain P14-A, with a functional *plcA-prfA* operon on the chromosome and a Gly145Ser PrfA mutant background (Table 4). Introduction of additional copies of the mutant *prfA* allele had, as

TABLE 3. PrfA-dependent virulence factor expression in BHI by PrfA-deficient mutants complemented with the Gly145Ser (pRG145S constructs) or the wild-type (pRWT constructs) *prfA* alleles

Strain	Plasmid[a]	Expression[b]	
		hly	*plcB*
MS5	None	1 (31.99)	1 (0.14)
	pMK4	1.0	1.1
	pRG145S	4.0	174.0
	pRG145S-b	4.0	175.1
	pRWT	0.7	1.2
	pRWT-b	5.6	12.5
P14[c]	None	0.7	1.0
P14-A[c]	None	4.0	177.8
OD43	None	1 (3.99)	1 (0.05)
	pMK4	1.0	1.2
	pRG145S	32.1	497.4
	pRG145S-b	64.1	498.4
	pRWT	12.7	7.6
	pRWT-b	25.4	68.8
Δ*prfA* mutant	None	1 (2.51)	1 (0.07)
	pMK4	1.0	1.2
	pRG145S	101.9	356.8
	pRG145S-b	102.2	387.7
	pRWT	9.1	4.3
	pRWT-b	50.9	262.9

[a] The suffix -b designates bicistronic constructs.
[b] *hly* and *plcB* expression was measured by determining hemolytic (LLO) and lecithinase (PLC) activities in the culture supernatant. Results are expressed as relative activities with respect to background expression levels (those for MS5, OD43, and the Δ*prfA* mutant without a plasmid) (absolute values are in parentheses). Data are mean values from at least three independent determinations. The differences in activation shown by the MS5 mutant, on the one hand, and the OD43 and Δ*prfA* mutants, on the other, are attributable to their different background levels of *hly* and *plcB* expression (1 order of magnitude higher in MS5), because absolute activity values after complementation with pRG145S are very similar in the three strains. In accordance with the model proposed here for PrfA-mediated virulence gene expression, production of low, residual levels of an activated form of PrfA could explain why the PrfA mutant MS5 (from a Gly145Ser background) expresses 10 times more hemolysin and lecithinase than OD43 (from a wild-type PrfA background) in BHI despite having all of the known *prfA* promoters abolished by a transposon insertion into the *plcA-prfA* intergenic region.
[c] Control strain.

expected, no significant effect on *hly* and *plcB* expression. In contrast, and very interestingly, complementation with pRWT drastically reduced *hly* and, particularly, *plcB* expression (14-fold decrease in lecithinase activity) (Table 4). Thus, the activation of virulence genes by the presumably elevated levels of mutant PrfA in P14-A seems to be neutralized by the relatively moderate amounts of wild-type PrfA protein that would be synthesized from the monocistronic mRNA transcribed from pRWT. This titration effect could be explained if two PrfA forms with different virulence gene activation capacities competed for their target promoters.

Nevertheless, no significant inhibition was observed after complementation with pRWT-b. Although apparently in contradiction with the above-described explanation, this result may be attributed to a masking of the titration effect by the presumably high levels of wild-type PrfA generated by the autoamplification loop in pRWT-b (which would be constantly stimulated in the P14-A background), as elevated levels of wild-type PrfA can also induce virulence factor overexpression in *L. monocytogenes* (see above) (Table 3).

TABLE 4. PrfA-dependent virulence factor expression in BHI by the *L. monocytogenes* Gly145Ser mutant P14-A complemented with additional copies of the Gly145Ser mutant *prfA* (pRG145S constructs) or with wild-type *prfA* (pRWT constructs)[a]

Plasmid	Expression	
	hly	*plcB*
None	100 (132.00)	100 (25.54)
pMK4	122.2	99.8
pRG145S	193.9	98.0
pRG145S-b	193.9	100.2
pRWT	76.9	6.9
pRWT-b	122.1	104.4

[a] See Table 3 footnotes for details.

The Gly145Ser substitution in PrfA mimics CRP mutations that cause cAMP independence. Transcriptional regulation by CRP is dependent on intracellular cAMP levels in *E. coli* (22). Upon binding to the N-terminal domain of CRP, cAMP induces a conformational change that is transmitted to the C-terminal DNA-binding domain of the regulatory protein. It is generally thought that this leads to hinge reorientation and concomitant protrusion of the F α-helix bearing the HTH motif, allowing it to interact with target DNA with increased sequence specificity (17, 22). CRP mutations that mimic the allosteric conformational change induced by cAMP, rendering the regulatory protein cAMP independent, have been characterized. A cluster of such mutations (called *crp** mutations) maps to the D α-helix of CRP (16, 17, 19, 22). Most interestingly, the Gly145Ser mutation is in a PrfA region (residues 141 to 151) highly similar (50% identity and 70% similarity) to the D α-helix of CRP. The PrfA undecapeptide and the homologous CRP stretch are located at identical positions in the corresponding proteins (Fig. 4).

One of the mutations causing CRP to be functionally active in the absence of cAMP is an Ala→Thr substitution at position 144 (16, 17, 19). This Ala-144 precisely aligns with Gly-145 of PrfA (Fig. 4). From the known tertiary structure of CRP (48), residue 144 in the D α-helix faces residue 190 in the F α-helix. Bulkier side chains at position 144 may consequently repel the F α-helix from the D α-helix, thus facilitating the interaction of the CRP HTH motif with target DNA (20, 22). Another *crp** mutation is at position 141 and consists, like in our case, of a Gly→Ser substitution (16, 22). In wild-type CRP, residue 141 faces the C α-helix across the hinge connecting the N- and C-terminal domains. A larger, polar side group at this position may alter the hinge orientation, causing protrusion of the F α-helix (20, 22). The structural and functional similarities between PrfA and CRP (25, 40) suggest that the region spanning residues 141 to 151 in PrfA may have a role in transcriptional regulation similar to that of the D α-helix region of CRP.

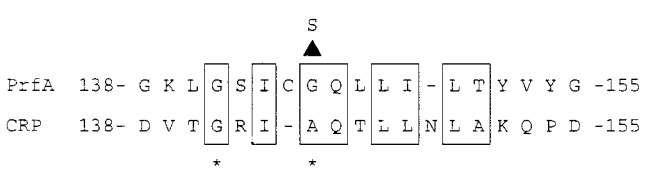

FIG. 4. Alignment of amino acids 138 to 151 of PrfA with the corresponding residues of CRP (D α-helix, residues 140 to 151). Identical or similar amino acids are boxed. The Gly145Ser substitution in PrfA is indicated by an arrowhead. Asterisks denote the positions of substitutions in *crp** mutants (Gly141Ser and Ala144Thr) that cause cAMP independence in CRP. The sequences are from references 19 and 30.

Indeed, P14-A has lost its capacity to downregulate the transcription of virulence genes in response to several environmental stimuli, which, by analogy with CRP* mutants, could be explained by a locking of its PrfA in a "switched-on", cofactor-unresponsive state.

Model for PrfA-mediated virulence gene regulation in *L. monocytogenes*. We have recently demonstrated that the transcription of virulence genes in wild-type strains of *L. monocytogenes* is very low in BHI but is strongly induced by a change in the extracellular medium composition (i.e., in charcoal-treated medium) (36). Here we show that a substitution in PrfA results in high, constitutive production of virulence factors in BHI (i.e., under nonactivating conditions) comparable to that by wild-type strains in charcoal-treated BHI (36). An explanation of these observations is that PrfA shifts from an inactive to an active conformation upon interaction with an environmentally regulated PrfA-binding signal molecule, and the Gly145Ser mutation locks PrfA in a cofactor-independent, transcriptionally active form.

From this hypothesis, a model for PrfA-mediated virulence gene expression can be established. In this model, the levels of virulence factor expression do not depend primarily on the concentration of the PrfA protein, as previously suggested (13, 14, 41), but on the functional conformation of the PrfA protein itself, as follows. Constitutive transcription of *prfA* from its own promoters in the *plcA-prfA* intergenic region leads to the synthesis of moderate levels of an inactive PrfA form in the absence of activating environmental signals. If appropriate changes in the environment (e.g., an increase in temperature and a change in composition of the extracellular medium) occur, the inactive PrfA protein synthesized from the monocistronic transcripts is rapidly converted into its active form. As long as the activating conditions persist, this leads to a sharp increase in the concentration of transcriptionally active PrfA protein through the positive feedback mediated by the PrfA-dependent *plcA-prfA* bicistronic transcripts, concomitantly triggering a rapid activation of the remaining PrfA-dependent virulence genes. A rapid drop in the transcription levels of the PrfA-dependent virulence genes, including *prfA* itself, follows the conversion of PrfA back into its inactive conformation as soon as the activating environmental signals cease.

This model is compatible with all experimental data and characteristics of PrfA-mediated virulence gene regulation. In particular, it explains the following observations: (i) repression or activation of virulence gene transcription can be observed without concomitant changes in monocistronic *prfA* levels (3, 4, 21, 27); (ii) complementation of PrfA-deficient mutants (with a nonfunctional autoamplification loop) with the monocistronic Gly145Ser *prfA* allele fully activates transcription of virulence genes in *L. monocytogenes* grown under nonactivating conditions, whereas complementation with the monocistronic wild-type allele has no such effect (Table 3); and (iii) introduction of the monocistronic wild-type *prfA* allele strongly reduces hemolysin and lecithinase expression in the mutant strain P14-A (with "natural" Gly145Ser background) grown in BHI (Table 4), which could be due to competition between inactive wild-type PrfA and active mutant PrfA forms.

In summary, our observations support the hypothesis that at least some environmental signals that modulate virulence gene expression in *L. monocytogenes* could be transduced to the PrfA system by an effector molecule or cofactor. This as-yet-unidentified molecule may interact with the listerial regulatory protein through an allosteric transition mechanism similar to that underlying cAMP-mediated functional activation of CRP.

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