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Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*

(intracellular pathogen/hemolysin/transcriptional activator)

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ABSTRACT **We have isolated, by molecular cloning and genetic complementation of a listeriolysin-negative mutant, a gene required for the expression of this virulence factor in *Listeria monocytogenes*. The mutant strain SLCC53, which was nonhemolytic and avirulent, harbored a deletion of 450 base pairs located ≈1500 base pairs upstream of the listeriolysin gene. No transcripts corresponding to the listeriolysin gene were detected in the mutant. DNA sequencing of this region from the hemolytic strain EGD revealed that the region deleted in the mutant would abrogate expression of a 27-kDa polypeptide. Introduction of a recombinant plasmid expressing this 27-kDa polypeptide restored hemolytic activity to the mutant and increased the hemolytic activity of the wild-type *L. monocytogenes* strain EGD. We have designated the gene encoding the 27-kDa polypeptide *prfA*, for positive regulatory factor of listeriolysin (*lisA*) expression. The *prfA* gene regulates transcription of the *lisA* gene positively.**

The hemolytic toxin listeriolysin has long been recognized as an important virulence factor of *Listeria monocytogenes*, a ubiquitous Gram-positive microorganism that is responsible for, in its severest form, a meningoencephalitis in animals and human beings (1). Clinical isolates of *L. monocytogenes* are invariably hemolytic and virulent when tested in a rodent model of infection (2). Transpositional inactivation of the listeriolysin gene in pathogenic *L. monocytogenes* strains leads to loss of hemolytic activity, avirulence in the animal model (3, 4), and failure to grow in tissue culture cells (5–7). It now appears that secretion of listeriolysin is an essential prerequisite to bacterial growth and spread in host tissues where bacteria are trapped within the host phagocytic cells (8, 9). Specifically, it has been suggested that listeriolysin participates in the lysing of the phagolysosomal membrane, allowing the invading bacteria free access to the eukaryotic cytoplasm. Although much progress has been made in understanding the role of listeriolysin in the pathogenesis of listerial infections, little is known at the molecular level about factors that regulate its expression.

The listeriolysin genes from two different serotypes of *L. monocytogenes* have been cloned and sequenced (10, 11). Despite large differences in their hemolytic phenotype, the sequences corresponding to the respective listeriolysin gene and its promoter region are identical in both strains. Hence, variation in the hemolytic activity is likely to be determined by differences in regions external to the listeriolysin gene. Recently, we reported on the detection of a small deletion of 450 base pairs (bp) in strain SLCC53, a nonhemolytic mutant of a *L. monocytogenes* serotype 1/2a strain that was located to 1.6 kilobases (kb) upstream of its listeriolysin gene (12). The deletion rendered a strain avirulent and thus defined a locus that not only affected listeriolysin expression but may also regulate virulence in *L. monocytogenes* as a whole.

In this study, we show that the ahemolytic phenotype of strain SLCC53 is due to a lack of transcription of the listeriolysin gene. Hemolytic activity could be restored by transcomplementation of the mutant with a plasmid harboring a chromosomal segment from a hemolytic wild-type strain that flanks and covers the deletion present in strain SLCC53. Introduction of the same plasmid into the hemolytic *L. monocytogenes* strain EGD led to an increase in the hemolytic activity of this strain. Molecular cloning, sequencing, and maxicell analysis of recombinant plasmids containing this region revealed that a polypeptide of 27 kDa would be removed by the deletion in the ahemolytic strain SLCC53. The data presented provide evidence for a factor that is necessary for listeriolysin production and that positively regulates its expression at the transcriptional level. We have designated this gene *prfA*, for positive regulatory factor of listeriolysin (*lisA*) production.†

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *L. monocytogenes* serotype 1/2a strains SLCC53, a spontaneously occurring nonhemolytic strain that is avirulent for mice, and EGD, a weakly hemolytic strain virulent for mice, have been described (12, 13). The *Escherichia coli* strain DH5α was used for transformation and cloning. Maxicell labeling of plasmid-encoded polypeptides was performed by using strain CSH26ΔF6 (13).

The cloning vectors pERL1 and pERL3 are plasmid-shuttle vectors capable of replication in both *E. coli* and *Listeria* spp. A detailed description of their construction and properties will be presented elsewhere. Briefly, the plasmid is a composite of two plasmids, one carrying the origin of replication of the plasmid pAMβ1 and an erythromycin-resistance gene (14) joined to a fragment harboring the pBR322 replicon (15) and the kanamycin gene from Tn*5* (16). A polylinker cassette in opposite orientations is present in the plasmids pERL1 and pERL3, respectively. pERL1 51-1 contains the 3.45-kb *Bam*HI fragment harboring the *lisA* gene present on plasmid pLM51. A 1.6-kb *Pst* I/*Xba* I fragment from plasmid pLM50 is present in pERL3 50-1.

Media and Antibiotics. *L. monocytogenes* was grown in brain–heart infusion broth (Difco) at 37°C. *E. coli* strains were grown routinely in Luria–Bertani broth at the same temperature. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml for *E. coli*; erythromycin, 300 μg/ml for *E. coli* and 5 μg/ml for *L. monocytogenes*. Restriction enzymes and ligase were purchased from Boeh-

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Abbreviation: ORF, open reading frame.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55160).

ringer Mannheim and were used as recommended by the manufacturer.

Transformation. Plasmids were transformed into *L. monocytogenes* following the method of Lucas and Levine (17).

Primer-Extension Analyses. A synthetic oligonucleotide corresponding to 20 nucleotides immediately upstream and including the initiation codon of the *lisA* gene was 5'-end-labeled and used as primer. Labeled primer (2 pmol) was hybridized to 100 μg of cellular RNA in 6 μl of a solution containing 50 mM Tris·HCl (pH 8.3), 60 mM NaCl, 10 mM dithiothreitol. Subsequently, 9 μl of reaction mixture was added, resulting in a solution containing 0.5 μl (20 units) of avian myeloblastosis virus reverse transcriptase (Pharmacia), 50 mM Tris·HCl (pH 8.3), 60 mM NaCl, 10 mM dithiothreitol, 6 mM magnesium acetate, 2.5 units of RNase inhibitor (Sigma), and 0.5 mM each dNTP. The samples were incubated at 42°C for 90 min, vacuum dried, and resuspended in 10 μl of formamide dye mixture. Three-microliter aliquots were then heated at 95°C for 3 min and immediately loaded onto 6% polyacrylamide/urea sequencing gels for electrophoresis. Dideoxynucleotide sequencing reactions (18) using the same primer and an appropriate plasmid DNA template were run in parallel to allow determination of the endpoints of the extension products.

Nucleotide Sequence. The nucleotide sequence of the *prfA* gene was determined on plasmid pLM50 by a modification of the Sanger method for supercoiled templates as described by Zagursky *et al.* (18). Homology searches were performed on the National Biomedical Research Foundation data base by using the FASTP algorithm (19).

PAGE and Electrophoretic Analysis of Plasmid-Coded Polypeptides. SDS/PAGE was performed with a 12.5% separating gel using 10 μl of trichloroacetic acid-concentrated supernatants (1 ml) of *L. monocytogenes* strains in sample buffer. Polypeptides were visualized by staining the gel with Coomassie blue R-250.

Analysis of plasmid-coded translational products in a maxicell system has been described (20). Labeling of polypeptides was carried out in methionine assay medium (Difco) containing [^{35}S]methionine (Amersham; Buchler). Samples were analyzed as described above, dried, and used to expose Fuji RX-X-ray film.

Hemolysin Assays. Hemolytic titers were determined as described (12). Where appropriate supernatant fluids were supplemented with either 10 mM dithiothreitol or cholesterol (25 μg/ml).

RESULTS

The Ahemolytic Strain SLCC53 Contains a Transcriptionally Silent Listeriolysin Gene. The nonhemolytic *L. monocytogenes* strain SLCC53 is deficient in the production of listeriolysin. In a previous study, we showed that this strain harbored sequences corresponding to the entire listeriolysin gene, and we attributed the lack of listeriolysin production to a deletion located 1600 bp 5' to the listeriolysin gene (*lisA*) (12). Still, the Southern hybridization analysis performed would not have ruled out the possibility of the presence of single site mutations within the listeriolysin gene that could abrogate its activity. Hence, if the listeriolysin gene in SLCC53 is mutated, introduction of a plasmid harboring an intact copy of the listeriolysin gene would restore hemolytic activity. To test this hypothesis, we introduced a recombinant plasmid pERL1 51-1, harboring the listeriolysin gene, including its promoter and terminator regions cloned from the hemolytic *L. monocytogenes* strain EGD, into strain SLCC53 and assayed for the reappearance of hemolytic activity. No hemolytic activity was detected in the strain SLCC53 harboring plasmid pERL1 51-1. When the plasmid carrying the listeriolysin gene was introduced into the hemolytic *L. monocytogenes* strain EGD, a 4-fold increase in hemolytic activity was detected in its supernatant fluids (Table 1). Thus, strain SLCC53 does not produce listeriolysin even if intact copies of the *lisA* gene are supplied in trans within the strain. SDS/PAGE analysis of trichloroacetic acid-precipitated supernatants of strains SLCC53 and EGD, both with and without plasmid pERL1 51-1, showed that the amount of secreted listeriolysin increased when the *lisA* gene was introduced into strain EGD (Fig. 1*B*, lanes 1 and 4); no protein was produced in the SLCC53 strains (Fig. 1*A*, lanes 1 and 2).

Table 1. Hemolytic activity of *L. monocytogenes* strains harboring plasmids expressing the *lisA* and *prfA* genes

Strain	Plasmid	Hemolytic titer
EGD	pERL3	8
EGD	pERL1 51-1 (*lisA*)	32
EGD	pERL3 50-1 (*prfA*)	32
SLCC53	pERL3	—
SLCC53	pERL1 51-1 (*lisA*)	—
SLCC53	pERL3 50-1 (*prfA*)	32

Hemolytic titer is expressed as complete hemolytic units, which is defined as the reciprocal of the highest dilution at which complete lysis of erythrocytes is observed.

To further analyze the basis for the lack of expression of the listeriolysin gene in SLCC53, primer-extension analysis of RNA was performed. RNA obtained from *L. monocytogenes* 1/2a strains EGD and SLCC53 showed that in strain EGD, RNA transcripts corresponding to the *lisA* gene are initiated at two distinct sites 10 nucleotides apart located 130 nucleotides upstream of its initiation codon. Both transcription initiation sites were entirely absent in the nonhemolytic strain (Fig. 2). The deletion present 1.6 kb upstream of the listeriolysin gene in strain SLCC53 is therefore associated with the concomitant absence of RNA transcripts corresponding to the listeriolysin gene and suggests a functional linkage between these two loci.

Molecular Cloning of the Region Corresponding to the Deletion in Strain SLCC53. To understand the nature of the molecular defect present and its relationship to the loss of

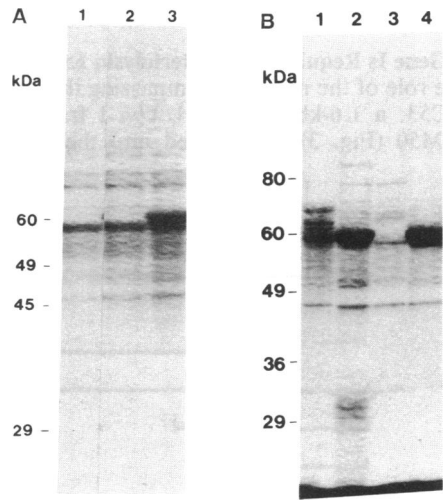

FIG. 1. SDS/PAGE of secreted polypeptides of *L. monocytogenes* strains harboring plasmids carrying the *lisA* and *prfA* genes. Proteins were precipitated from culture supernatant fluids with 10% trichloroacetic acid and separated on a 10% SDS/polyacrylamide gel. (*A*) Lanes: 1, SLCC53 (pERL3); 2, SLCC53 (pERL1 51-1 *lisA*); 3, SLCC53 (pERL3 50-1 *prfA*). (*B*) Lanes: 1, EGD (pERL3); 2, EGD (pERL3 50-1 *prfA*); 3, SLCC53 (pERL3); 4, EGD (pERL1 51-1 *lisA*). Arrow indicates position of the listeriolysin gene product. Molecular mass standards (in kDa) are indicated.

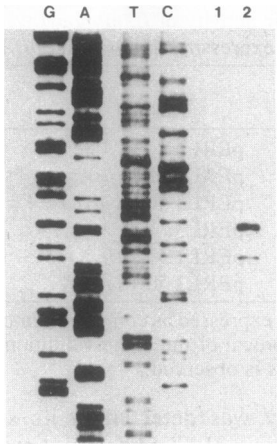

FIG. 2. Mapping and detection of *lisA* transcripts by primer-extension analysis. A ^{32}P-end-labeled primer, corresponding to 20 nucleotides preceding and including the initiation codon of the listeriolysin gene, was used for reverse transcription of the *lisA* transcript. The DNA products were separated on an 8% polyacrylamide gel simultaneously with a dideoxynucleotide sequencing reaction ladder using the same DNA primer and plasmid pLM48 as template to allow determination of the end point of the extension product. Lanes G, A, T, and C are tracks of sequencing reaction; 1 and 2 denote primer-extension reactions done with total RNA from strains SLCC53 and EGD, respectively.

hemolytic activity in strain SLCC53, we cloned a region from a wild-type *L. monocytogenes* serotype 1/2a strain corresponding to the region absent in strain SLCC53. To do this, a 135-bp radiolabeled probe harboring upstream sequences most distal to the listeriolysin gene in pLM48 (ref. 13 and Fig. 3) was used to screen a plasmid-based gene library constructed from the hemolytic wild-type *L. monocytogenes* strain EGD. Three recombinants were obtained, all of which carried inserts of various lengths but contained regions common to the DNA probe used. Restriction endonuclease mapping of the three plasmids revealed that these recombinants pLM50, pLM51, and pLM52 contained 950, 400, and 700 bp, respectively, of additional listerial DNA 5′ to the probe used (Fig. 3). The restriction endonuclease sites that flank the deletion in SLCC53 are present in plasmids pLM50 and pLM52.

The *prfA* Gene Is Required for Listeriolysin Expression. To elucidate the role of the region encompassing the deletion in strain SLCC53, a 1.6-kb-long *Pst* I/*Xba* I fragment from plasmid pLM50 (Fig. 3) was cloned into the newly constructed shuttle-vector pERL3, capable of replication in both *E. coli* and *Listeria* species. The resulting plasmid, pERL3 50-1, was transformed into the *L. monocytogenes* serotype 1/2a strains EGD and SLCC53. As the *Hin*dIII site in plasmid pERL 50-1 is deleted in the mutant strain SLCC53, a mutant pERL3 50-2 lacking DNA sequences to the right of the *Hin*dIII site present in pERL3 50-1 was also constructed (see Fig. 3).

Introduction of the plasmid pERL3 50-1 into strain SLCC53 caused the reappearance of hemolytic activity in supernatant fluids (Table 1). As has been previously described for purified listeriolysin, this activity was enhanced by the addition of thiol-reducing agents and was completely inhibited when cholesterol was present in the assay. Furthermore, a 4-fold increase in activity, from 8 to 32 units, was detected when this plasmid was introduced into the weakly hemolytic strain EGD (Table 1). The presence of hemolytic activity in strain SLCC53 complemented with the plasmid pERL3 50-1 was associated with the appearance of secreted listeriolysin protein in its culture supernatant (Fig. 1*A*, lane 3). Similarly, increased amounts of this protein were seen in strain EGD harboring this plasmid (Fig. 1*B*, lane 4). When strains were transformed with the plasmid pERL3 50-2, neither the appearance of hemolytic activity in SLCC53 nor enhancement of the hemolytic activity of strain EGD was observed. These results confirm that the listeriolysin gene in strain SLCC53 is not mutated and is capable of expressing functionally active listeriolysin. This expression is absolutely dependent on the region located ≈1500 bp upstream of the listeriolysin gene. This transactivating factor is also involved in regulating the expression of the *lisA* gene in strain EGD.

DNA Sequence Analysis of the Region from *L. monocytogenes* Serotype 1/2a Strain EGD. The nucleotide sequence of the 1560-bp-long *Pst* I fragment from pLM50 was determined (Fig. 4). Computer analysis of the nucleotide sequence identified one open reading frame (ORF) of 705 nucleotides. The ATG at position 412 from the *Pst* I site is the probable methionine start codon, since it is preceded by a ribosome binding site centered 7 bp upstream. A possible consensus sequence described for prokaryotic promoters is located ≈130 nucleotides upstream of the initiation codon and may represent the transcriptional initiation site of this gene. The predicted primary translation product is a 235-amino acid polypeptide with a molecular mass of 27.1 kDa and a pI of 7.3. Downstream of the termination codon are sequences of dyad symmetry resembling rho-independent transcription terminator sequences of *E. coli*. The *Hin*dIII restriction endonuclease site identified within the SLCC53 deletion is located

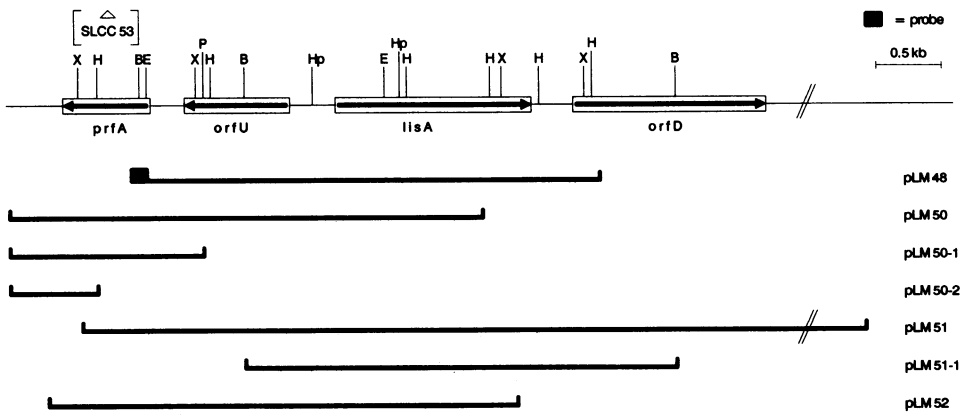

FIG. 3. Restriction map of the chromosomal region around the listeriolysin gene (*lisA*) of *L. monocytogenes* strain EGD. Heavy lines represent various lengths of *L. monocytogenes* chromosomal DNA inserted into the plasmid pUC18 vector. Solid block represents the DNA probe used to isolate sequences upstream of plasmid pLM48. Regions following the double-slashed lines are not drawn to scale. The locations of the *prfA*, ORF U, *lisA*, and ORF D are indicated together with the direction of transcription of these genes. The deletion present in SLCC53 is shown at the top of the figure in square brackets. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa* I; P, *Pst* I; X, *Xmn* I.

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GCTGCAGCATTAAACAACAAAGTAGAGCAATTCGTACTCAACTTAACATCGGAAAAAGTT   60
AlaAlaAlaLeuAsnAsnLysValGluGlnPheValLeuAsnLeuThrSerGluLysVal

CGAGGATTAGGCATACTAATCATGGACTTCCCCGAAAAACAAACAATTAAAAACATCATA  120
ArgGlyLeuGlyIleLeuIleMetAspPheProGluLysGlnThrIleLysAsnIleIle

AAAAACAATAAATTCAACTAACATATATTATTCCTACAAAAAAGGGTTAGTATATCTCCG  180
LysAsnAsnLysPheAsnEND

AGCAACCTCGGAACCATATACTAACTCTATTTCAATTTTAACATCTAAATAAATCCGTTT  240

TTAAATATGTATGCATTTCTTTTGCGAAATCAAAATTTGTATAATAAAATCCTATATGTA  300
                -35              -10

AAAAACATCATTTAGCGTGACTTTCTTTCAACAGCTAACAATTGTTGTTACTGCCTAATG  360

TTTTTAGGGTATTTTAAAAAAGGGCGATAAAAAACGATGGGGGGATGAGACATGAACGCT  420
                                RBS      METAsnAla      3

CAAGCAGAAGAATTCAAAAAATATTTAGAAACTAACGGGATAAAACCAAAACAATTTCAT  480
GlnAlaGluGluPheLysLysTyrLeuGluThrAsnGlyIleLysProLysGlnPheHis   23

AAAAAAGAACTTATTTTTAACCAATGGGATCCACAAGAATATTGTATTTTTCTATATGAT  540
LysLysGluLeuIlePheAsnGlnTrpAspProGlnGluTyrCysIlePheLeuTyrAsp   43

GGTATCACAAAGCTCACGAGTATTAGCGAGAACGGGACCATCATGAATTTACAATACTAC  600
GlyIleThrLysLeuThrSerIleSerGluAsnGlyThrIleMetAsnLeuGlnTyrTyr   63

AAAGGGGCTTTCGTTATAATGTCTGGCTTTATTGATACAGAAACATCGGTTGGCTATTAT  660
LysGlyAlaPheValIleMetSerGlyPheIleAspThrGluThrSerValGlyTyrTyr   83

AATTTAGAAGTCATTAGCGAGCAGGCTACCGCATACGTTATCAAAATAAACGAACTAAAA  720
AsnLeuGluValIleSerGluGlnAlaThrAlaTyrValIleLysIleAsnGluLeuLys  103

GAACTACTGAGCAAAAATCTTACGCACTTTTTCTATGTTTTCCAAACCCTACAAAAACAA  780
GluLeuLeuSerLysAsnLeuThrHisPhePheTyrValPheGlnThrLeuGlnLysGln  123

GTTTCATACAGTCTAGCTAAATTTAATGATTTTTCGATTAACGGGAAGCTTGGCTCTATT  840
ValSerTyrSerLeuAlaLysPheAsnAspPheSerIleAsnGlyLysLeuGlySerIle  143

TGCGGTCAACTTTTAATCCTGACCTATGTGTATGGTAAAGAAACTCCTGATGGCATCAAG  900
CysGlyGlnLeuLeuIleLeuThrTyrValTyrGlyLysGluThrProAspGlyIleLys  163

ATTACACTGGATAATTTAACAATGCAGGAGTTAGGATATTCAAGTGGCATCGCACATAGC  960
IleThrLeuAspAsnLeuThrMetGlnGluLeuGlyTyrSerSerGlyIleAlaHisSer  183

TCAGCTGTTAGCAGAATTATTTCCAAATTAAAGCAAGAGAAAGTTATCGTGTATAAAAAT 1020
SerAlaValSerArgIleIleSerLysLeuLysGlnGluLysValIleValTyrLysAsn  203

TCATGCTTTTATGTACAAAATCTTGATTATCTCAAAAGATATGCCCCTAAATTAGATGAA 1080
SerCysPheTyrValGlnAsnLeuAspTyrLeuLysArgTyrAlaProLysLeuAspGlu  223

TGGTTTTATTTAGCATGTCCTGCTACTTGGGAAAATTAAATTAAATCAAAAACAGTATTC 1140
TrpPheTyrLeuAlaCysProAlaThrTrpGluAsnEND                       235

CTCAATGAGGAATACTGTTTTATATTTTATTCGAATAAAGAACTTACAGAAGCATTTTCA 1200

TGAACGCGTACGATTGCTTCACCAAGAAGAGCTGCAACAGATAGTTGTTCCATTTTGTCA 1260

ATCCATTTTTCTTCTGGAAGAGCGATGGAGTTTGTAACAACTAATTTTTCGATTGGCGAT 1320

TCTTCAATACGTTTCATAGCCGACCTGAAAGAACTGGGTGCGAACAACATGCGTAAACTT 1380

TTGTTGCGCCAGCTATAACCACCGCAATTCCTGGAGTTATTTTTTCTACCAAGAGAAGTG 1440

GCAACATAATAATCGTCGCGATAATCGATACTAAAAAGCCAATCGCAACAATTATTAAAA 1500

CCCATTTTAGTAACATGAAAAATAAGTGCTTTTGCGGGTATTTCCAACTTTTTCGGATAG 1560
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FIG. 4. DNA sequence of the chromosomal region harboring the *prfA* gene. Shown are 1560 nucleotides located between the *Pst* I and endpoint of the insert DNA in plasmid pLM50-1. The deduced amino acid sequences corresponding to the ORFs of ORF U and the *prfA* gene are also shown. Potential sites corresponding to the −35 and −10 regions of the promoter and a ribosome-binding box are underlined and are marked −35, −10, and RBS, respectively. A putative terminator sequence is also shown underlined following the termination codons of the ORF U and *prfA* genes. The additional nucleotide A present in this sequence and absent from the DNA sequence of the corresponding region in a serotype 1/2c strain is indicated with an asterisk.

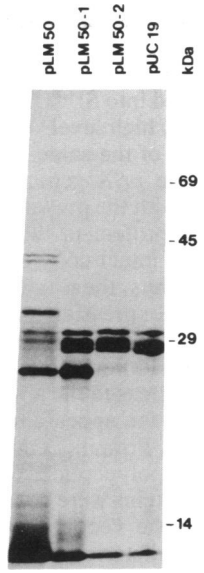

FIG. 5. Autoradiographs of ^{35}S-labeled polypeptides encoded by pLM50 and its derivatives in maxicells. The 27-kDa polypeptide in lanes pLM50 and pLM50-1 is the product of the *prfA* gene. Molecular mass standards (in kDa) are indicated.

within this open reading frame, and its deletion in plasmid pERL3 50-2 would remove the first 138 N-terminal amino acids of this open reading frame. This result clearly indicates that expression of the *lisA* gene is dependent on the presence of this 27-kDa protein. We designate this gene *prfA* for positive regulatory factor of listeriolysin (*lisA*) production.

The remaining portion of an open reading frame and its putative termination signal was detected in the nucleotide sequence located upstream of the *prfA* gene (Fig. 4). This sequence is identical to that previously reported for the ORF U polypeptide and its putative terminator from the *L. monocytogenes* serotype 1/2c LO28 strain (21). Both the ORF U and *prfA* reading frames are located 5′ to the listeriolysin gene and transcribed in the opposite orientation.

Identification of the prfA Protein. To identify the gene product encoded by the *prfA* gene, the inserts present in pERL3 50-1 and 50-2 were cloned into plasmid pUC19 and transformed into the maxicell strain CSH26ΔF6 (20). Polypeptides encoded by the various plasmids were detected by radioactive labeling with [^{35}S]methionine. Although listerial proteins are poorly expressed in *E. coli* strains (13), the A+T-rich region upstream from its predicted start codon contains a region showing strong homology to a consensus *E. coli* promoter. The presence of a ribosome binding site suggested that *prfA* would also be translated.

A 27-kDa polypeptide was expressed in strains harboring plasmids with an intact copy of the *prfA* gene. This polypeptide was lacking in strain pLM50-2, which carries a deletion within the *prfA* gene (Fig. 5). Synthesis of the other identifiable proteins is directed by the vector plasmid.

DISCUSSION

Listeriolysin is currently the only listerial gene product that has unequivocally been assigned a role as a virulence factor in pathogenic *Listeria*. In this study, we demonstrated that a gene, denoted *prfA*, located ≈1500 bp upstream of the listeriolysin gene is required for its expression. The presence of a deletion in this region of strain SLCC53 abolishes listeriolysin production and concomitantly abolishes virulence. Primer-extension analysis of RNA showed that the deletion within the *prfA* gene in SLCC53 affected production of listeriolysin at the transcriptional level. Hence, *prfA* is the

first regulatory protein to be reported that regulates expression of a virulence factor in pathogenic *Listeria*.

When the listeriolysin gene cloned from the hemolytic *L. monocytogenes* strain EGD was introduced into SLCC53, no listeriolysin was produced. Nevertheless, high levels of the toxin were detected in supernatant fluids of the same strain transformed with plasmids harboring the *prfA* gene. This increase in hemolytic activity concurred with the presence of larger amounts of secreted listeriolysin protein in the medium. These results clearly show that an intact copy of the listeriolysin gene is present in SLCC53. Thus, the product of the *prfA* gene positively regulates the expression of the listeriolysin gene.

We observed that the introduction of additional copies of the *prfA* gene into strain EGD not only increased the amount of secreted listeriolysin but also induced the appearance of several protein species in the supernatant. Prominent among them were proteins of 90 kDa and smaller polypeptides of 49, 31, and 29 kDa (Fig. 1*B*, lane 2). These proteins were not seen when the strain was transformed with the vector plasmid alone or with a plasmid harboring the listeriolysin gene. Hence, the *prfA* gene may also be involved in regulating the expression of other secreted proteins.

Recently, Mengaud *et al.* (21) have published the sequence of the region extending from the listeriolysin gene up to and including the first eight amino acids of *prfA* from a strongly hemolytic *L. monocytogenes* serotype 1/2c strain. The 435-bp overlap between these two sequences is identical except for the presence of an additional nucleotide A (indicated with an asterisk in Fig. 4) at nucleotide 269 of the sequence presented here. Indeed, this is the only difference in the 4223 bp of nucleotide sequence that is now available for these two strains (refs. 10 and 21; unpublished results). This change is within the putative promoter region of the *prfA* gene and the higher hemolytic activity of strain LO28 could be the result of increased *prfA* production. This would be consistent with our observation that increasing the gene dosage of *prfA* in strain EGD led to an increase in hemolytic activity. By extension, the variation in hemolytic activity observed with clinical isolates of *L. monocytogenes* could simply reflect expression differences and/or allelic variation in the *prfA* gene of these isolates.

The *prfA* gene encodes a protein of 235 amino acids with a predicted molecular mass of 27 kDa. A search of sequence similarities to other proteins in the National Biomedical Research Foundation data base using the FASTP algorithm of Lipman and Pearson revealed no significant homologies to known regulatory proteins. It is possible that the *prfA* gene may be a coactivating factor, acting in association with RNA polymerase to promote specificity of transcription.

A recurring observation that is emerging from molecular studies of pathogenic bacteria is the detection of deletions and mutations within loci involved in coordinate control of well-characterized virulence factors in strains that have been maintained for various periods of time under laboratory conditions. In *Vibrio cholera*, a deletion of the *toxS* gene in the avirulent laboratory strain 569B has recently been characterized (22). The absence of the *regB* gene in the prototypical *Pseudomonas aeruginosa* strain PAO1 appears to account for its relatively low yields of exotoxin A (23). In *Bordetella pertussis* and *Bordetella bronchiseptica*, spontaneous mutations and deletions of between 50 and 500 bp have been detected within the *bvg* locus in strains undergoing phase variation from the virulent to the avirulent states (24). Similarly, morphological changes observed with group A streptococci grown under laboratory conditions are associated with a loss of virulence. These stably locked avirulent-phase mutants have all been shown to harbor small deletions within the *virR* locus (25). The data presented here add *L. monocytogenes* to this list of pathogenic bacteria exhibiting lowered virulence due to a deletion within a locus regulatory for a virulence factor. It appears that the production of virulence factors represents a metabolic drain of resources within the bacterial cell growing under laboratory conditions; mutations within a regulatory locus for these factors would be the simplest mechanism to abolish or reduce their coordinate expression.

The results presented in this study identify a single gene that positively regulates the expression of the listeriolysin gene in pathogenic *Listeria*. Although our results lead us to conclude that *prfA* affects listeriolysin production at the transcriptional level, its molecular mechanism of action remains to be elucidated. *In vitro* studies with purified *prfA* protein are required to elucidate the function of *prfA*.

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