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Transcriptional Activation of the *Listeria monocytogenes* Hemolysin Gene in *Bacillus subtilis*

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The *prfA* gene of *Listeria monocytogenes* was recently reported to be required for expression of *hly*, which encodes a pore-forming hemolysin essential for pathogenicity (M. Leimeister-Wachter, C. Haffner, E. Domann, W. Goebel, and T. Chakraborty, Proc. Natl. Acad. Sci. USA 87:8336–8340, 1990). We demonstrate here that a *hly-lacZ* fusion introduced into *Bacillus subtilis* is strongly activated when the *prfA* gene product is supplied in *trans* under the control of an isopropyl-β-D-thiogalactopyranoside-inducible promoter, Pspac. Moreover, the PrfA-dependent activation of *hly* is abolished by point mutations in a 14-bp DNA palindromic sequence present in the 5′ upstream region of *hly*. This indicates that PrfA is both necessary and sufficient for *hly* transcriptional activation and establishes the palindrome as the likely target sequence for PrfA interaction. The presence of a palindrome in the upstream regions of three additional *L. monocytogenes* genes clustered near *hly* suggests that PrfA may serve as a transcriptional activator for a major virulence regulon of *L. monocytogenes*. In addition, the ability of PrfA to activate its target promoters effectively in *B. subtilis* suggests that further analysis of this regulon and perhaps other aspects of *L. monocytogenes* gene regulation might be carried out in part through reconstruction experiments in *B. subtilis*.

Like many other intracellular parasites, including rickettsiae, shigellae, *Trypanosoma cruzi*, and *Theileria parva* (32), the bacterial facultative intracellular pathogen *Listeria monocytogenes* is able to escape from phagocytic vacuoles and grow directly in the host cell cytoplasm (12, 39). Ultrastructural studies have revealed fascinating details of the infectious cycle, which involves destruction of the phagosomal membrane, nucleation of actin filaments that propel the bacteria through the cytoplasm, and formation of bacterium-tipped pseudopodial extensions that facilitate cell-to-cell spread (6, 33, 39). However, few of the bacterial gene products involved in these events have been identified. An exception is listeriolysin O, the product of *hly*, which is known to promote lysis of the phagosomal membrane (12, 39). This was established in part by demonstrating that *hly* engineered for expression in *Bacillus subtilis* conferred on this common soil bacterium the ability to lyse phagosomal membranes after it was ingested by a macrophagelike cell line (2). In the course of these experiments, it became apparent that the natural promoter for *hly* was not active in *B. subtilis*, despite the close phylogenetic relatedness of *B. subtilis* and *L. monocytogenes* (25). This raised the possibility that expression of *hly* required the participation of a specific transcription activator not normally present in *B. subtilis*.

Two recent reports presented evidence that the *prfA* gene of *L. monocytogenes* encodes a positive activator of *hly* (24, 27). A spontaneously occurring *prfA* deletion mutant that blocked transcription of *hly* was characterized (24). This is consistent with our own studies involving Tn*917*-generated small-plaque mutants of *L. monocytogenes* (38). Several of these mutants were caused by insertional disruption of *prfA*, which blocked expression not only of *hly* but also of genes encoding two phospholipase activities that may potentiate the ability of listeriolysin to disrupt host membranes (4, 14, 22, 23, 26). These mutants were absolutely defective for intracellular growth (38) and were 5 orders of magnitude less virulent for mice (1).

Mengaud et al. (30) have previously noted the presence of a 14-bp palindromic sequence within or near the apparent promoters for *hly* and two neighboring transcription units, and they suggested a possible regulatory role for the palindromes (30). In the present work, we show that expression of *prfA* in *B. subtilis* is sufficient to activate transcription from the *hly* promoter and that single point mutations within the upstream palindrome can abolish this PrfA-mediated activation. This definitively establishes a direct role for PrfA as an activator of virulence-associated transcription in *L. monocytogenes* and indicates that the palindrome is probably a specific recognition sequence for the PrfA protein. The observations that *plcA* (which encodes a phosphatidylinositol-specific phospholipase C) is divergently transcribed from *hly* and shares the *hly* palindrome and that nearly identical palindromes are present upstream from other genes clustered near *hly* support the model that PrfA is the activator protein for a major virulence regulon of *L. monocytogenes* and that the palindrome is an essential *cis*-acting feature of this region.

MATERIALS AND METHODS

Bacterial strains and growth media. *L. monocytogenes* 10403S (3) belongs to serotype 1, is resistant to streptomycin, and has a 50% lethal dose for mice of 3×10^4 (35). *L. monocytogenes* was stored at −70°C in brain heart infusion broth (Difco) containing 50% (vol/vol) glycerol. *B. subtilis* KY42, provided by Karen York, contains a silent transposon Tn*917* insertion in which the *ermC* region was replaced with a chloramphenicol resistance gene (41). *B. subtilis* ZB307A (SPβc2del2::Tn917::pSK10Δ6) has been previously described (18). *Escherichia coli* DH5α-MCR (BRL) was used as a host for recombinant plasmids. All strains were grown in Luria-Bertani (LB) broth (36). Antibiotics were used at the

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following concentrations: chloramphenicol, 5 μg/ml; phleomycin (Bristol Laboratories), 0.8 μg/ml; erythromycin, 1 μg/ml; and ampicillin, 50 μg/ml. The gene nomenclature used here corresponds to that recently adopted (34): *hly* for *hlyA* and *lisA*; *plcA* for ORFU, *pic*, and *plcA*; *prfA* for *prfA*; *mpl* for ORFD, *prtA*, and *mpl*; *actA* for *prtB*; and *plcB* for *prtC*.

Construction of promoter fusions in *B. subtilis*. An 821-bp *Sau*96-*Hin*dIII fragment containing the *hly* promoter (29) was cloned into the expression vector pTKlac (20) upstream of a promoterless derivative of the *lacZ* gene from *E. coli*, which contained a *B. subtilis* ribosome-binding site. This plasmid was recombined into a specialized SPβ prophage after transformation of *B. subtilis* ZB307A. SPβ phage (SPβ::*hly-lacZ*) were generated by heat induction as described previously (19).

L. monocytogenes genomic DNA, isolated as described by Flamm et al. (10), was used as a template for the polymerase chain reaction (16) in the presence of the primers described below to generate a promoterless copy of the *prfA* gene flanked by two unique restriction sites. Primer A (GENOSYS Biotechnologies, Inc.), 5′-GGTCTAGACGAT TGGGGGATGAGAC-3′, creates an *Xba*I site upstream of *prfA* coding sequences and is complementary to sequences beginning 18 nucleotides 5′ to the start of translation (24). Primer B, 5′-GGGTCGACCAGCTCTTCTTGGTGAAG-3′, creates a *Sal*I site at the 3′ end of *prfA* and is complementary to sequences 114 nucleotides downstream from the TAA stop codon (24). Following amplification, the DNA product was digested with *Sal*I and *Xba*I and subcloned into pAG58-ble-1 (43). The resulting plasmid, Pspac-*prfA*, places *prfA* under the control of the isopropyl-β-D-thiogalactoside (IPTG)-inducible Pspac promoter (40). Pspac-*prfA* was introduced into *B. subtilis* KY42 by transformation, selecting for phleomycin (DP-B1443). A control strain transformed with pAG58-ble-1 alone was designated DP-B1445. DP-B1443 and DP-B1445 were then transduced with SPβ::*hly-lacZ* to produce strains DP-B1451 (containing Pspac-*prfA* and SPβ::*hly-lacZ*) and DP-B1450 (containing Pspac and SPβ::*hly-lacZ*).

Primer extension. RNA was purified by centrifugation through CsCl by a modification of a procedure described by Kenney et al. (21). *B. subtilis* strains were grown in 100 ml of LB medium containing appropriate antibiotics. IPTG was added to a final concentration of 1 mM to logrithmically growing bacteria at an optical density at 600 nm of 0.2 to 0.3. The cells were harvested after 2 h of additional growth at 37°C. *L. monocytogenes* 10403S, grown in 100 ml of LB medium containing 2.5 mM $CaCl_2$, 20 mM $MgCl_2$, and 20 mM $MgSO_4$, was harvested after growth with shaking at 37°C for 5 h. Cell pellets were suspended in 7.5 ml of guanidine isothiocyanate buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate [pH 5.0], 0.5% Sarkosyl, 1 M 2-mercaptoethanol). The cells were broken by two passages through a French pressure cell at 12,000 lb/in^2. The lysate was layered on a 2.75-ml cushion of 5.7 M CsCl–0.025 M sodium acetate (pH 5.0), in SW41 polyallomer tubes (14 by 89 mm). The tubes were centrifuged at 32,000 rpm at 18°C for 22 h in a Beckman SW41 rotor.

The resulting RNA pellet was suspended in TE buffer (0.01 M Tris HCl [pH 8.0], 0.001 M EDTA), extracted three times with phenol-chloroform (1:1 [vol/vol]) with 1% (vol/vol) diethyl pyrocarbonate, and extracted once with chloroform. The RNA was precipitated with ethanol, dissolved in 50 to 75 μl of TE, and stored at −70°C.

High-pressure liquid chromatography-purified primer 5′-GCCAAATACCGTTTGCCACCCCTCTCT-3′ (10 pmol) was end labeled by using 50 μCi of [γ-^{32}P]ATP (>3,000 Ci/mmol) and phage T4 polynucleotide kinase (BRL) as previously described (36). Approximately 0.03 pmol of labeled primer was added to 20 μg of *L. monocytogenes* or *B. subtilis* RNA in 10-μl reaction volumes containing 0.05 M Tris HCl (pH 8.3) and 0.1 M KCl. Reaction mixtures were incubated at 90°C for 1 min, 60°C for 2 min, and then on ice for 15 min to allow annealing of primer to template. The annealed reaction mixes (6 μl) were added to tubes containing 1 μl of 2.5 mM dATP, dCTP, dGTP, and dTTP mix; 2 μl of reverse transcriptase buffer (0.25 M Tris HCl [pH 8.3], 0.2 M KCl, 0.036 M Mg acetate, 0.01 M dithiothreitol, 2 U of RNasin [Promega Biotech] per μl), and 1 μl (3 U) of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.). Reaction mixes were incubated at 52°C for 45 min, and then 5 μl of Sequenase stop buffer (USB) was added to terminate the reactions. The primer extension reaction mixtures (4 μl) were subjected to electrophoresis in 8% polyacrylamide gels containing 8 M urea followed by autoradiography.

Mutagenesis of the *hly* promoter palindrome. Three primers were designed to be used in combination with the polymerase chain reaction (16) to allow mutagenesis and cloning of the *hly* promoter upstream of the promoterless *lacZ* gene in pTKlac. Each of these three primers was located 193 bp upstream from the ATG initiation codon of *hly* (29). The first primer (P1), 5′-ATGTGGATCCATTAACATTTGTTAATGA-3′, created a *Bam*HI site 5′ of the palindrome. The second primer (P2), 5′-ATGTGGATCCATTAAAATTTGTTAAT GA-3′, created a *Bam*HI site and a C→A substitution within the palindrome. The third primer (P3), 5′-ATGTGGATCCATT AAAATTTTTTAATGA-3′, created a *Bam*HI site and C→A and G→T substitutions within the *hly* palindrome. Each primer described above was used in a polymerase chain reaction amplification reaction in conjunction with a fourth primer (P4), 5′-TTTGGATAAGCTTGAGCATATT-3′, located 585 bp downstream from the ATG initiation codon of *hly* and including an endogenous *Hin*dIII site, and *L. monocytogenes* genomic DNA to generate *hly* promoter palindrome mutant products of 788 bp, which were subcloned upstream of the promoterless *lacZ* gene in pTKlac (20). The *hly* promoter mutant constructs were sequenced to verify the nucleotide substitutions and then integrated into SPβ in *B. subtilis* ZB307A to generate SPβ::*hly*(P1)-*lacZ*, SPβ::*hly*(P2)-*lacZ*, and SPβ::*hly*(P3)-*lacZ*. These three phage preparations were used to transduce DP-B1443 and DP-B1445 to erythromycin resistance.

β-Galactosidase assay. *B. subtilis* strains were grown in 50 ml of LB broth with appropriate antibiotics at 37°C to an optical density at 600 nm of approximately 0.1. The cultures were halved, and IPTG was added to one culture to a final concentration of 1 mM. Cultures were incubated at 37°C with shaking, and at the indicated time intervals, 1.5-ml aliquots were removed and frozen in a dry ice-ethanol bath. After being thawed on ice, samples were assayed by a fluorometric method using 4-methylumbelliferyl-β-D-galactoside as a substrate as previously described (41).

RESULTS

The *prfA* gene product activates *hly* transcription. To determine whether the *prfA* gene product directly activates transcription from the *hly* promoter, we constructed an expression system utilizing a related gram-positive bacterium, *B. subtilis*, as a host. The *prfA* structural gene was cloned into a Pspac expression vector (40), thereby placing

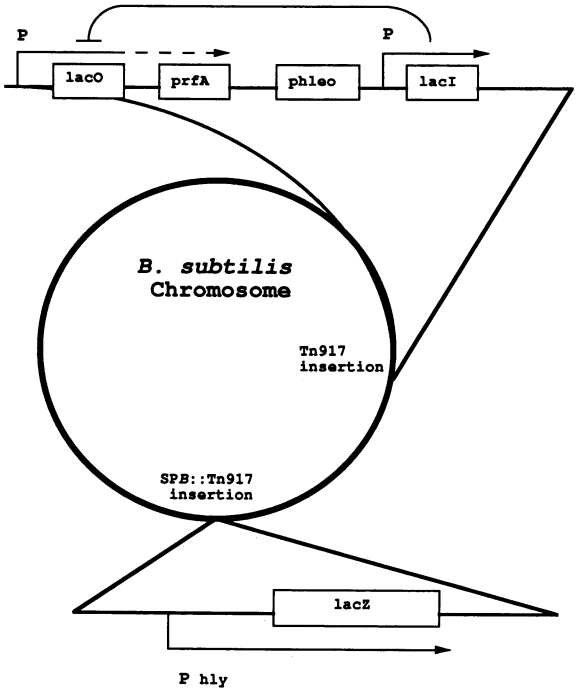

FIG. 1. Strategy for testing the ability of PrfA to activate the *hly* promoter. Expression of *prfA* in *B. subtilis* was placed under the control of the IPTG-inducible promoter Pspac by insertion of a DNA fragment containing the *prfA* coding sequence into the SPAC expression cassette of pAG58-*phleo* (43). The expression cassette was then integrated by recombination at the site of a phenotypically silent Tn*917* insertion (20). Activity of the *hly* promoter was monitored through the use of a *lacZ* transcriptional fusion integrated by recombination into an SPβ prophage (44).

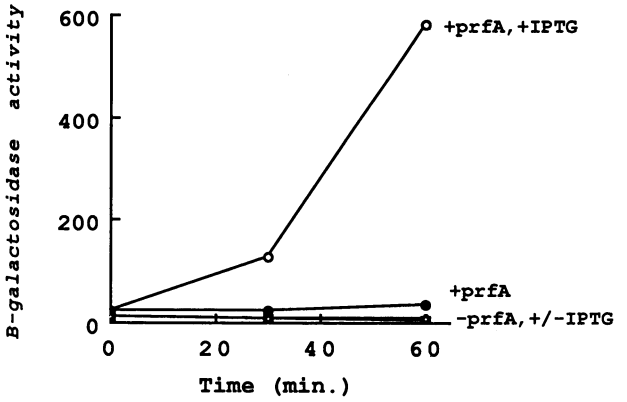

FIG. 2. Effects of *prfA* on *hly-lacZ* expression. β-Galactosidase activity was measured at the indicated time intervals in cultures of *B. subtilis* strains containing the *hly-lacZ* fusion and Pspac-*prfA* (+prfA, circles) or Pspac alone (−prfA, squares) in the presence or absence of IPTG. Units of β-galactosidase activity are as described by Youngman (41).

prfA under the control of an IPTG-inducible promoter and providing for regulated expression of the *prfA* gene product in *B. subtilis*. A transcriptional reporter gene fusion between *hly* promoter sequences and the *lacZ* gene of *E. coli* was introduced into the *B. subtilis* chromosome through the use of an SPβ specialized transducing phage. The resulting *B. subtilis* strain, DP-B1451 (shown in Fig. 1), contained a single copy of *prfA* under the control of an IPTG-inducible promoter and a transcriptional fusion of *hly* promoter sequences to the *lacZ* structural gene. Transcriptional activation of the *hly-lacZ* fusion in *B. subtilis* was measured by monitoring expression of β-galactosidase activity following induction of *prfA*.

Expression of β-galactosidase from the *hly-lacZ* fusion was completely dependent on the presence of *prfA* coding sequences (Fig. 2). β-Galactosidase levels were over 100-fold higher in the presence of *prfA* following IPTG induction than in the absence of *prfA*. It should be noted that β-galactosidase levels were higher in uninduced cells in the presence of *prfA* than in its absence, indicating that there is a low level of *prfA* expression from the Pspac promoter in the absence of IPTG induction. However, addition of IPTG increased β-galactosidase activity in these cells over 20-fold, indicating that the *prfA* gene product directly activates transcription from the *hly* promoter and that the presence of *prfA* is sufficient for this activation.

Mapping of *hlyA-lacZ* transcription initiation sites in *B. subtilis*. Two transcriptional initiation sites for the *hly* promoter in *L. monocytogenes* were previously identified (30). The sites are separated by 10 nucleotides and located 122 bp (p1) and 133 bp (p2) from the *hly* translation initiation codon. To determine whether the *prfA* gene product directs *hly* transcription in *B. subtilis* from the same initiation sites as are used in *L. monocytogenes*, we used primer extension analysis (Fig. 3). Two *hly* transcripts which mapped to positions 123 (p1) and 133 (p2) relative to the translation initiation codon (lanes 1 and 2, respectively) were reproducibly identified in *L. monocytogenes* and IPTG-induced *B. subtilis*. These transcripts were undetectable in *B. subtilis* in the absence of IPTG induction (lane 3) or in *B. subtilis* lacking *prfA* coding sequences (lane 4). The *prfA* gene product therefore directs transcriptional initiation from the *hly* promoter in *B. subtilis* at initiation sites identical to those used in *L. monocytogenes*. The single nucleotide discrepancy between our data for the initiation site of transcript p1 and that previously reported for *L. monocytogenes* likely reflects a *L. monocytogenes* strain difference.

The *prfA* gene product recognizes a DNA palindrome upstream of *hly*. A 14-bp DNA palindrome is present in the *hly* promoter region located 20 bp upstream from the −10 region of the *hly* P2 transcript (30) (Fig. 4). To determine whether the *prfA* gene product activates *hly* transcription by recognizing this 14-bp DNA palindrome, we created single- and double-base-pair substitutions in conserved regions of the palindromic sequence by using polymerase chain reaction (37) (Fig. 4). A single-base-pair change of C→A in one half of the palindrome resulted in complete abolition of *prfA*-activated transcription (Fig. 5). The double-substitution mutation which restores base pairing, *hly*(C:A,G:T), was likewise noninducible. These results indicate that the *prfA* gene product mediates its effects through the recognition of the palindromic DNA sequence present in the *hly* promoter region. Single nucleotide substitutions can eliminate recognition of the palindrome by the *prfA* gene product, and this recognition is not restored by the introduction of a complementary mutation which restores palindrome base pairing.

DISCUSSION

L. monocytogenes requires the action of the pore-forming hemolysin listeriolysin O to escape from the phagosome and grow within the host cell cytoplasm (2, 12, 39). This *hly*-

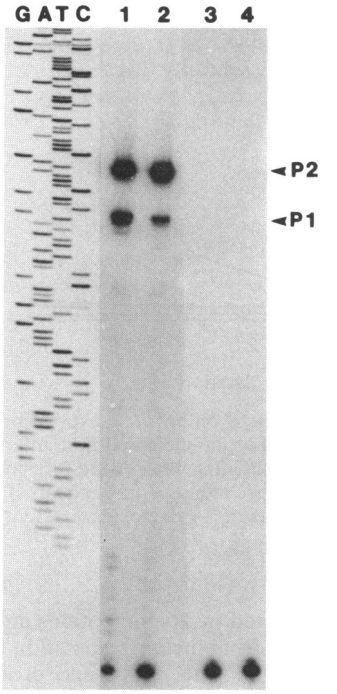

FIG. 3. Primer extension analysis of *hly-lacZ* transcripts. RNA was isolated from *L. monocytogenes* 10403S, DP-B1451 (with *prfA*) with and without IPTG induction, and DP-B1450 (without *prfA*) with IPTG induction. A radiolabeled oligonucleotide was incubated with the RNA, and reverse transcriptase was added to produce a DNA copy of the transcript, as described in Materials and Methods (lanes 1 to 4). The same oligonucleotide was used to prime dideoxy sequencing products from a DNA template that contained the *hly-lacZ* fusion. The letters above each lane indicate the dideoxynucleotide used to terminate each reaction. Lane 1, DP-B1451 with IPTG; lane 2, *L. monocytogenes* 10403S; lane 3, DP-B1451 without IPTG; lane 4, DP-B1450 with IPTG.

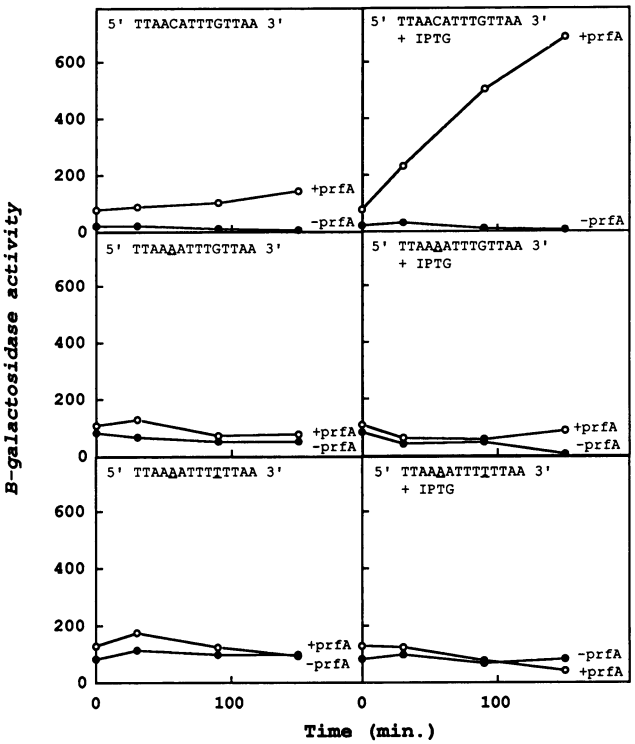

FIG. 5. Effect of mutations within the *hly* palindrome on *prfA*-directed *hly-lacZ* expression. Each panel shows the accumulation of β-galactosidase in the presence (open circles) or absence (closed circles) of *prfA*. The DNA sequence of the palindrome present in the upstream region of the *hly-lacZ* fusion in each strain is shown in the left-hand corner of each panel. β-Galactosidase was measured at the indicated time points in the presence or absence of IPTG.

encoded hemolytic activity represents an essential component of *L. monocytogenes* pathogenesis (5, 13, 17, 35). Expression of hemolysin is dependent on the presence of a second gene product, which is encoded by *prfA* (24, 27). The results of this study provide strong evidence that PrfA functions as a direct activator of *hly* transcription. Expression of the *L. monocytogenes prfA* gene product in *B. subtilis* in *trans* was necessary and sufficient for over 100-fold activation of transcription from the *hly* promoter. PrfA function required the presence of a 14-bp DNA palindrome in the upstream region of *hly*, and this palindrome apparently functions as a specific recognition sequence for PrfA binding. Single nucleotide substitutions within this palindrome resulted in the complete elimination of PrfA-directed activation of *hly* transcription. These experiments establish a definitive link between the presence of the 14-bp DNA palindrome and PrfA-directed activation of *hly* transcription.

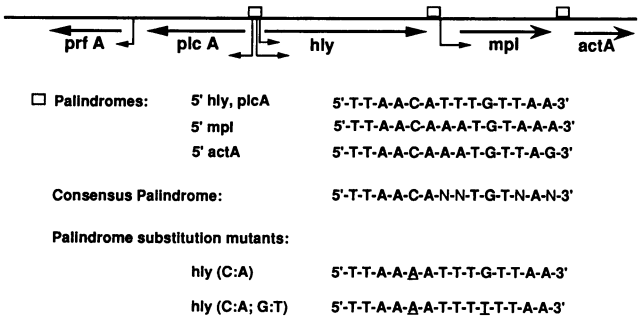

FIG. 4. Physical locations of DNA palindromes and nucleotide substitutions. The sequences and approximate locations of three 14-bp DNA palindromes in *L. monocytogenes* are shown. Nucleotide substitutions within the palindrome sequences were generated within the *hly* promoter as described in Materials and Methods and fused to *lacZ*.

The predicted amino acid sequence of PrfA contains a region (residues 133 to 152) which resembles the helix-turn-helix motif found in some bacterial and phage DNA-binding proteins, such as the *E. coli* CAP-activator protein (7) and bacteriophage lambda repressor and Cro proteins (15). These proteins bind as homodimers to palindromic DNA sequences located within their target promoters. The *hly* palindrome resembles the binding sites of these and other helix-turn-helix regulatory proteins. It is nearly identical to the palindrome present in the O_L3 operator site of phage 434 recognized by Cro and phage 434 repressor proteins, and its repeated 6-bp 5′-TGTTAA-3′ sequence is also present in the *lac* operator site (15). These protein-DNA recognition site similarities and the potential helix-turn-helix structural motif of PrfA suggest that the PrfA protein may bind as a homodimer to the *hly* palindrome via specific protein-DNA contacts. Confirmation of this hypothesis awaits purification of the PrfA protein.

The 14-bp *hly* palindrome recognized by PrfA is located between *hly* and *plcA*, which are divergently transcribed (30). In addition, similar palindromes exist in the 5′ upstream regions of two other *L. monocytogenes* genes clustered near *hly*, namely, the *mpl* gene, which bears sequence homology to bacterial metalloproteases (9, 28), and *actA*, which is located downstream of *mpl* (27). Recently, Mengaud et al. have demonstrated that transformation of a *L. monocytogenes prfA* deletion strain with a plasmid carrying *prfA* increases transcription from the *plcA*, *hly*, *mpl*, and *actA* promoters (27). Thus, the *prfA* gene product functions as a transcriptional activator for several *L. monocytogenes* virulence factors. The presence of the 14-bp palindrome in the promoter regions of *hly*, *plcA*, *mpl*, *actA* and perhaps other yet-unidentified *L. monocytogenes* genes appears to define members of a virulence regulon under the transcriptional control of PrfA. The single base differences in the *mpl* and *actA* palindromes in comparison to the palindrome present in the *hly* and *plcA* promoter region may function to provide an additional level of transcriptional control. For example, if PrfA recognizes the *mpl* or *actA* palindromes with less affinity than that observed for *hly*, greater amounts of PrfA protein would be required to activate transcription of *mpl* or *actA*. Activation of *prfA*-dependent genes in *L. monocytogenes* would then depend on the relative amounts of PrfA protein present in the cell.

Initiation of *hly* transcription following PrfA-mediated induction occurs at identical sites in *B. subtilis* and *L. monocytogenes*. The relative abundances of the *hly* p1 and p2 transcripts were also similar in the two strains, suggesting that the mechanism of PrfA-mediated induction of *hly* transcription in *B. subtilis* resembles the process as it occurs in *L. monocytogenes*. It had previously been postulated that the p1 transcript may have originated from a constitutively acting *hly* promoter and that the p2 transcript was expressed from an inducible promoter (30). Our results indicate that PrfA is capable of directing the initiation of both transcripts in *B. subtilis* and may therefore direct both in *L. monocytogenes*. Previous work has indicated that no *hly* transcripts are detectable in *L. monocytogenes prfA* deletion strains (24), an observation which supports the role of PrfA in directing initiation of both transcripts.

Since PrfA is capable of functioning as a strong transcriptional activator in *B. subtilis*, it is possible that the *prfA* gene product requires no posttranslational modification for its own activity. However, we cannot yet rule out the possibility that endogenous *B. subtilis* regulatory factors contribute to activation of PrfA-directed transcription. One striking aspect of the results reported here is the efficiency with which PrfA works in concert with *B. subtilis* RNA polymerase to activate promoters under its control. This raises the possibility that other aspects of *L. monocytogenes* virulence gene regulation (e.g., factors that modulate PrfA expression or activity) might be investigated through a reconstruction of regulation in *B. subtilis* in much the same way that *E. coli* has served as a heterologous test system for analysis of *Vibrio cholerae* regulatory factors (8, 31).

PrfA is clearly a transcriptional activator of a gene(s) required for the escape of *L. monocytogenes* from the host cell vacuole. There is evidence that PrfA also regulates genes required for nucleation of actin filaments and cell-to-cell spread. A Tn*917* insertion upstream of *prfA* results in decreased hemolytic expression and secretion of PI-PLC. However, unlike *prfA* structural gene mutations, this mutant retains enough activity to lyse the phagocytic vacuole and enter the cytoplasm. Interestingly, this mutant is defective in nucleating actin filaments and consequently is defective in cell-to-cell spread (11, 38). This suggests that PrfA is required for events that occur in the host cytoplasm as well as in the vacuole. An intriguing possibility exists that regulation of *prfA* expression is mediated by exposure of *L. monocytogenes* to the specific environments found within the different compartments of the host cell.

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