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The Expression of Virulence Genes in *Listeria monocytogenes* Is Thermoregulated

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The expression of listeriolysin, a major virulence factor of the gram-positive facultative intracellular pathogen *Listeria monocytogenes*, is positively regulated by a transcriptional activator, the *prfA* gene product. We had previously shown that mutations within the *prfA* gene lead to loss of listeriolysin production. In this communication, the regulation of expression of listeriolysin by a specific environmental condition, namely, temperature, was studied in wild-type strains of *Listeria monocytogenes*. We found that expression of the hemolysis phenotype was thermoregulated. A *lisA*::*lacZ* fusion was constructed, and its expression in the wild-type strain was studied at various growth temperatures. The results showed that the fusion β-galactosidase activity was expressed only when cultures were grown at temperatures above 30°C. This activity could be either specifically repressed or induced, depending on growth temperature. No change in activity was detected in a strain harboring a control β-galactosidase fusion at the various growth temperatures tested. Northern (RNA) blot analysis of *lisA*-specific RNA transcripts showed that thermoregulation is manifested at the level of transcription. We also found that the transcription of other PrfA-regulated virulence genes in *L. monocytogenes* was similarly affected by growth temperature. Hence, as in other facultative intracellular pathogens, *Shigella* and *Yersinia* spp., temperature is an important cue in the induction of expression of virulence genes in *L. monocytogenes*. Our studies revealed that a higher level of regulation is imposed on the PrfA-mediated activation of virulence genes in pathogenic *L. monocytogenes*.

The gram-positive facultative intracellular bacterium *Listeria monocytogenes* is an opportunistic pathogen, particularly in immunocompromised persons, pregnant women, and newborns (32). The consumption of contaminated food has been shown to be a significant infection route for human listeriosis, and several recent epidemics have underscored the significance of *L. monocytogenes* in food-borne infections (8, 19, 31). Listerial infections can result in septicemia, meningitis, meningoencephalitis, and death. The virulence of *L. monocytogenes* is attributed to its ability to survive and replicate in cells of the infected host (20). Following oral uptake of infected food, *L. monocytogenes* is able to cross the intestinal barrier and spread to the internal organs, where it causes the formation of granulomatous foci. In severe infections, the endothelial barriers to the brain or the placenta may be bridged. Hence, the intruding bacterium must possess an array of factors that permit invasion and assist and promote evasion from immune defenses.

The best-characterized listerial virulence factor necessary for intracellular survival is listeriolysin, a hemolytic cytolysin, that allows the invading bacterium to escape from the phagosome by lysing the phagolysosomal membrane (9, 11, 27). We have recently localized the listeriolysin gene (*lisA*) in a cluster of genes that is uniquely present in the pathogenic species *L. monocytogenes*. This region includes the *mpl* gene encoding a metalloprotease and a gene encoding a phosphatidylinositol-specific phospholipase C (*pic*) located downstream and upstream of the listeriolysin gene (*lisA*), respectively (5, 17). Mutations within any one of these genes lead to a loss of virulence of *L. monocytogenes* (4, 9, 11, 13, 22). These virulence genes are positively and coordinately regulated at the transcriptional level by the product of the *prfA* gene, which is itself located 1.5 kb 5′ of the *lisA* gene (3, 17, 18). Hence, like virulence gene determinants of several gram-positive and gram-negative pathogens, such as *Staphylococcus aureus* (28), *Streptococcus pyogenes* (33), *Shigella* spp. (30), *Vibrio cholerae* (26), *Bordetella pertussis* (29), and *Yersinia* spp. (36), expression of virulence genes in *L. monocytogenes* is coordinately regulated. Little is known of the expression of virulence determinants in *L. monocytogenes* as responses to specific environmental conditions. Since *L. monocytogenes* is a ubiquitous organism that is capable of growth even at very low temperatures, we examined the effect of temperature on the expression of virulence genes. In this communication, we report that the production of pathogenic factors in *L. monocytogenes* is dependent on growth temperature. Indeed, expression of all virulence factors known to be positively regulated by the PrfA protein is also coordinately repressed at lowered temperatures of growth.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. monocytogenes* EGD serovar 1/2a and SLCC 4013 serovar 4b have been described previously (15). *L. monocytogenes* SLCC 5348 serovar 1/2c and SLCC 5214 serovar 4a were obtained from the special *Listeria* culture collection of the Institute of Hygiene and Microbiology, University of Würzburg, Würzburg, Germany. *Listeria* spp. were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at various temperatures with aeration. For plasmid-containing *Listeria* strains, the medium was supplemented with 5 μg of erythromycin per ml.

Plasmids. Plasmids pMG14 and pGK20 were obtained from J. Kok (University of Groningen, Groningen, The

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Netherlands) (12). Plasmid pMG14 harboring a β-galactosidase (*lacZ*) gene lacking both transcriptional and translational signals was used to construct a *lisA*::*lacZ* translational fusion. A 852-bp *Eco*RI fragment from plasmid pLM47 (15) extending from the *Eco*RI site in the multiple cloning site of the vector and containing the promoter region and the first 399 bp up to the *Eco*RI site within the *lisA* gene was inserted proximal to the *lacZ* gene on plasmid pMG14 to generate plasmid pMG47-1. Plasmid pGK20 harbors a 345-bp *Eco*RI fragment carrying a strongly expressed streptococcal proteinase promoter (*prtP*) translationally fused to the *lacZ* gene on pMG14 (Fig. 2).

Transformation in *L. monocytogenes*. Plasmids were introduced into *L. monocytogenes* by the protoplast transformation protocol as described previously (38).

Polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 10% separating gels, using trichloroacetic acid-precipitated supernatants from 500 μl of a culture grown overnight (optical density at 600 nm [OD_{600}], 1.5). Proteins were electroblotted onto nitrocellulose filters (14) and detected with anti-listeriolysin rabbit antisera. Blots were developed by using horseradish peroxidase-conjugated anti-rabbit antisera, 0.015% hydrogen peroxide, and 4-chloro-naphthol as substrates.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated from stationary cultures (OD_{600}, 1.5 to 1.6) of *L. monocytogenes* by the hot phenol extraction method (18). RNA analysis was performed by formaldehyde gel electrophoresis (1). RNA samples (10 μg) were electrophoresed in a 1.2% agarose gel, transferred onto nylon filters (Hybond N; Amersham), and hybridized with appropriate radiolabeled DNA fragments (7), using conditions as specified by the vendor.

Primer extension studies. The synthetic oligonucleotide 5′-CTGCTTGAGCGTTCATGTCTCATCCCCC-3′ including the initiation codon of the *prfA* gene was used as the primer and labeled at its 5′ end (1). Primer extension analysis with avian myeloblastosis virus reverse transcriptase (Pharmacia) was performed as described previously, except that 50 μg of total RNA was used (18).

Preparation of DNA probes. Specific DNA probes for the *prfA*, *pic*, *lisA*, and *mpl* genes were obtained after digestion of plasmid pLM51 (18) with the appropriate restriction endonucleases and electroelution of the required DNA fragment following agarose gel electrophoresis. The regions covered by the gene probes are depicted in Fig. 3.

Hemolysin and β-galactosidase assays. The hemolysin titer assay was performed as described previously (15). In this study, we used 0.5% erythrocytes to assay for the very low hemolytic activity in culture supernatants. β-Galactosidase activity was assayed by the method of Miller (25).

RESULTS

Production of listeriolysin at various growth temperatures. Listeriae are ubiquitous bacteria in soil and are capable of growth even at very low temperatures, a property that has been previously used as an enrichment technique in the isolation and identification of *Listeria* spp. (37). Hence, the hemolytic activities expressed by several *L. monocytogenes* strains comprising different serovars and expressing various levels of hemolytic activity in cultures grown at either 20, 30, or 37°C was determined (Table 1). Previous studies had demonstrated that the expression of hemolytic activity was highest when bacteria were grown to stationary phase (10, 16). In cultures that had been grown overnight at either 20 or 30°C, little or no hemolytic activity was detected. When the same strains were grown at 37°C, this hemolytic activity was 8- to 16-fold higher, depending on the strain studied (Table 1). Immunoblot analysis using rabbit anti-listeriolysin antisera confirmed that increased levels of hemolytic activity directly corresponded to enhanced production of the listeriolysin polypeptide in the supernatant fluids of these strains (Fig. 1).

TABLE 1. Hemolytic titers of culture supernatants from *L. monocytogenes* strains grown at various temperatures[a]

L. monocytogenes strain	Serovar	Hemolytic titer (hemolytic units) of strain grown at:		
		20°C	30°C	37°C
EGD	1/2a	1	2	32
SLCC 5348	1/2c	1	4	64
SLCC 4013	4b	1	4	32
SLCC 5214	4a		2	16

[a] Bacteria were grown overnight in brain heart infusion broth. Hemolytic titers of supernatant fluids were determined as described in Materials and Methods. The corresponding immunoblot developed with anti-listeriolysin rabbit antisera is depicted in Fig. 1.

Influence of the growth temperature on the expression of a *lisA*::*lacZ* fusion protein. In order to examine the influence of growth temperature on listeriolysin production, we constructed plasmid pMG47-1. This plasmid harbored the dual promoters and the proximal 399 bp of the *lisA* gene translationally fused to the *lacZ* gene of *Escherichia coli* on plasmid pMG14. Its expression was compared with that of a plasmid pGK20 harboring the promoter and the first 120 bp of the streptococcal protease (*prtP*) fused at the same *Eco*RI site to

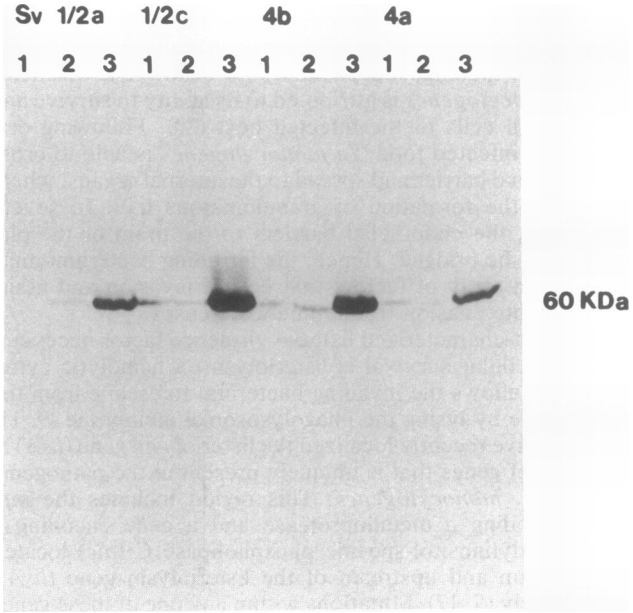

FIG. 1. Immunoblot of trichloroacetic acid-precipitated *L. monocytogenes* culture supernatants developed with anti-listeriolysin antiserum. Each lane contains the equivalent of 50 μg of protein. The *L. monocytogenes* strains tested were EGD, SLCC 5348, SLCC 4013, and SLCC 5214. The serovar (Sv) is indicated over the lanes. The cultures were grown at 20°C (lanes 1), 30°C (lanes 2), and 37°C (lanes 3).

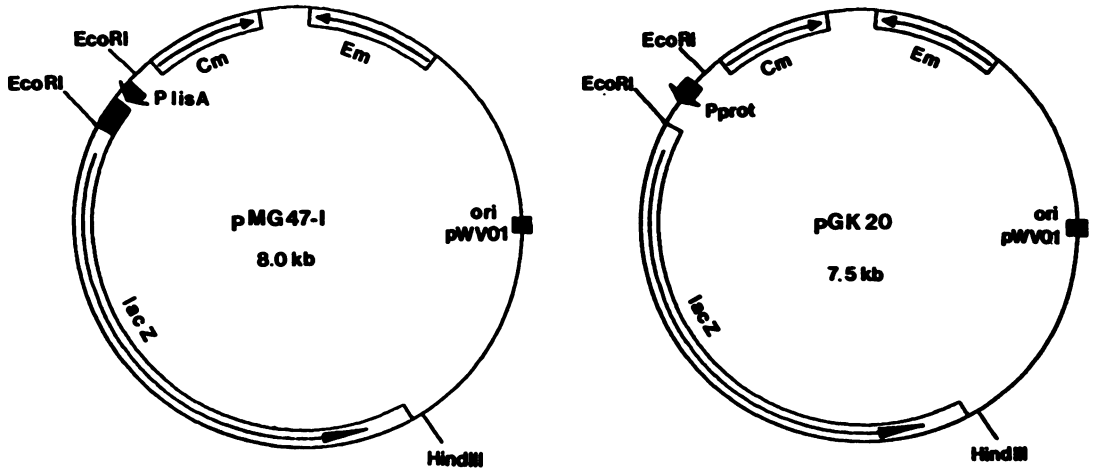

FIG. 2. Physical maps of recombinant plasmids pMG47-1 and pGK20. Plasmid pMG47-1 expressed a listeriolysin::β-galactosidase fusion protein, and a streptococcal protease::β-galactosidase fusion polypeptide is produced in bacteria harboring plasmid pGK20. The positions of some relevant markers on these plasmids and the direction of transcription are given. The cutting sites for the restriction endonucleases *Eco*RI and *Hin*dIII are indicated. Cm, gene conferring chloramphenicol resistance in *L. monocytogenes*; Em, gene conferring erythromycin resistance in *L. monocytogenes* and *E. coli*; *lacZ*, promoterless copy of the *E. coli* β-galactosidase gene; *ori* pVW01, origin of replication from the lactococcal plasmid pWV01. The size of each plasmid is given in kilobases. P_{lisA}, listeriolysin promoters P_1 and P_2 (18); P_{prot}, streptococcal protease promoter (12).

the *lacZ* gene. Hence, these plasmids are identical to each other, except for the *Eco*RI fragments inserted in front of the *lacZ* genes (Fig. 2). The fusion β-galactosidase polypeptide expressed by pGK20 was used as a control, because it was constitutively expressed in *L. monocytogenes* (see below). The results of β-galactosidase activity determined for *L. monocytogenes* strains harboring either plasmid and grown overnight at the different temperatures are depicted in Table 2. These data clearly showed that little or no β-galactosidase activity was detectable in the *L. monocytogenes* strain carrying the *lisA*::*lacZ* fusion at temperatures between 4 and 33°C. A 40-fold-higher level of activity was, however, observed in cultures that were grown at 37°C. Unlike the *lisA*::*lacZ* fusion, the *prtP*::*lacZ* fusion was strongly expressed at all the temperatures tested (Table 2).

In the next series of experiments, we examined the effect of changes of growth temperatures on the expression of β-galactosidase activity in *L. monocytogenes* strains harboring either plasmid pMG47-1 or pGK20. Bacterial cultures were first grown at 37°C to an OD_{600} of 1.3, and portions were shifted to 30 and 42°C or kept at 37°C. At various time points following the shift, the level of fusion β-galactosidase activity was determined. In cultures that were shifted down to 30°C, no activity was detectable within 1 h of the downshift. In contrast, β-galactosidase activity increased by three- and fourfold, respectively, in cultures that were maintained at 37°C or shifted to 42°C (Table 3). Strains harboring the streptococcal *prtP*::*lacZ* fusion showed no significant change in β-galactosidase activity at all the temperatures tested. These data indicated that expression of listeriolysin is modulated by changes in growth temperature.

Thermoregulation of virulence genes in *L. monocytogenes* is mediated at the level of transcription. To determine the molecular level at which thermoregulation exerted its influence, we analyzed the mRNA level of *lisA* transcripts in *L. monocytogenes* grown overnight at either 20 or 37°C. RNAs extracted from strain EGD (wild type) grown at both tem-

TABLE 2. Expression of fusion β-galactosidase activities in *L. monocytogenes* EGD harboring plasmid pMG47-1 or pGK20

Strain	β-Galactosidase activity[a] (Miller units) in cultures grown at:					
	4°C	20°C	30°C	33°C	35°C	37°C
L. monocytogenes [pMG47-1] (*lisA*::*lacZ*)			2	2	15	94
L. monocytogenes [pGK20] (*prtP*::*lacZ*)	360	590	890	900	950	1,030

[a] Results shown represent the mean values for three experiments. The standard deviation was <10% of the mean for all experiments.

TABLE 3. Effects of temperature shifts on β-galactosidase activity in *L. monocytogenes* EGD containing plasmids harboring the *lisA*::*lacZ* and *prtP*::*lacZ* fusions[a]

Strain	New temperature (°C)	β-Galactosidase activity[b] at the following time (min) after temperature shift		
		30	60	120
L. monocytogenes [pMG47.1] (*lisA*:*lacZ*)	30	18	ND	ND
	37	32.5	53	86
	42	34	56	112
L. monocytogenes [pGK20] (*prtP*:*lacZ*)	30	710	750	835
	37	690	742	810
	42	661	680	650

[a] Bacteria were grown in brain heart infusion broth to an OD_{600} of 1.3. The β-galactosidase values at time zero for *L. monocytogenes* carrying either pMG47.1 or pGK20 were 28 and 660 U, respectively.

[b] The β-galactosidase units represent the mean values for three experiments. ND, not detectable.

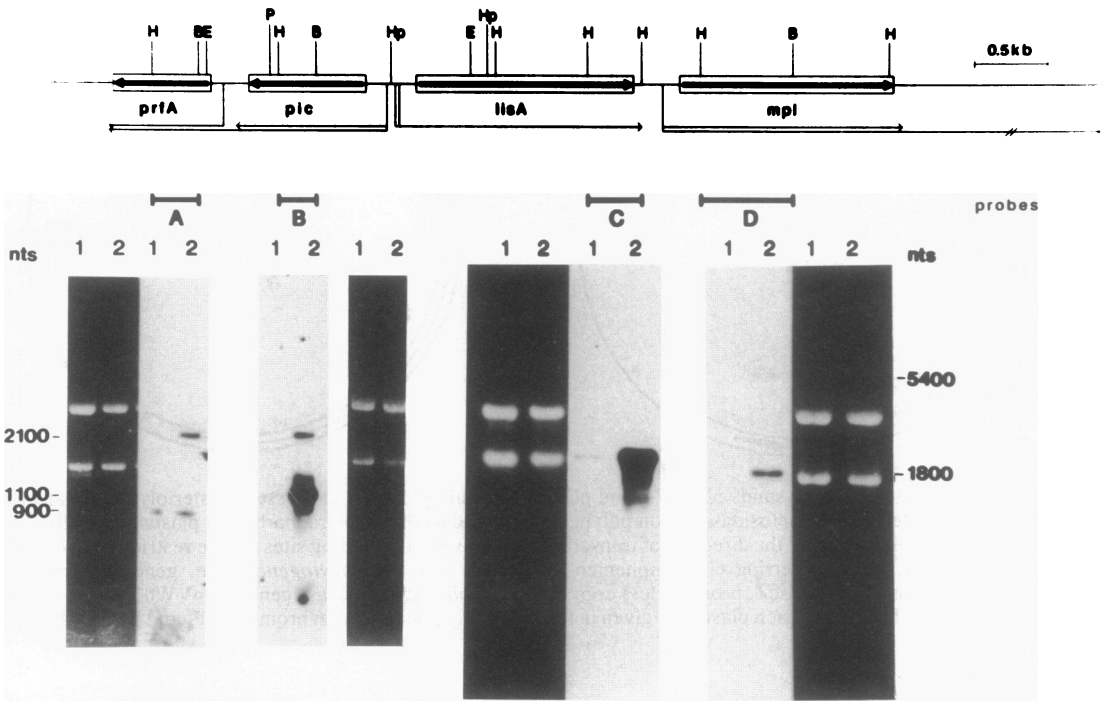

FIG. 3. (Upper) Physical map of the chromosomal region of *L. monocytogenes* EGD harboring the *prfA*, *pic*, *lisA*, and *mpl* genes. The direction of transcription of each gene is indicated. DNA probes used in the detection of the various transcripts depicted are lettered A, B, C, and D. (Lower) Agarose gel electrophoresis of total RNA and the corresponding autoradiograms from Northern blot analysis of transcripts produced by strain EGD at 20 (lanes 1) and 37°C (lanes 2). The following transcript sizes (in nucleotides [nts]) were detected: 900 and 2,100 for *prfA*, 1,100 and 2,100 for *pic*, 1,800 for *lisA*, and 1,800 and 5,400 for *mpl*.

peratures were subjected to Northern blot hybridization analysis using a radiolabeled probe specific for the *lisA* gene (Fig. 3). Low levels of the 1,800-nucleotide *lisA*-specific transcript were detected only at the lower temperature. This level increased markedly in cultures that were grown at 37°C. These results demonstrated that expression of the *lisA* gene is modulated at the transcriptional level at different growth temperatures.

Transcription of the listeriolysin gene in *L. monocytogenes* is positively regulated by the product of the *prfA* gene (18). This prompted us to examine whether the *prfA* regulator is itself subject to thermoregulation. Northern blot analysis of total RNA from cultures grown at either 20 or 37°C showed that the 900-nucleotide-long transcript corresponding to the *prfA* gene was equally well transcribed at either growth temperature (Fig. 3). Using a primer extension assay, we confirmed this result and demonstrated that transcription of the *prfA* gene initiated at the same nucleotide at both temperatures (Fig. 4). Nevertheless, in cultures grown at 37°C, another transcript of 2,100 nucleotides in length was observed in addition to the 900-nucleotide *prfA* transcript. Further experiments showed that the longer transcript was the result of transcription originating from the promoter of the *pic* gene (see below). Hence, the *prfA* gene is transcribed from two different promoter regions at higher growth temperatures, and it is the longer bicistronic transcript that is subject to temperature regulation.

In *L. monocytogenes*, listeriolysin is coordinately expressed with the *pic* and *mpl* genes whose products encode phosphatidylinositol-specific phospholipase C (17) and metalloprotease, respectively (5). We therefore also analyzed mRNA levels of both these genes in cultures grown at either 20 or 37°C. Little or no specific RNA transcripts were seen for either gene in cultures grown at 20°C. However, the level of transcription of these genes increased strongly in cultures grown at 37°C. We obtained evidence that both the *pic* and *mpl* genes are proximal genes in each of two operons flanking the monocistronic *lisA* gene. Of the two transcripts of 1,100 and 2,100 nucleotides that were detected by using a

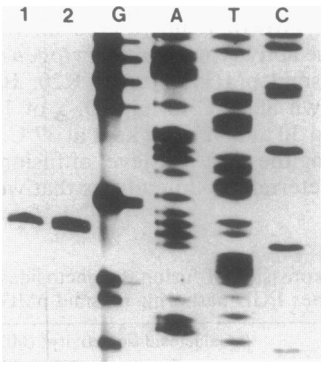

FIG. 4. Mapping and detection of *prfA* transcripts in *L. monocytogenes* EGD by primer extension analysis. An α-^{32}P-end-labeled 28-nucleotide-long primer (see Materials and Methods) was used for reverse transcription of the transcript. The DNA products were separated on a 6% polyacrylamide gel simultaneously with a dideoxynucleotide sequencing reaction ladder, using the same DNA primer and plasmid pLM47 as templates to allow determination of the extension product. Lanes 1 and 2 show results of the primer extension reaction done with total RNA from strain EGD grown at 37 and 20°C, respectively. Lanes denoted G, A, T, and C are tracts of the sequencing reaction ladder.

DNA probe internal to the *pic* gene at 37°C (Fig. 3), the larger transcript contains both the *pic* and *prfA* genes, while the smaller transcript contains only the *pic* gene. For the *mpl* gene, two transcripts were also detected. The smaller 1,800-nucleotide transcript corresponded to only *mpl* mRNA, while the larger transcript of 5,400 nucleotides includes the *mpl* gene and a large open reading frame located downstream. These results clearly demonstrated that the expression of *lisA*, *pic*, *mpl* and at least one further gene located downstream of the *mpl* gene in *L. monocytogenes* is thermoregulated. Regulation is effected by changes in the transcription levels of the respective genes at different growth temperatures.

DISCUSSION

In this communication, we presented results demonstrating that the expression of several virulence genes in pathogenic *L. monocytogenes* was coordinately regulated by growth temperature. In our initial experiments, we found that the hemolytic activity of various *L. monocytogenes* serotype strains varied with growth temperature. These results were confirmed and extended by examining expression of a listeriolysin::β-galactosidase fusion at various growth temperatures and comparing the results obtained with a constitutively expressed streptococcal protease::β-galactosidase fusion under the same conditions. The process of temperature-mediated modulation of expression of the listeriolysin gene was specific and rapid and was already observed 1 h after cultures were shifted to higher or lower growth temperatures. Finally, Northern blot analysis revealed that temperature regulation is exerted at the level of transcription of the gene.

Using gene fusion technology, we found that the expression of *lisA*::*lacZ* β-galactosidase activity was affected by growth temperature. *L. monocytogenes* grown at temperatures prevalent in the environment had lower β-galactosidase activity compared with those grown at the temperature of the infected host, i.e., 37°C (Tables 2 and 3). The further increase in activity observed at 42°C can be best explained as a adaptive mechanism of the invading bacteria to stress as a result of increased body temperatures or inflammation resulting from infection of the host. This is in agreement with studies showing listeriolysin expression in *L. monocytogenes* EGD to be inducible by stress factors, like hydrogen peroxide or elevated temperatures (34, 35).

In the studies reported here, the various gene fusions were found to be thermoregulated, even though these genes were harbored in *trans* on vector plasmids. This is in contrast with the situation observed in *Shigella* spp. in which the effect of temperature on temperature-regulated genes is alleviated when they are cloned onto multicopy plasmids (21, 30). The results obtained here could have been due to the fact that the streptococcal pWV01 replicon present on these plasmids is maintained at a low copy number (between 5 and 10 copies per chromosomal equivalent [16]) in *L. monocytogenes*. The finding in this study that the *prtP* streptococcal promoter present on plasmid pGK20 is constitutively expressed at a level more than 10 times higher than that of the listeriolysin promoter suggests its usefulness in the construction of expression vectors in *Listeria* spp.

Apart from the listeriolysin gene, we discovered that several other genes clustered in this region of the listerial chromosome were also thermoregulated. Coordinate expression of the *pic* and *mpl* genes has been recently shown to be mediated by the *prfA* gene product (17, 18). Both the *pic* and *mpl* genes, like the *lisA* gene, encode de facto virulence factors of pathogenic *Listeria* spp. (4, 22). Furthermore, an isogenic *L. monocytogenes* strain, mutated in the open reading frame located downstream of the *mpl* gene, is incapable of inducing actin accumulation following invasion of the eukaryotic cell and is avirulent when tested in a mouse model of infection (3). Northern blot analysis of transcripts indicated that the *pic* and *mpl* genes are part of two divergent operons flanking the listeriolysin gene (Fig. 4). Nevertheless, each gene itself is also transcribed monocistronically. A major finding in this study is that all transcripts originating from the *pic*, *lisA*, and *mpl* promoters are thermoregulated, regardless of whether they give rise to mono- or polycistronic mRNAs. In this respect, it is interesting to note that a consensus palindromic sequence is present in the −44 region of all promoters studied here except the *prfA* gene (23).

The pattern of transcription of the *prfA* gene itself was more complex with regard to thermoregulation. While there were virtually no changes in the level of the 900-nucleotide-long transcript corresponding to the *prfA* gene alone at either 20 or 37°C, the larger 2,100-nucleotide-long bicistronic transcript consisting of both the *pic* and *prfA* genes was present only at 37°C. A termination sequence has been postulated for the *pic* gene so that either there is antitermination at this putative terminator or the termination sequence is too weak to prevent strong transcripts originating from an activated *pic* promoter (17). Hence, there are more transcripts corresponding to the *prfA* gene at 37°C than at 20°C.

Although the *prfA* gene is transcribed from its own promoter constitutively at either temperature, there is little or no transcription of PrfA-regulated genes at low temperatures. Hence, the production of other cellular components that act in concert with the PrfA polypeptide to induce transcription may be limiting at lower temperatures. On the other hand, the PrfA polypeptide could alternate between active and inactive forms, depending on growth temperature. This could result from either temperature-dependent conformational changes or posttranslational chemical modification of the PrfA polypeptide which would allow it to activate transcription from the temperature-regulated promoters of the *pic*, *lisA*, and *mpl* genes. Alternatively, temperature regulation of virulence genes may be mediated by changes in the superhelicity of DNA as in *Shigella* spp., where such changes are postulated to be induced by a histonelike protein, VirR (6). Studies aimed at elucidating the molecular action of the PrfA polypeptide and its quantification under different growth conditions will permit clarification of its role in temperature regulation.

Coordinate expression of virulence genes of many intracellular bacterial pathogens is regulated by environmental stimuli (21, 24). The results presented herein revealed two levels of regulation of virulence genes in pathogenic *L. monocytogenes*, both of which operate at the transcriptional level. Virulence gene expression in *L. monocytogenes* is very efficient at temperatures existing inside the mammalian host but barely detectable at environmental temperatures. This is a physiologically economic principle also used by other bacterial pathogens, such as *Bordetella*, *Yersinia*, or *Shigella* species (21). Hence, the regulatory factor *PrfA* may be seen as a factor evolved to potentiate the expression of virulence genes in pathogenic *L. monocytogenes* under conditions such as those that might exist in the eukaryotic host.

Clearly, none of the listerial genes examined in this study are required for growth in the environment; nonpathogenic *Listeria* spp. do not harbor these genes and are nevertheless ubiquitous in nature. The signals and molecular mechanisms involved in sensing and transducing these environmental

changes in pathogenic *L. monocytogenes* will provide insight as to how such adaptive processes have evolved in intracellular parasites.

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