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Differential Activation of Virulence Gene Expression by PrfA, the *Listeria monocytogenes* Virulence Regulator

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PrfA is a pleiotropic activator of virulence gene expression in the pathogenic bacterium *Listeria monocytogenes*. Several lines of evidence have suggested that a hierarchy of virulence gene activation by PrfA exists. This hypothesis was investigated by assessing the ability of PrfA to activate the expression of virulence gene fusions to *lacZ* in *Bacillus subtilis*. Expression of PrfA in this heterologous host was sufficient for activation of transcription at the *hly*, *plcA*, *mpl*, and *actA* promoters. Activation was most efficient at the divergently transcribed *hly* and *plcA* promoters. The putative PrfA binding site shared by these promoters is perfectly symmetrical and appears to represent the optimum sequence for target gene activation by PrfA. The activation of *actA* and *mpl* expression was considerably weaker and occurred more slowly than that observed at the *hly* and *plcA* promoters, suggesting that greater quantities of PrfA are required for productive interaction at these promoters. Interestingly, expression of an *inlA-lacZ* transcriptional fusion was very poorly activated by PrfA in *B. subtilis*, suggesting that other *Listeria* factors, in addition to PrfA, are required for PrfA-mediated activation at this promoter. Further support for the involvement of such factors was obtained by constructing and analyzing a *prfA* deletion mutant of *L. monocytogenes*. We observed that, in contrast to that of the other genes of the PrfA regulon, expression of *inlA* is only partially dependent on PrfA.

Listeria monocytogenes is a ubiquitous, gram-positive, facultative intracellular bacterium. It is responsible for infrequent, but often serious, opportunistic infections in humans and animals. *L. monocytogenes* is capable of invading and multiplying within a wide range of phagocytic and nonphagocytic mammalian cells. Its intracellular lifestyle is characterized by rapid phagolysosomal lysis and bacterial multiplication in the eukaryotic cytoplasm. The bacteria can spread within tissues by passing directly from an infected cell to adjacent cells by a mechanism involving actin-based bacterial propulsion. A number of genes involved in *Listeria* pathogenesis have been identified (reviewed in references 6, 7, 39, and 41). These genes include *hly*, which encodes a pore-forming cytolysin, the first virulence determinant described in *L. monocytogenes* (reviewed in reference 8); *plcA* and *plcB*, which encode respectively, a phosphatidylinositol-specific phospholipase and a broad substrate-range phospholipase (21, 22); *mpl*, which encodes a polypeptide homologous to metalloproteases from other bacterial pathogens (12, 34); *actA*, which encodes a surface protein required for actin assembly (13, 27); and *inlA*, which encodes a protein, internalin, required for entry of *L. monocytogenes* into cultured epithelial cells (20). All of these genes, with the exception of *inlA*, are clustered at a single locus consisting of three transcriptional units (Fig. 1), and all are coordinately regulated by PrfA, the transcriptional activator encoded by the *prfA* gene (4, 15, 33).

The *prfA* gene lies downstream from, and is cotranscribed with, *plcA* (Fig. 1). As PrfA activates transcription from the *plcA* promoter, *prfA* positively regulates its own expression. In addition to the 2.2-kb *plcA-prfA* transcript, *prfA* is also expressed as a monocistronic message originating from promoters in the intergenic *prfA-plcA* region. Transposon insertions within the *prfA* structural gene increase transcription from the latter *prfA* promoters, suggesting that *prfA* also negatively regulates its own synthesis (17, 18). Gel retardation analysis with purified PrfA and a DNA fragment containing the *hly* promoter has demonstrated that PrfA is a sequence-specific DNA binding protein (18, 40a). Although the precise mechanism of transcriptional activation-repression by PrfA is not known, significant homologies have recently been observed between the 27-kDa PrfA protein and members of the CAP-FNR family of pleiotropic transcription regulators (23, 29, 41). As many other bacterial pathogens, the expression of virulence genes in *L. monocytogenes* is environmentally modulated and is subject to growth phase and thermal regulation (30, 33, 38).

Several observations suggest that PrfA interacts differentially at its various target promoters. Blocking readthrough transcription of *prfA* from the *plcA* promoter had a significantly greater effect on expression of the phospholipase activity encoded by *plcB* than on listeriolysin levels (3). Freitag et al. (18) found that reduced expression of *prfA* allowed sufficient expression of listeriolysin for phagolysosome lysis in infected cells, yet the cytoplasmic bacteria were unable to nucleate actin, suggesting that *actA* expression was more severely affected. However, it is also possible that the levels of listeriolysin required to fulfill its physiological role are much lower than those of ActA.

A 14-bp dyad-symmetric sequence plays a crucial role in the interaction of PrfA with its target promoters. The PrfA-dependent activation of *hly* expression in *Bacillus subtilis* was abolished by point mutations in that sequence located between the divergently transcribed *hly* and *plcA* genes (19). It had been previously proposed, on the basis of sequence conservation (15, 35, 45), that the similar regions of dyad symmetry centered at approximately −40 in the *mpl*, *actA*, and *inlA* promoters

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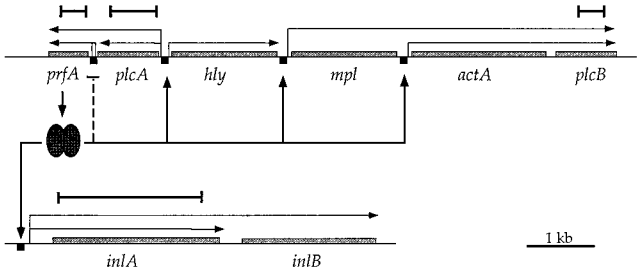

FIG. 1. Coordinate regulation of virulence gene expression by PrfA in *L. monocytogenes*. The putative PrfA binding sites are represented by solid squares. The proposed negative regulation of the intergenic *prfA* promoters by PrfA (17, 18) is represented by the broken line. Note that PrfA is shown functioning as a dimer; this, although not formally proven, is considered highly likely on the basis of the homologies between PrfA and members of the CAP family of transcriptional regulators. Fragments used as probes for Northern blot analysis are represented by the bracketed lines above the corresponding genes.

(Fig. 2) are involved in the coordinate regulation of virulence gene expression. Sequence variability within these putative PrfA sites has led to the suggestion that PrfA may recognize or interact differentially with these promoters, lending an additional level of control to the system (18, 35). Thus, increases in PrfA levels would result in sequential activation of PrfA-dependent genes. To test this "PrfA-site hierarchy" model directly, we studied the activation of virulence gene expression by PrfA in a heterologous host, *B. subtilis*. Our results support the notion of differential activation by PrfA and strongly suggest that this differential activation is determined by the target promoter sequences themselves. Furthermore, our analysis indicates that other *L. monocytogenes* factors, in addition to PrfA, are involved in the activation of *inlA* expression.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are described in Table 1. *Escherichia coli* MC1061 and DH5αF′ (Bethesda Research Laboratories) were used as hosts for recombinant plasmids. All strains were grown in Luria-Bertani broth (40) or brain heart infusion broth (BHI; Difco) supplemented with the following antibiotics as required: ampicillin, 50 μg/ml; chloramphenicol, 5 μg/ml; erythromycin, 5 μg/ml; kanamycin, 5 μg/ml; phleomycin, 0.8 μg/ml.

Transformation. Plasmids were transformed into *E. coli* strains made competent by treatment with $CaCl_2$ and into *B. subtilis* strains by a one-step procedure (28). Plasmids were introduced into *L. monocytogenes* strains by electroporation by a modification of the method described by Michel et al. (36). Briefly, a saturated overnight culture of *L. monocytogenes* was diluted 1/100 in BHI and incubated at 37°C with agitation until the absorbance at 600 nm (A_{600}) of the culture approached 0.2. Penicillin G (Sigma) was then added to a final concentration of 0.12 μg/ml, and growth was continued as before for a further 2 h. Bacteria (100 ml) were harvested by centrifugation at 3,200 × *g* for 20 min, washed once in an equal volume of ice-cold electroporation buffer (816 mM sucrose, 3 mM $MgCl_2$) and twice in 0.5 volume of ice-cold electroporation buffer, and then resuspended in a final volume of 1 ml. Electroporation was performed with 100 μl of bacteria mixed with 1 μg of DNA at 2.4 kV, 25 μF, and 200 Ω. The electroporated cells were immediately removed from the cuvette, transferred to 2 ml of prewarmed BHI, and incubated at 37°C with agitation for 1 to 2 h. Aliquots (200 μl) were then plated on antibiotic-containing BHI agar. Transformants generally appeared after 2 days at 37°C. The efficiency of transformation varied considerably, depending on the plasmid used, and was maximal (approximately 5×10^3/μg) with pMK4 (42).

Construction of promoter fusions. To monitor gene expression, we constructed transcriptional fusions between the promoter regions of the various *prfA*-activated genes and the *lacZ* reporter gene contained in expression vector pAZ7. Plasmid pAZ7 (Fig. 3a) is a derivative of translational fusion plasmid pAC7 (47) in which the truncated *lacZ* gene from pAC7 was replaced with the *lacZ* gene and *B. subtilis* ribosome-binding site from pHT304′*lacZ* (43). Plasmid pAZ7 carries the ColE1 replicon from pBR322 for replication in gram-negative hosts but does not replicate in gram-positive bacteria. Sequences from the *B. subtilis amyE* gene flank the promoterless *lacZ* gene and the divergently transcribed kanamycin resistance determinant in pAZ7, facilitating single-copy integration of the *lacZ* cassette at the *amyE* locus by homologous recombination, selecting for kanamycin resistance.

The divergently transcribed *hly* and *plcA* promoters were isolated on a ca. 2-kb *Sau*3A fragment from pLis3 (9) and cloned into the *Bam*HI site of pAZ7 to create pVGF1 (*hly-lacZ*) and pVGF2 (*plcA-lacZ*; Fig. 3b). Similarly, the promoter for the *mpl* gene was isolated as a 1.4-kb *Sau*3A fragment from pLis3 and cloned into the *Bam*HI site of pAZ7 to generate pVGF3. *inlA-lacZ* fusion plasmid pVGF4 was constructed by introducing a 946-bp *Hin*cII-*Bgl*II promoter-bearing fragment from pPE2 (20) into pAZ7 digested with *Sma*I and *Bam*HI. The promoter located upstream from the *actA* and *plcB* genes was isolated from p*actA*1, a pBR322-derived plasmid harboring a 2.7-kb, *actA*-containing *Hin*dIII insertion (27). p*actA*1 was digested with *Eco*RI and *Bsa*A1 to release a 400-bp fragment, containing 25 bp of pBR322 sequences between the *Hin*dIII and *Eco*RI sites. This fragment was subcloned into *Eco*RI-*Sma*I-digested pAZ7 to create pACTLAC1. Strains of *E. coli* and *B. subtilis* harboring this plasmid produced high levels of β-galactosidase, independently of the PrfA status of the cell (data not shown). Much of this activity may have originated from the pBR322 *tet* promoter which lies between the *Eco*RI and *Hin*dIII sites of pBR322 (and, consequently, pACTLAC1). Thus, to remove these sequences, the *actA* promoter was further subcloned into pDG793 (16) on a 400-bp *Hin*dIII-*Bam*HI fragment (pACTLAC2). pVGF5, the *actA-lacZ* fusion used in the studies described below, was constructed by digesting pACTLAC2 with *Eco*RI and *Bam*HI and ligating the purified 400-bp *actA* promoter fragment to appropriately digested pAZ7 (Fig. 3).

Construction of *L. monocytogenes* Δ*prfA*. To delete the *prfA* gene in *L. monocytogenes* LO28, sequences flanking *prfA* were cloned into *E. coli* vector pBluescript KS+. Sequences downstream from *prfA* were cloned on a 1.8-kb *Kpn*I-*Eco*NI fragment from pLis29 (33). This region, which contains the previously described *prs* gene and the 3′ end of the *tms* gene, was cloned into pBluescript KS+ digested with *Kpn*I and *Hin*cII (pB*prs*). Sequences upstream from *prfA* were isolated on a 1.3-kb *Eco*RI-*Hin*cII fragment from pLis8 (35) and cloned into the unique *Eco*RI and *Sma*I sites of pB*prs* to generate plasmid pBΔ*prfA*. To permit allele exchange in *L. monocytogenes*, the LO28-derived sequences were subcloned from pBΔ*prfA* into thermosensitive shuttle vector pGhost5 (32) by digestion with *Kpn*I and *Not*I. The resultant plasmid, pGΔ*prfA*, was transformed into LO28 by electroporation as described above. The thermosensitive origin of replication carried by pGhost5 permits plasmid replication in *L. monocytogenes* at 37°C but not at 42°C. Homologous recombination, followed by plasmid elimination, was obtained by overnight growth of cultures at the restrictive temperature in the presence of antibiotic, followed by growth (18 h) at the permissive temperature in the absence of antibiotic selection. Finally, suitable dilutions were plated onto Columbia agar containing 5% sheep blood and incubated for 48 h at the restrictive temperature. Colonies of recombinant bacteria carrying deletions in *prfA* were readily identifiable because of the absence of *prfA*-regulated hemolysin activity in these strains. Plasmid absence in these strains was verified by screening for erythromycin resistance, and the presence of the deletion was confirmed by Southern blotting. The strain was designated BUG802. It lacks *prfA* sequences between nucleotides 298 and 1009 according to the numbering of Mengaud et al. (33). The deleted sequence contains all but the first 8 amino acids of PrfA.

Preparation and analysis of RNA from *L. monocytogenes*. To prepare total *L. monocytogenes* RNA, approximately 1.5×10^{10} bacteria (i.e., 10 ml of a culture at an A_{600} of 1) grown in Luria-Bertani broth were resuspended in 1 ml of 15 mM NaCl–1.5 mM Na citrate and centrifuged at 13,000 × *g*. The pellet was resuspended in 1 ml of 10 mM Na_2HPO_4–20% sucrose (pH 7.2) containing 4 mg of lysozyme and incubated at 37°C for up to 60 min. Protoplasts were pelleted at 4,000 × *g* for 5 min. They were lysed with 0.5 ml of 4 M guanidinium isothiocyanate–25 mM Na citrate–0.5% Sarkosyl per sample. Samples were further treated as previously described (5). Finally, the RNA was dissolved in 50 to 100 μl of sterile diethyl pyrocarbonate-treated water and stored at −70°C.

Northern (RNA) blot analysis. RNA samples (6 μg) were denatured in RNA sample buffer by heating to 65°C for 5 min and separated on a 1% agarose gel containing 2.2% formaldehyde for approximately 6 h. The RNA was transferred to Hybond-N (Amersham) by capillary blotting and hybridized with ^{32}P-labelled DNA probes (Megaprime; Amersham). The *plcA* probe contained 696 bp of

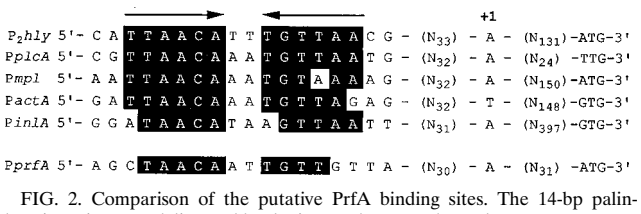

FIG. 2. Comparison of the putative PrfA binding sites. The 14-bp palindromic regions are delineated by the inverted arrows above the sequences. Base pair substitutions relative to the *hly-plcA* palindromes are shown, as are the distances between the inverted repeats and the transcriptional start points (+1) and initiation codons of each gene. P*prfA* corresponds to the *prfA* P2 promoter identified by Freitag et al. (18), while P*inlA* refers to P$_2$*inlA*, which was identified by Dramsi et al. (15).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Reference or source
L. monocytogenes strains		
LO28	Virulent wild-type strain (serotype 1/2c)	46
BUG802	LO28Δ*prfA*	This study
B. subtilis strains		
QB4503	*B. subtilis* 168*trpC2 sacP*::Cm^r	10
BUG1170	*B. subtilis* 168*trpC2 sacP*::Cm^r *amyE*::*plcA-lacZ*	This study
BUG1195	*B. subtilis* 168*trpC2 sacP*::Cm^r *amyE*::*hly-lacZ*	This study
BUG1196	*B. subtilis* 168*trpC2 sacP*::Cm^r *amyE*::*actA-lacZ*	This study
BUG1197	*B. subtilis* 168*trpC2 sacP*::Cm^r *amyE*::*mpl-lacZ*	This study
BUG1198	*B. subtilis* 168*trpC2 sacP*::Cm^r *amyE*::*inlA-lacZ*	This study
BUG1171	BUG1170 Pspac-*prfA*	This study
BUG1199	BUG1195 Pspac-*prfA*	This study
BUG1203	BUG1196 Pspac-*prfA*	This study
BUG1210	BUG1197 Pspac-*prfA*	This study
BUG1214	BUG1198 Pspac-*prfA*	This study
BUG1218	QB4503(pAZ7)	This study
Plasmids		
pAZ7	*lacZ* transcriptional fusion vector incorporating *spoVG* ribosome-binding site; pBR322 replicon, does not replicate in gram-positive hosts; *lacZ* flanked by *amyE*; Ap^r Km^r	This study
pVGF1	pAZ7 with Phly on 2.1-kb *Bam*HI-*Sau*3A insert from pLis3	This study
pVGF2	pAZ7 with PplcA on 2.1-kb *Bam*HI-*Sau*3A insert from pLis3	This study
pVGF3	pAZ7 with Pmpl on 1.4-kb *Sau*3A insert from pLis3	This study
pVGF4	pAZ7 with PinlA on 946-bp *Hin*cII-*Bgl*II insert from pPE2	This study
pVGF5	pAZ7 with PactA on 400-bp *Eco*RI-*Bam*HI insert from pACTLAC2	This study
pspac-*prfA*	IPTG-inducible Pspac-*prfA* fusion; does not replicate in gram-positive hosts; Ap^r Cm^r Ph^r	19
pDG793	Transcriptional fusion-suicide vector for integration at *B. subtilis thrC* locus; Ap^r Em^r	16
pBluescript KS+	Cloning vector; Ap^r	Stratagene
pB*prs*	pBluescript KS+ with *tms* and *prs* genes on 1.8-kb *Kpn*I-*Eco*NI fragment from pLis29	This study
pBΔ*prfA*	pB*prs* with *plcA* on 1.3-kb *Eco*RI-*Hin*cII fragment from pLis8	This study
pGΔ*prfA*	pGhost5 with 2.1-kb *kpn*I-*Not*I fragment from pBΔ*prfA*	This study
pGhost5	Thermosensitive suicide vector; Em^r	Appligene

[a] Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Ph, phleomycin.

plcA, beginning 30 bp upstream from the initiation codon of the gene, the *actA-plcB* probe extended from nucleotides 2625 to 2988 (45), the 2.2-kb *inlA* probe comprised sequences encoding amino acids 36 to 766 of the revised Internalin sequence (14), and the *prfA* probe extended from nucleotides 321 to 690 (33). Filters were prehybridized (for at least 45 min) and hybridized (for at least 2 h) at 67.5°C by using the Rapid Hybridization System (Amersham). They were washed at the same temperature, once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS), once in 1× SSC–0.1% SDS, and twice in 0.7 × SSC–0.1% SDS, for a total time of at least 1 h. Gene expression was quantified by densitometric scanning of appropriately exposed autoradiograms with a Bio-Rad GS-670 imaging densitometer.

Virulence assays. The 50% lethal dose of strain LO28 and that of *prfA* mutant BUG802 were determined after intravenous injection of pathogen-free female C3H mice as described previously (26).

β-Galactosidase assays. β-Galactosidase production in *B. subtilis* strains was measured by a fluorimetric assay with the substrate 4-methylumbelliferyl-β-D-galactopyranoside (Sigma). *B. subtilis* strains were grown in 25 ml of Luria-Bertani broth with appropriate antibiotics at 37°C to an A_{600} of 0.1 to 0.2. The cultures were divided equally, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to one culture to a final concentration of 1 mM. Incubation was continued at 37°C, and aliquots (0.2 to 1 ml, depending on the strain) were removed and immediately frozen in liquid nitrogen. After thawing, the assay was performed as previously described (26).

RESULTS

PrfA activates *L. monocytogenes* virulence gene promoters in *B. subtilis*. PrfA is both necessary and sufficient, in the context of a heterologous *B. subtilis* host, for activation of transcription at the *hly* promoter (19). To determine whether this is true for other PrfA-regulated genes, the ability of PrfA to activate all known PrfA-regulated promoters (*hly*, *plcA*, *mpl*, *actA*, and *inlA*) was assessed in *B. subtilis*.

Plasmid pAZ7 and the fusion constructs pVGF1 to pVGF5 were transformed into *B. subtilis* QB4503 (168*trpC2 sacP*::Cm^r; 10) by the method of Kunst and Rapoport (28). Since these plasmids are unable to replicate in gram-positive hosts, selection for Km^r allowed the isolation of strains in which the integration cassettes (depicted in Fig. 3) had recombined onto the chromosome by homologous recombination at the *amyE* locus. The *prfA* gene product was supplied in *B. subtilis* under the control of the IPTG-inducible Pspac promoter. The IPTG-inducible plasmid Pspac-*prfA* (19) was integrated into the chromosome of each of the *B. subtilis* strains described above by a single recombination event between the pC194-derived chloramphenicol resistance determinant on the plasmid and the chloramphenicol cassette in the *sacP* gene (Table 1 is a list of the strains used and their genotypes). Thus, in all of the strains, both the regulatory gene (*prfA*) and the putative target sequences are present in single copies on the *B. subtilis* chromosome (Fig. 4). As the *sacP* operon is not expressed in the absence of sucrose (10), expression of *prfA* in this system should originate exclusively from the synthetic Pspac promoter. In our experiments, transcriptional activation was measured as the difference in *lacZ* expression between IPTG-induced *prfA*-proficient cells and *prfA*-deficient cells, as we (and others [19]) observed that even in the absence of IPTG, residual expression of *prfA* from the uninduced Pspac promoter was sufficient to cause some activation of the *hly* and *plcA* promoters.

The activity of *lacZ* fusions with a subset of PrfA-activated promoters (*hly*, *plcA*, *mpl*, and *actA*) was significantly increased following *prfA* induction in *B. subtilis* (Fig. 5 and Table 2). The

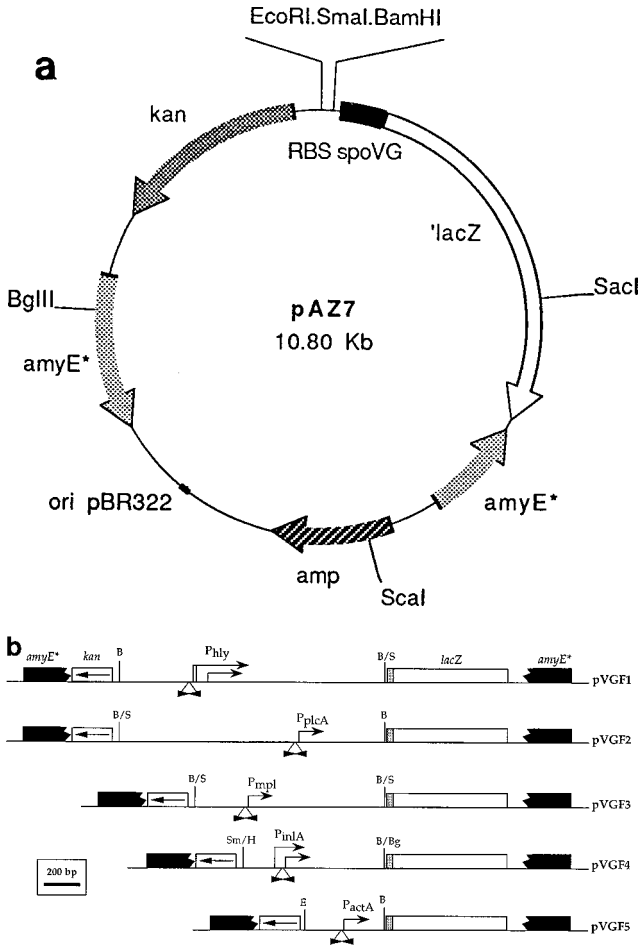

FIG. 3. (a) Physical map of plasmid pAZ7. This vector allows the construction of transcriptional fusions with *lacZ* and single-copy integration at the *B. subtilis amyE* locus. The ribosome-binding site (RBS) from the *B. subtilis spoVG* gene ensures efficient expression of β-galactosidase in gram-positive hosts. Unique and other relevant restriction sites are shown. (b) Schematic representation of *L. monocytogenes* promoter-*lacZ* fusions in pAZ7. The integration cassette flanked by *amyE* sequences is shown. Putative PrfA binding sites are shown by inverted arrowheads. The truncated *amyE* sequences are designated *amyE**. The *aphAIII* kanamycin resistance gene is transcribed divergently from *lacZ*, and the *spoVG* ribosome-binding site is represented by the shaded box immediately upstream from *lacZ*. Distances between the putative PrfA-regulated promoters and *lacZ* are as follows: P_{hly}, 1,449 bp; P_{plcA}, 560 bp; P_{mpl}, 1,013 bp; P_{inlA}, 669 bp; P_{actA}, 393 bp. Restriction site abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*cII; S, *Sau*3A; Sm, *Sma*I. Only *Listeria*-derived sequences are shown to scale.

magnitude of transcriptional activation and the time course of the effect were not identical at all promoters and were in the relative order *plcA* > *hly* ⩾ *actA* = *mpl* (Fig. 5 and Table 2). The *plcA* and *hly* promoters were most affected by *prfA* expression. Less than 30 min after induction of *prfA*, transcription at both of these promoters was increased over 70-fold (Fig. 5a and b and Table 2). At later time points, expression of the *plcA-lacZ* fusion was 1,000-fold higher in the presence of *prfA* than in its absence. Expression of β-galactosidase from the *hly-lacZ* fusion was consistently 200- to 300-fold higher in PrfA-producing cells (Fig. 5a). The *hly-lacZ* construct was the most highly expressed virulence gene fusion to *lacZ* in *B. subtilis*; the lower induction of *hly* expression in *B. subtilis* relative to *plcA* reflects higher basal expression from the *hly* promoter (Table 2).

Significant activation of transcription at the *actA* and *mpl* promoters occurred 60 to 120 min following the induction of *prfA* expression with IPTG (Fig. 5c and d). The magnitude of transcriptional activation in the presence of PrfA (5- to 11-fold for both promoters) was considerably less than that observed for the *hly* and *plcA* promoters. Note that of the five *lacZ* fusions tested, *mpl-lacZ* had the highest basal (PrfA-independent) activity (Table 2). Interestingly, transcription of the *inlA-lacZ* fusion was poorly activated by PrfA in *B. subtilis*. Expression of β-galactosidase from the *inlA-lacZ* fusion was maximally induced only two- to threefold by the presence of *prfA* in *B. subtilis* (Fig. 5e and Table 2), demonstrating that, in this context, PrfA alone is insufficient to activate expression from the *inlA* promoter.

PrfA regulation of virulence gene expression in *L. monocytogenes*. The activation of virulence gene expression by PrfA has been documented from the study of a variety of *L. monocytogenes* strains defective in PrfA expression because of transposon insertions or spontaneous deletions of *prfA* coding sequences (4, 18, 31, 33). Intrigued by the apparent inability of PrfA to activate transcription of an *inlA-lacZ* fusion in *B. subtilis*, we decided to construct a complete deletion of the *prfA* coding sequences in *L. monocytogenes* and to examine the effect of such a lesion on virulence gene activation. *prfA*-defective strain BUG802, a derivative of wild-type strain LO28, was constructed as described in Materials and Methods. RNA was isolated from the wild-type and deletion mutant strains at various points during in vitro growth and hybridized with probes derived from *plcA* (strongly activated by PrfA in *B. subtilis*), *actA* (intermediate activation), and *inlA* (poor activation). The expression of the *prfA* gene (in LO28) was monitored by using a *prfA*-specific probe (see Materials and Methods). The previously described growth phase regulation of virulence gene expression is apparent in Fig. 6 (15, 33). In the wild-type strain, maximum expression of the *prfA*-regulated *plcA*, *actA-plcB*, and *inlA* genes occurs in exponential-phase cultures and corresponds to maximum transcription of the *prfA* gene (Fig. 6). In the Δ*prfA* strain, transcripts originating from the *plcA* and *actA* promoters were virtually undetectable, demonstrating an absolute requirement for PrfA for expression. Compared with that in the wild type, transcription of these genes in the mutant strain was reduced more than 100-fold

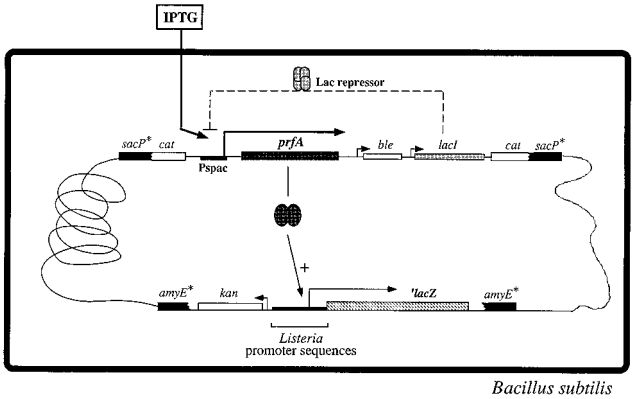

FIG. 4. Heterologous expression system. The main features of this system are described in the text. Expression of *prfA* is repressed by binding of the constitutively expressed Lac repressor to its operator site in the Pspac promoter. Addition of IPTG causes a conformational change in the repressor, allowing transcription of *prfA* to proceed. Plasmid Pspac-*prfA* was integrated at the *cat*-mutated *sacP* gene of *B. subtilis* QB4503, while the *Listeria* promoter-*lacZ* constructs were integrated by recombination at the *amyE* locus. Truncated gene fragments are indicated by asterisks.

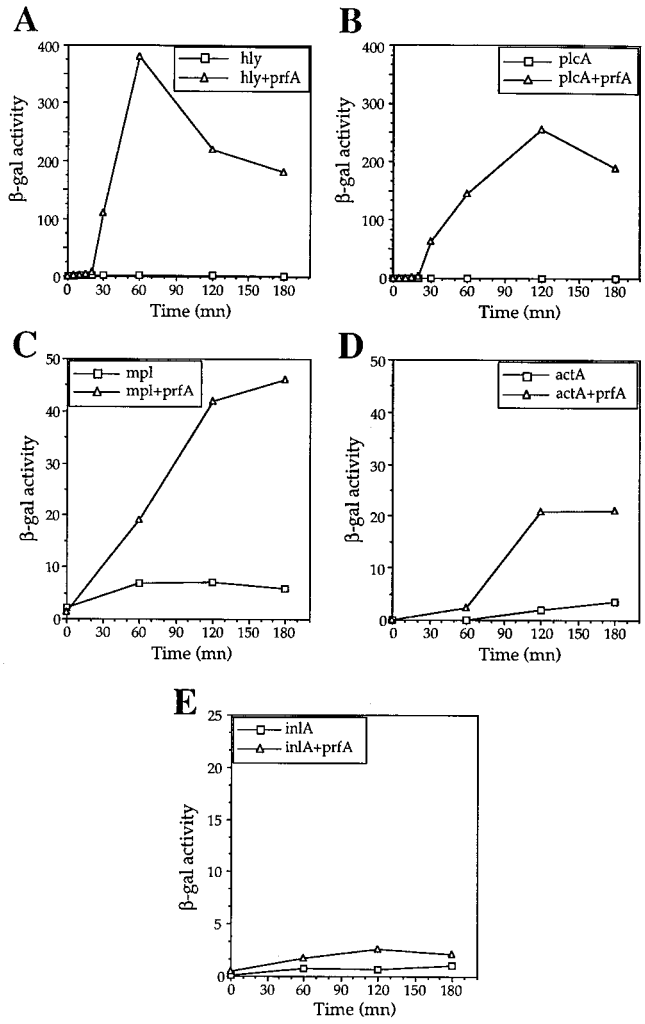

FIG. 5. Effects of *prfA* on *Listeria* promoter-*lacZ* expression in *B. subtilis*. Expression of *prfA* was induced at time zero by addition of IPTG to 1 mM, and β-galactosidase activity was measured at the times indicated as described in Materials and Methods. A single representative experiment is shown for each of the five fusions tested; similar curves were obtained in at least two independent experiments. β-Galactosidase activity is given as 10^5 fluorescence units per hour per bacterium. A, *hly-lacZ*; B, *plcA-lacZ*; C, *mpl-lacZ*; D, *actA-lacZ*; E, *inlA-lacZ*.

(*plcA*) and more than 20-fold (*actA*), as judged by densitometric scanning of the blots.

In *L. monocytogenes*, exponential-phase expression of the *inlAB* locus was enhanced 10- to 15-fold in the presence of *prfA* (compare Fig. 6, lanes 1 and 2). However, in the early stationary phase, considerable *inlAB* expression was observed in Δ*prfA* mutant BUG802 (Fig. 6, *inlA* panel, lane 4). Thus, while the *inlAB* locus requires PrfA for maximal expression in exponentially growing cultures of *L. monocytogenes*, our results demonstrate that *inlA* expression is only partially dependent on PrfA, with significant PrfA-independent *inlAB* transcription.

The Δ*prfA* mutant has severely reduced virulence. Previous analyses of the role of PrfA in the virulence of *L. monocytogenes* have employed a variety of strains carrying transposon-induced or spontaneously occurring mutations in the *prfA* gene (4, 31, 33). We assayed the virulence of wild-type strain LO28 and its isogenic *prfA* mutant strain, BUG802, in the well-characterized mouse model of infection. The *prfA* defect in BUG802 resulted in severe attenuation of virulence after intravenous infection, the 50% lethal dose increasing by 4 orders of magnitude, from 8×10^3 to more than 8×10^7.

DISCUSSION

The results presented here demonstrate that a hierarchy of virulence gene activation by PrfA exists. Previous work has shown that in a heterologous *B. subtilis* host, PrfA is necessary and sufficient for activation of *hly* expression (19). We have confirmed these findings and extended them to include the promoters for the *plcA*, *actA*, and *mpl* genes. In contrast, PrfA alone is not sufficient for activation of the *inlA* promoter in *B. subtilis*. The finding that considerable *inlA* expression occurs in the absence of *prfA* in *L. monocytogenes* BUG802, but not in *B. subtilis*, further reveals a role for additional, *Listeria*-specific factors in the regulation of *inlA* expression.

In *B. subtilis*, activation of transcription occurred most rapidly at the divergently transcribed *hly* and *plcA* promoters. The putative PrfA-binding site shared by these promoters is perfectly symmetrical and may represent the optimum sequence for transcriptional activation by PrfA. Thus, low PrfA concentrations would be sufficient for activation at the *hly-plcA* promoter, whereas activation at the *actA* and *mpl* promoters may require the accumulation of greater amounts of PrfA. This differential activation may be due to the single base substitutions in the putative PrfA target sites present in these promoters (Fig. 2), which may either reduce the affinity of PrfA for these promoters or affect the ability of bound PrfA to activate gene expression. Significantly, activation of transcription at the *plcA* promoter switches on an autocatalytic circuit causing rapid accumulation of *prfA*-encoding mRNA and ultimately resulting in the differential induction of target gene expression. Such a system would allow sequential activation of virulence genes during the course of *L. monocytogenes* infection, with factors involved in phagolysosomal lysis being expressed earlier in the infectious cycle than those required for actin nucleation and cell-to-cell spread (17–19). While such a model is consistent with the in vitro data, it may well be an oversimplification of the regulatory events which occur in vivo. It has been shown that ActA is the bacterial protein most highly expressed during

TABLE 2. *L. monocytogenes* virulence gene activation by PrfA in *B. subtilis*

Genotype	Mean β-galactosidase sp act[a]									
	hly-lacZ		*plcA-lacZ*		*mpl-lacZ*		*actA-lacZ*		*inlA-lacZ*	
	30 min	180 min	30 min	180 min	30 min	180 min	30 min	180 min	30 min	180 min
Pspac-*prfA*	165	225	55.3	200	6.1	36.4	4.8	10.1	1.2	2.4
Control (no *prfA*)	1.9	0.7	0.8	0.21	3.8	5.9	0.9	0.9	1.4	0.8
Mean activation ratio ± SEM	85 ± 16	320 ± 73	74 ± 11	950 ± 140	1.68 ± 0.1	6.1 ± 1.0	5.4 ± 0.3	10.9 ± 0.8	0.8 ± 0.1	2.9 ± 0.2

[a] β-Galactosidase activities represent the mean values obtained in two to five independent experiments, after 30 and 180 min of IPTG induction.

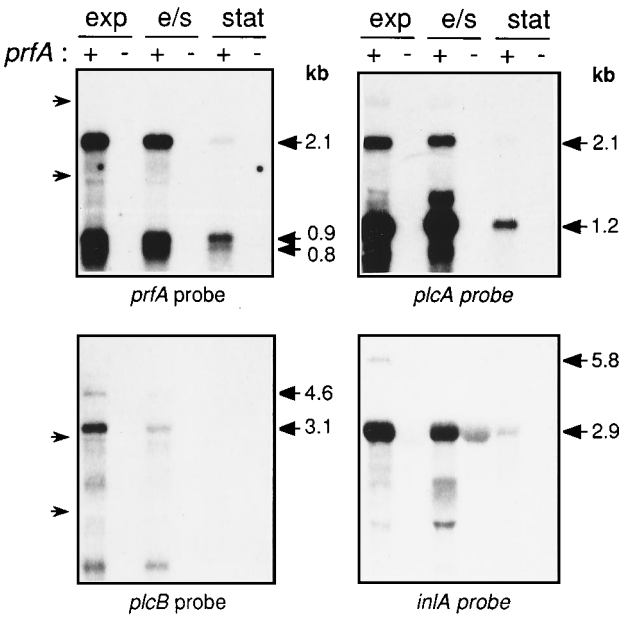

FIG. 6. Northern blot analysis of virulence gene expression in *L. monocytogenes* LO28 and its isogenic Δ*prfA* derivative, BUG802. RNA was prepared from cultures of wild-type LO28 (*prfA*$^+$) or BUG802 (*prfA*) grown in Luria-Bertani broth and harvested at the following time points: 2 h (A_{600} of 0.2 to 0.3, exponential phase [exp]); 3.5 h (A_{600} of 0.5 to 0.7, early stationary phase [e/s]); 7 h (A_{600} of 0.6 to 0.7, stationary phase [stat]). mRNA sizes were derived from the positions of the 16S and 23S bacterial rRNAs present in the samples (short arrows), as well as from the 18S and 28S rRNAs of a mouse RNA sample run in parallel (not shown). The probe used is indicated below each panel. The two smaller *prfA* transcripts, originating from promoters in the *plcA-prfA* region, were described by Camilli et al. (3). The 3.1-kb band in the *plcB* panel corresponds to the *actA-plcB* transcript. Additional bands may reflect instability of *mpl*-encoding mRNAs (1). The relative intensity of the two *inlA*-hybridizing mRNAs is different from that reported previously (15), and this may be due to differences in RNA preparation protocols. Note that in samples in which a given mRNA is strongly expressed, the corresponding probe may appear to hybridize at positions immediately below the rRNA bands.

intracellular growth in J774 macrophages (1, 2). However, other factors, including mRNA stability (1), may also account for high ActA levels in vivo, and it is unknown whether this intracellular induction of *actA* expression is PrfA dependent. We have recently reported that a transcriptional fusion between promoters in the intragenic *plcA-prfA* region and *lacZ* is equally well expressed during growth in vitro (BHI) and in vivo (J774 macrophages), while in contrast, expression of the PrfA-dependent *plcA* promoter is strongly induced during intracellular growth (26). This uncoupling between expression of the monocistronic *prfA*-specific mRNA and the activation of *prfA*-dependent gene expression suggests a role for additional regulatory components (and/or posttranslational modification of PrfA) in the intracellular induction of *plcA*, and possibly *actA*, expression (see below).

The existence of accessory regulatory factors is suggested by our studies on the activation of *inlA* expression in *B. subtilis* and *L. monocytogenes*. The inability of PrfA to significantly activate transcription from the *inlA* promoter in *B. subtilis* contrasts with the situation observed in *L. monocytogenes*, where *inlAB* expression in the exponential phase is 10- to 15-fold higher in the presence of PrfA. It should be noted that under the conditions of the present work, PrfA levels observed on immunoblots are at least fivefold lower in *B. subtilis* than in *L. monocytogenes* (our unpublished results). The putative PrfA target site in the *inlA* promoter contains three base substitutions relative to the *hly-plcA* PrfA site, and interaction of PrfA with this imperfect target sequence may require either the higher levels of PrfA reached in listeriae or another *Listeria*-specific factor(s) absent from the *B. subtilis* system. Moreover, *inlA* expression in *L. monocytogenes* is only partially dependent on PrfA. The existence of additional regulatory elements contributing to *inlA* expression may be inferred from the finding that considerable transcription of the *inlAB* locus occurs in Δ*prfA* mutant strain BUG802. This *prfA*-independent expression is maximal during the early stationary phase of growth and may originate from a sigma 28 consensus promoter identified upstream from the putative PrfA-dependent *inlA* promoter(s) (15). Internalin-mediated invasion of cultured intestinal epithelial cells is known to be maximal in the exponential phase, suggesting that this PrfA-independent transcription of *inlA* plays only a minor role in the invasive phenotype, at least for this cell type. Indeed, the Δ*prfA* mutant strain was found to be 50- to 100-fold less invasive than wild-type LO28 in a standard Caco2 cell infection assay (15a).

Recently, sequences resembling the putative PrfA-binding site have been identified in the intergenic *plcA-prfA* region, and a direct role for PrfA in autorepression (with implications for virulence gene activation) through interaction with this sequence has been proposed (29). We investigated the ability of PrfA to modulate the expression of transcriptional and translational fusions of this promoter region to *lacZ* in our heterologous system. Preliminary experiments indicate that induction of PrfA expression has no negative effect on expression of the *prfA-lacZ* fusions in *B. subtilis*, suggesting that PrfA does not interact directly with this sequence or that if it does, significantly higher concentrations of PrfA, or additional factors, are required for stationary-phase repression of PrfA-dependent gene expression. We have recently analyzed *prfA* point mutations which affect the activating function of PrfA, and, when introduced into *L. monocytogenes*, result in altered PrfA levels (40a). These mutations do not alter the levels of the monocistronic *prfA* transcripts, making questionable the role of PrfA in the negative regulation of its own expression.

In addition to the growth phase regulation of virulence gene expression, PrfA-dependent genes are also modulated by temperature and the plant-derived disaccharide cellobiose. Growth of listeriae at low temperatures inhibits transcription of the major virulence determinants (30). Similarly, growth of listeriae in the presence of 5 to 10 mM cellobiose represses transcription of the *hly* and *plcA* genes (38). Intriguingly, expression of the monocistronic *prfA*-specific message is unaffected by these environmental stimuli (26, 30). This uncoupling of *prfA* transcription from virulence gene activation and our findings on the poor activation of *inlA* expression by PrfA in a heterologous host indicate that virulence gene regulation in *L. monocytogenes* is mediated by a network of regulatory components including PrfA. This theme of hierarchical activation of virulence genes is found in other pathogenic bacteria including *Bordetella pertussis*, *Shigella flexneri*, *Vibrio cholerae*, and the gram-positive bacterium *Staphylococcus aureus* (11, 24, 25, 37, 44).

The pivotal role of *prfA* in virulence gene expression is highlighted by the severe attenuation of *prfA*-defective bacteria in the mouse model of infection. Nonetheless, it seems likely that virulence gene regulation by PrfA in *L. monocytogenes* requires the recruitment of additional *Listeria* factors, at least for activation of transcription at the *inlA* promoter and for stationary-phase repression of *prfA*, and PrfA target gene, expression. The *inlA-lacZ* fusion described in this report could be used to clone these additional *Listeria* factors in *B. subtilis*. Such accessory factors lend an additional level of control to the

system and may help to explain why, in response to certain environmental stimuli, it is possible to activate virulence genes without increasing monocistronic *prfA* message levels.

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