JOURNAL OF BACTERIOLOGY, Jan. 1992, p. 568–574
0021-9193/92/020568-07$02.00/0

Vol. 174, No. 2

# Coordinate Regulation of Virulence Genes in *Listeria monocytogenes* Requires the Product of the *prfA* Gene

TRINAD CHAKRABORTY,[1]†* MICHAELA LEIMEISTER-WÄCHTER,[2] EUGEN DOMANN,[2] MARIA HARTL,[2] WERNER GOEBEL,[2] THOMAS NICHTERLEIN,[3] AND SERVE NOTERMANS[4]

*Medizinische Universitäts Klinik, Klinische Biochemie,[1] and Institut für Genetik und Mikrobiologie,[2] 8700 Würzburg, and Institut für Medizinische Mikrobiologie der Universität Jena, 0-6900 Jena,[3] Federal Republic of Germany, and National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 Bilthoven, The Netherlands[4]*

Received 6 August 1991/Accepted 6 November 1991

**The *prfA* gene of *Listeria monocytogenes* encodes a protein that activates transcription of the listeriolysin gene (*lisA*). In order to explore the role of the *prfA* gene product in the pathogenesis of listerial infection, we constructed a site-directed insertion mutation in *prfA* by the chromosomal integration of a novel suicide vector containing a portion of the *prfA* coding region. This mutation not only transcriptionally silenced the listeriolysin (*lisA*) gene but also abrogated production of specific RNA transcripts corresponding to the phosphatidylinositol-specific phospholipase C (*pic*) and metalloprotease (*mpl*) genes, two further virulence gene products expressed only by pathogenic *Listeria* strains. The strain was also found to be avirulent when tested in a mouse model of listerial infection. The concomitant loss of multiple characteristics such as production of LisA, Pic, Mpl, and loss of virulence in a mouse infection model is the result of a mutation in a single gene and demonstrates that the *prfA* gene product is a positive regulator of multiple virulence determinants in *L. monocytogenes*.**

*Listeria monocytogenes*, a gram-positive facultative intracellular bacterium, is an opportunistic pathogen for animals and humans (31). The recent increase in incidence of human listeriosis and its association with several food-borne epidemics (6, 22, 23, 30) have prompted study of the characteristics of pathogenic *Listeria* strains that result in invasive disease following consumption of *Listeria*-contaminated food. Much progress has recently been made in identifying genes and genetic loci required for uptake and intracellular survival of this bacterium within eucaryotic host cells (7, 8, 12, 16, 17, 29, 33). Nevertheless, little is known of the factors that regulate the expression of these genes.

Growth of pathogenic *Listeria* strains within host tissues is dependent upon the production of the hemolytic exoprotein listeriolysin (7, 16), a 58-kDa protein that is encoded by a single gene, *lisA* (3, 18, 26). The hemolysin is essential for intracellular growth of the bacterium within the host phagocytic cells, where it participates in the lysing of the phagolysosomal membrane, allowing the invading bacterium free access to the host cytoplasm (28, 34). Expression of the listeriolysin gene is positively regulated at the transcriptional level by the product of the *prfA* gene (21). Recently, we have shown that the *prfA* gene also regulates expression of a gene encoding phosphatidylinositol-specific phospholipase C (*pic* [19]). Hence, the *prfA* gene product may play an important role in controlling multiple virulence properties of *Listeria monocytogenes*. The *prfA* gene encodes a 27,100-Da protein, and little is known of its molecular mechanism of action. Alteration of the *prfA* gene in *L. monocytogenes* through the construction of specific missense and deletion mutants might provide a means of elucidating the mechanism of action, as well as help in the identification of other gene products that might be under *prfA* control.

* Corresponding author.
† Present address: GBF, Mascheroder Weg 1, Braunschweig, Germany.

An ahemolytic *L. monocytogenes* strain, SLCC 53, harboring a deletion within the *prfA* coding region has recently been characterized (20). However, this strain is a spontaneously occurring mutant, and secondary mutations affecting hemolysin production may also exist. Hence, we wished to construct an isogenic mutant of a virulent wild-type isolate in order to evaluate the wider role of the *prfA* gene product in the pathogenesis of listerial infections. Here, we report the construction of a site-directed *prfA* insertion mutant constructed by chromosomal integration of a novel suicide plasmid containing a portion of the *prfA* coding sequence. Our studies suggest that coordinate regulation of virulence in *L. monocytogenes* is mediated by the PrfA protein.

## MATERIALS AND METHODS

**Bacterial strains, media, and reagents.** The weakly hemolytic *L. monocytogenes* serotype 1/2a strain EGD has been described previously (18). The *Escherichia coli* strain DH5α was used for cloning and transformation. *L. monocytogenes* was grown in brain heart infusion (BHI) broth (Difco) at 37°C. *E. coli* strains were routinely grown in Luria-Bertani (LB) 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 Boehringer Mannheim and used as recommended by the manufacturer. All other salts and ancillary agents were purchased from Sigma (Deisenhofen, Federal Republic of Germany).

**Southern hybridization.** Bacterial chromosomal DNA was isolated as described previously (18). Total genomic DNA was cleaved by *Eco*RI restriction endonuclease and electrophoresed overnight at 30 V through a 0.7% agarose gel. The DNA was transferred to nitrocellulose sheets by the method of Southern (32). DNA probes were labeled with [α-$^{32}$P]dATP by the random priming technique (5). Hybrid-

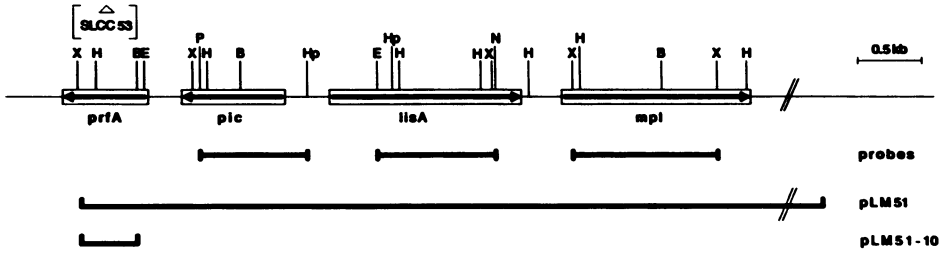


FIG. 1. Restriction map of the chromosomal region harboring the *prfA*, *plc*, *lisA*, and *mpl* genes of *L. monocytogenes* EGD. The locations of these genes are indicated, together with their direction of transcription. DNA probes used in the detection of the various RNA transcripts are indicated. Heavy lines represent various lengths of *L. monocytogenes* chromosomal DNA inserted into the plasmid pUC18 vector. Regions following the double-slashed lines are not drawn to scale. The deletion present in strain SLCC 53 is shown at the top of the figure in brackets. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nhe*I; P, *Pst*I; X, *Xmn*I.

ization conditions used for the detection of the various listerial genes were as described previously (18).

**Isolation of mRNA and Northern (RNA blot) hybridization.** Bacterial cultures were grown overnight with vigorous aeration at 37°C. Total RNA was isolated from *L. monocytogenes* by a modification of the hot phenol extraction method as published earlier (11). The concentration of RNA was determined spectrophometrically, and the RNA was stored at −70°C until further use.

RNA was analyzed by formaldehyde gel electrophoresis (1) following denaturation in 50% formamide–2.2 M formaldehyde–20 mM morpholinepropanesulfonic acid (MOPS)–5 mM sodium acetate–1 mM EDTA and heating for 65°C for 10 min. A total of 10 μg of RNA was electrophoresed through 1.2% agarose gels. The electrophoresis buffer consisted of 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA. Size-fractionated RNA was transferred onto nylon sheets (Hybond N; Amersham) and hybridized with the appropriate radiolabeled probe under the conditions recommended by the vendor. Following extensive washing of the nylon sheets, bands were visualized by autoradiography.

**Preparation of DNA probes.** Plasmid pAUL51-10 was linearized by digestion with the restriction endonuclease *Kpn*I and radiolabeled with [d-$^{32}$P]dATP as described above. Specific gene probes for *lisA*, *plc*, and *mpl* were derived after digestion of plasmid pLM51 with the appropriate restriction endonucleases and electroelution of the required DNA fragment from the gel following agarose gel electrophoresis. The regions corresponding to the gene probes are indicated in Fig. 1.

**Hemolysin and phospholipase C assays.** Hemolytic titers were determined as described before (18). When appropriate, supernatant fluids were supplemented with either 10 mM dithiothreitol or cholesterol (25 μg/ml). The phosphatidylinositol-specific phospholipase C (PIPLC) activities of *Listeria* strains were detected by adding 1% L-d-phosphatidylinositol substrate (Sigma; catalog number P6636) in 0.7% agarose as an overlay after overnight incubation of the bacterial colonies on LB agar plates at 37°C. PIPLC-active strains show a turbid halo around the colony (19).

**Transformation.** Plasmids were introduced into *L. monocytogenes* by protoplast transformation as described previously (35).

**Mouse virulence assay.** The mouse bioassay was performed as described by Kaufmann (14). Mice (strain NIH) were infected intravenously with approximately $10^3$ listeriae diluted in 70 mM phosphate buffer (pH 7.2) containing 0.25% NaCl. At days 2 and 4, bacterial numbers present in the spleen were determined. Spleens were first homogenized, and 0.1-ml portions of appropriate dilutions were plated on BHI agar plates and counted after overnight incubation at 37°C.

**Construction of pAUL-A.** Plasmid pAUL-A was constructed as follows (Fig. 2). The 2.3-kb *Hpa*I fragment containing the temperature-sensitive origin of replication from plasmid pE194 was isolated from plasmid pTV32ts (36) and inserted into the same site of plasmid pJDC9 (2). Plasmid pJDC9 contains a *lacZa'* multiple cloning site surrounded by transcriptional terminators and an erythromycin resistance marker expressed in both *E. coli* and *Listeria* spp. Recombinant plasmids were selected by their ability to grow on erythromycin-containing LB plates and screened for the presence of the 2.3-kb *Hpa*I fragment from plasmid pTV32ts.

**Construction of derivatives of plasmid pAUL-A carrying internal sequences from *prfA*.** The fragment carrying internal sequences from *prfA* was derived from plasmid pLM51-10, which encodes only the first 183 codons of the *prfA* gene (Fig. 1). A 455-bp *Bam*HI fragment carrying only sequences

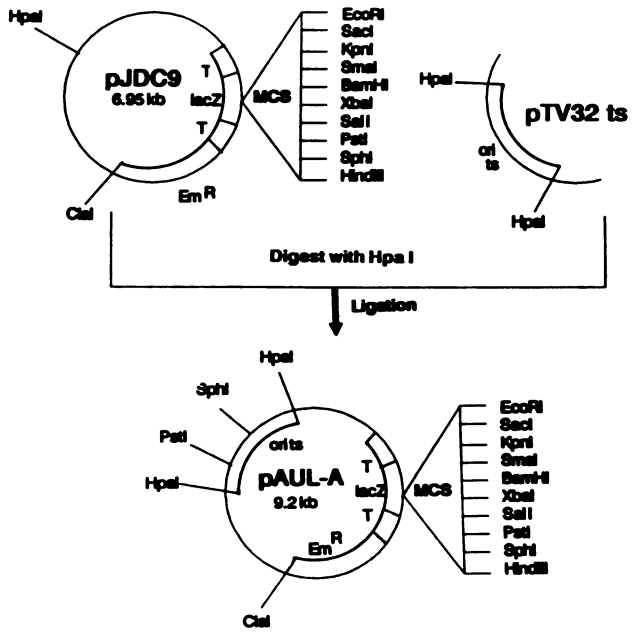


FIG. 2. Construction of the vector plasmid pAUL-A. MCS, multiple cloning site.

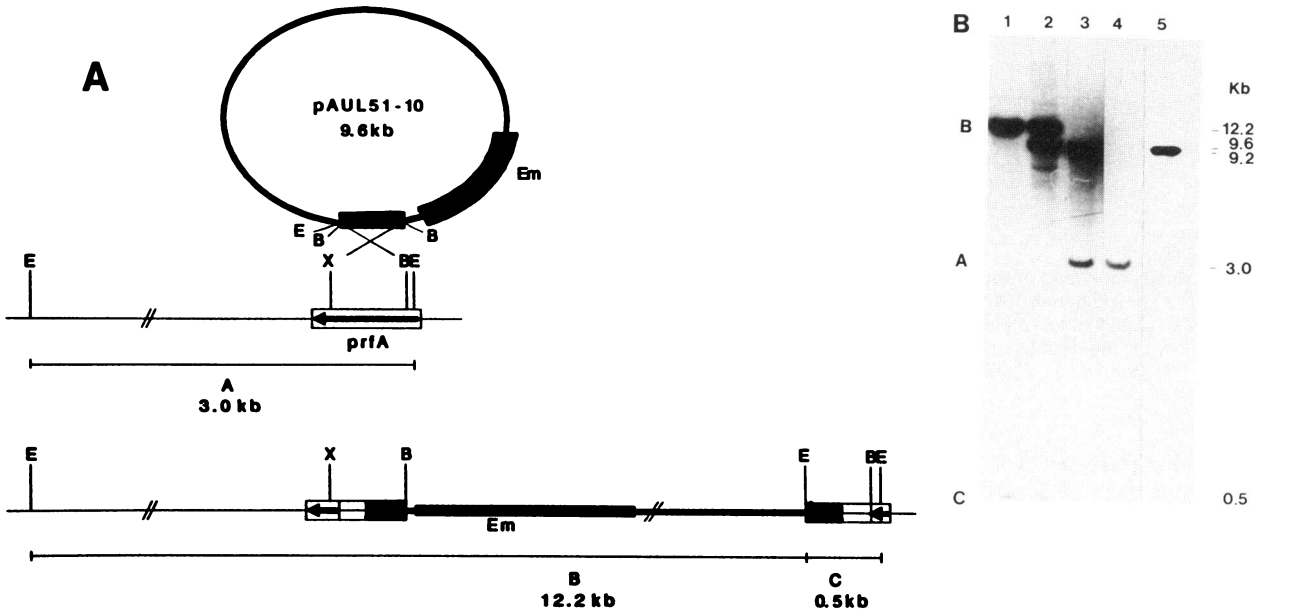


FIG. 3. Southern blot analysis of pAUL 51-10 integration events. (A) The region bracketed by the *Bam*HI sites on pAUL51-10 represents the internal fragment of the *prfA* gene from pLM51-10. In the absence of suitable cloning sites, the adjacent vector *Bam*HI site in pLM51-10 was used together with the *prfA*-internal *Bam*HI recognition site to permit cloning into pAUL-A. Homologous recombination (×) integrates pAUL51-10 into the chromosome to give the fragments indicated on the diagram. The thick arrows indicate the direction of transcription of the truncated *prfA'* gene. See Fig. 1 legend for abbreviations. (B) Chromosomal DNA samples from the strains indicated below were digested with *Eco*RI, electrophoresed in an agarose gel, transferred to nitrocellulose, and hybridized to $^{32}$P-labeled pAUL51-10. Bands appearing on the autoradiograph are labeled with letters that correspond to the fragments indicated on the schematic diagram given in panel A. Lanes: 1, strain EGD harboring plasmid pAUL51-10 and grown at 42°C; 2, EGD harboring plasmid pAUL51-10 and grown at 30°C; 3, EGD harboring pAUL-A and grown at 30°C; 4, EGD; 5, vector plasmid pAUL-A.

internal to the *prfA* gene was purified from an agarose gel slice by electroelution and ligated to pAUL-A. Recombinants were identified as Lac$^-$ colonies, and plasmid DNA was analyzed by using restriction endonucleases to identify plasmids carrying the correct insert. The resulting plasmid, pAUL51-10, was transformed into *L. monocytogenes* EGD. Integration of the plasmid was achieved by growing transformed strains at 42°C, a temperature restrictive for the replication of plasmid pAUL51-10.

## RESULTS

**Construction of a *prfA* insertion mutant.** A *prfA* mutant of *L. monocytogenes* EGD was constructed by insertion of a plasmid containing a portion of the *prfA* gene into the chromosomal copy of this gene by homologous recombination. To do this, we first constructed pAUL-A, a shuttle vector that is capable of autonomous replication in both *E. coli* and *Listeria* spp. (see Materials and Methods). However, in *Listeria* spp., this plasmid is incapable of replication at temperatures higher than 30°C because of the temperature-sensitive origin of replication from plasmid pE194ts (10). We found that less than 0.1% of the transformants harbor an autonomous plasmid when grown at the restrictive temperature of 42°C for 20 generations.

The insertional mutation in the *prfA* gene of *L. monocytogenes* EGD was isolated by first cloning a DNA fragment harboring an internal segment of the *prfA* coding sequence into plasmid pAUL-A to generate plasmid pAUL51-10 (Fig. 1 and 3). After transformation of the plasmid into *L. monocytogenes* and growth at 30°C overnight, colonies were streaked out onto plates and incubated at 42°C overnight. Several transformants were purified by restreaking colonies onto BHI plates containing erythromycin and further incubation at 42°C. Erythromycin-resistant transformants should contain the transformed plasmid integrated into the genome by homologous recombination between the *prfA* gene on the chromosome and the cloned sequence present on the plasmid.

This was shown to be the case by Southern blot hybridization of a representative *L. monocytogenes* strain transformed with the plasmid pAUL51-10. Genomic DNA from the parental *L. monocytogenes* strain EGD, harboring either the vector plasmid pAUL-A or pAUL51-10, was isolated after growth of the bacteria at different temperatures and cleaved with the restriction endonuclease *Eco*RI. Radiolabeled plasmid pAUL51-10 was used as a probe to detect integration events. The 3.0-kb *Eco*RI fragment (fragment A) detected in the wild-type strain and a derivative harboring only the vector plasmid pAUL-A is the original chromosomal fragment (Fig. 3B, lanes 3 and 4). The additional 9.2-kb *Eco*RI fragment seen in lanes 3 and 5 corresponds to the vector plasmid pAUL-A. Even when strains carrying pAUL51-10 were grown at 30°C, it was apparent that a high proportion of bacteria carried a disruption of the *prfA* gene. Hence, new *Eco*RI fragments of 12.2 and 0.5 kb (fragments B and C) created by the integration event are observed in addition to a 9.6-kb *Eco*RI fragment corresponding to plasmid pAUL51-10 (Fig. 3B, lane 2). At the higher temperature, integration of plasmid pAUL51-10 into the genome at the site of the *prfA* gene is efficient, and no fragment corresponding to free replicating plasmid is observed (Fig. 3B, lane 1).

It is apparent from Fig. 3B that the chromosomal insertion of plasmid pAUL51-10 creates two half-copies of the *prfA*

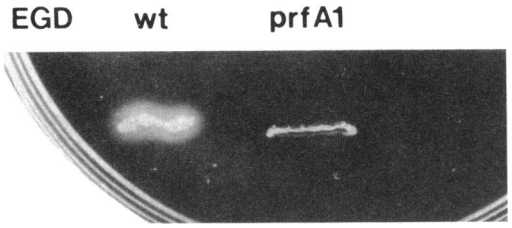


FIG. 4. Detection of PIPLC activity in *L. monocytogenes* EGD (wild type [wt]) and the isogenic EGD *prfA1* mutant. The assay was performed as described in Materials and Methods. PIPLC-active clones show a turbid halo around the colony as a result of release of insoluble diacylglycerol from the substrate.

gene, but only the promoter-proximal half of the copy carried sequences encoding the amino-terminal end of the PrfA protein. Hence, this half-copy (the *prfA1* allele) encodes a truncated PrfA peptide that lacks C-terminal amino acid residues.

**Characterization of the *prfA* insertion mutant.** The *prfA1* mutant was analyzed for the production of listeriolysin by the hemolysis assay after overnight growth in BHI medium. No differences in the growth rate were detected between the wild-type strain and the mutant strain in this medium. The mutant produced no detectable listeriolysin, whereas the wild-type strain produced a titer of 8 hemolytic units (HU) under identical conditions of growth. We also assayed for the presence of PIPLC activity, which, as we have recently shown, is coexpressed with listeriolysin (19). This experiment showed that PIPLC activity is entirely absent in the *prfA1* mutant (Fig. 4). Hence, the toxin-deficient phenotype of this mutant is similar to that of the previously described spontaneously occurring *prfA* deletion mutant.

The deletion within the *prfA* gene in the ahemolytic strain SLCC 53 affects production of listeriolysin at the transcriptional level. Northern blot analysis of total RNAs obtained from the *prfA1* mutant showed that the *lisA* transcript was also absent in this strain (Fig. 5). We further examined the ability of the *prfA* mutant to produce transcripts corresponding to the *pic* gene and a newly discovered metalloprotease gene (*mpl* [4]). These genes flank the *lisA* gene, are unique to pathogenic *L. monocytogenes*, have common sequences with *lisA* upstream of their respective promoters, and are de facto virulence factors of this organism (18, 24, 25; our unpublished results). In the parental EGD strain, two transcripts of 2.1 and 1.1 kb were obtained with the *pic* gene probe (Fig. 1 and 5). The smaller transcript corresponds to the *pic* gene itself, while the larger RNA species also includes the *prfA* gene that is located downstream. In the *prfA1* mutant strain, neither of these RNA transcripts were detected. Two transcripts of 1.8 and 5.4 kb were detected when total RNAs from the parental strain were probed for the presence of the *mpl* transcript. The shorter transcript contains only the *mpl* gene, while the longer transcript would result from transcription extending beyond the *mpl* gene (Fig. 5). As before, neither transcript was detected in the *prfA1* mutant strain. Hence, mutation of the *prfA* gene results in the concomitant abrogation of expression of the *lisA*, *pic*, and *mpl* genes in *L. monocytogenes*.

**Virulence of the *prfA1* mutant in mice.** The effect of the *prfA1* mutation on virulence was assessed by the ability of this mutant to grow in host tissues when injected intravenously in mice. In contrast to the wild-type strain, which showed significant growth in the spleens of infected hosts, the number of bacteria present in the spleens of mice infected with the *prfA* mutant strains EGD *prfA1* and SLCC 53 *prfA* declined rapidly at 2 days following infection and showed no further growth (Fig. 6). Hence, the absence of a functional *prfA* gene product in virulent *L. monocytogenes* renders the strain avirulent and incapable of growth in host tissues.

## DISCUSSION

The molecular cloning and identification of a number of listerial gene products, such as *lisA*, *pic*, *mpl*, *lmaA*, *iap*, and *prfA* (4, 9, 15, 18, 19, 21, 26), has made it imperative to obtain defined mutants in order to assess the role of the individual gene products in listerial infection. With this goal in mind, we constructed a novel shuttle vector, pAUL-A,

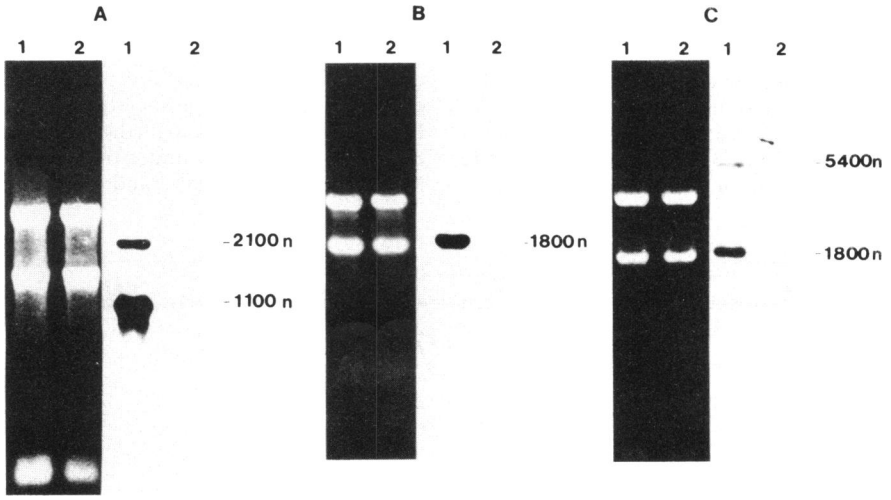


FIG. 5. Agarose gel electrophoresis of total RNA and the corresponding autoradiograms from Northern blot analysis of transcripts produced by strains EGD (lane 1) and the isogenic mutant EGD *prfA1* (lane 2) with the gene probes *pic* (A), *lisA* (B), and *mpl* (C). The probes used in the detection of transcripts corresponding to the respective genes are indicated in Fig. 1. The molecular sizes of the transcripts obtained are indicated (in nucleotides). Equivalent amounts of total cellular RNA were analyzed for each strain.

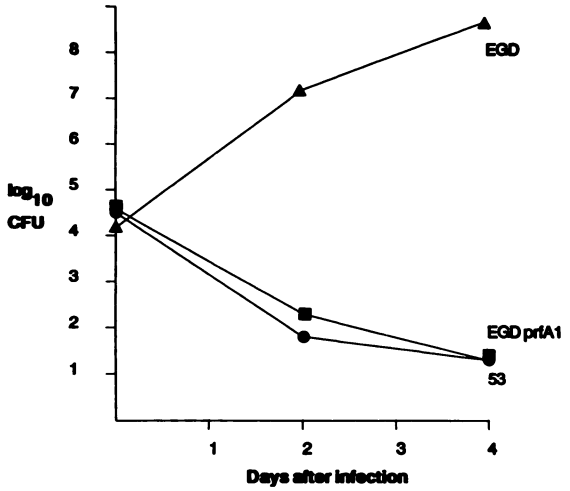


FIG. 6. Growth curves of *L. monocytogenes* strains EGD (▲), solid triangles EGD *prfA1* (■), solid squares and SLCC 53 *prfA* (●) solid circles in spleens of mice. Mice were infected with different numbers of viable listeriae, and the number of bacteria was determined on various days thereafter as described in the text. Each point represents the mean values for four mice.

capable of site directing virtually any nonlethal mutation into the listerial chromosome. Mutagenesis of the desired gene product is performed in an *E. coli* host in which cloning is facilitated by the presence of a *lacZa'* multiple cloning site flanked by transcriptional terminators and an erythromycin resistance marker that is expressed in both *E. coli* and *Listeria* spp. Replication of the plasmid in the latter host occurs at temperatures below 30°C because of the temperature-sensitive nature of the pE194ts replicon. The efficiency of the integration process is clearly demonstrated in lane 2 of Fig. 3B, where a substantial proportion of cells grown even at the permissive temperature carry integrated plasmids.

In this communication, we described the construction and characterization of a strain of *L. monocytogenes* carrying a chromosomal insertion within the *prfA* gene. The mutation was site directed by the use of a specific internal fragment of the *prfA* gene to target the integration of a nonreplicating plasmid into the chromosomal *prfA* gene by homologous recombination. The integration event truncates the *prfA* gene at its *Bam*HI site, removing 203 codons from its 3' end. The phenotype of this mutant is similar to that of the *prfA* deletion mutant characterized in the spontaneously occurring nonhemolytic mutant SLCC 53, which produced no detectable amounts of listeriolysin or PIPLC.

The *prfA* gene was originally defined as a positive regulatory factor required for listeriolysin expression (21). We have previously shown that transcomplementation of the ahemolytic SLCC 53 *prfA* mutant with the cloned *prfA* gene leads to the reappearance of both hemolytic and PIPLC activities in this strain (19, 21). In this study, using a site-directed *prfA* insertion mutant, we showed that the PrfA protein is essential for the transcription of the *lisA*, *pic*, and *mpl* genes and the product of a further open reading frame located 3' to the metalloprotease gene (Fig. 7). Northern blot analysis revealed that the genes flanking the listeriolysin gene are organized in two separate operons, one comprising the *pic* and *prfA* genes, and the other being the *mpl* gene and an open reading frame located immediately downstream from it. These genes are all clustered on a region of the chromosome of *L. monocytogenes* that is unique to this pathogenic species. Studies with mutants carrying site-directed insertions in individual genes identified in this region show that they all exhibit reduced virulence in the mouse model of infection (24, 25; our unpublished results). Hence, the PrfA protein appears to coordinately regulate a cluster of genes constituting a virulence regulon in pathogenic *L. monocytogenes*. A common palindromic sequence located at the spatial region corresponding to the −44 region of the promoters of these genes may be responsible for mediating regulation of the *prfA* gene product (27).

Evidence has recently been accumulating that the expression of virulence in pathogenic *Listeria* spp. is coordinately regulated. Kathariou and colleagues have described the isolation of transposon Tn*916*-induced mutations in a virulent *L. monocytogenes* strain that led to concomitant loss of the hemolytic phenotype, loss of production of lecithinase activity, and loss of the ability to enter mammalian cells (13). The point of insertion of the transposon and the gene affected, however, were not characterized. In an experimental analysis to determine the genes involved in intracellular survival of virulent *L. monocytogenes* in tissue culture cell lines, Sun et al. isolated a mutant, designated DAP-L973, which produced low levels of listeriolysin and phospholipase C, showed no association with F-actin in the eucaryotic cell, and is defective for cell-to-cell spread (33). An inspection of sequences available for insertion of the transposon in mutant DAP-L973 indicates that it is located precisely −13 bp proximal to the RNA start site of the *prfA* gene (21; our unpublished results). This could account for the low level of expression of the *lisA* and *pic* genes in this mutant strain. By extension, the lack of association of this mutant with cellular F-actin suggests that a further bacterial gene, involved in F-actin interaction, is under the control of the PrfA protein. More recently, Mengaud and colleagues have reported that

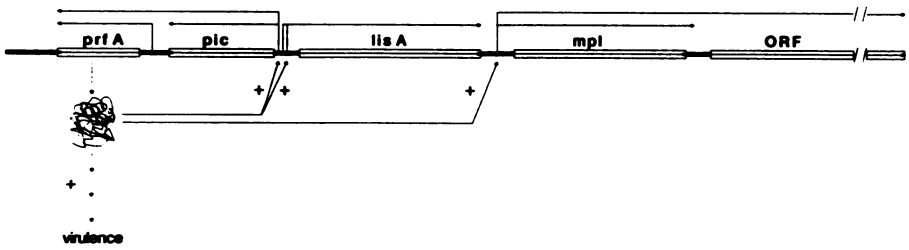


FIG. 7. Schematic diagram of virulence determinants regulated by the product of the *prfA* gene. Arrows indicate the direction of transcription of the respective genes. Note that the genes flanking the listerioysin gene are organized in operons. The potentiating effect of the PrfA protein on the expression of the various genes is indicated (+). Other gene loci required for the expression of virulence of these bacteria remain to be determined. ORF, open reading frame.

the production of lecthinase is also under the regulation of the PrfA regulator (24a).

The avirulence of the *prfA* mutant in the mouse model of infection is therefore not only the result of lack of listeriolysin, PIPLC, and metalloprotease production but also involves other genes required for intracellular replication and persistence. The results presented here and elsewhere (13, 21, 33) suggest that invasion, intracellular bacterial cell motility, and cell-to-cell spread may also be under *prfA* control.

The results of this study identify a single gene whose product is involved in positively regulating the expression of virulence in pathogenic *Listeria* strains. Genes under the control of the PrfA regulator also appear to be involved in processes operating very early in the infectious process and include the ability to invade and colonize gut mucosal surfaces prior to spreading into deeper tissues of the host (Fig. 7). The identification of the other genes coordinately regulated by *prfA* and a study of the environmental signals that modulate the expression of this important protein will provide us with further insights into the evolution in bacteria of the mechanisms which allow successful invasion of and survival within the eucaryotic host.

## ACKNOWLEDGMENTS

We thank Susanne Clemens-Richter and Brigitte Göpfert for excellent technical assistance. T.C. thanks D. A. Morrison (University of Illinois at Chicago) for the kind gift of plasmid pJDC9 and is deeply indebted to Thomas Jarchau for discussions on many aspects of this work and the ideas developed here.

This research was financed by a grant from the Bundesministerium für Forschung und Technologie.

## REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Chen, J.-D., and J. D. Morrison.** 1987. Cloning of *Streptococcus pneumoniae* DNA fragments in *Escherichia coli* requires vectors protected by string promoters. Gene **55**:179–187.
3. **Domann, E., and T. Chakraborty.** 1989. Nucleotide sequence of the listeriolysin gene from a *Listeria monocytogenes* serotype 1/2a strain. Nucleic Acids Res. **17**:6406.
4. **Domann, E., M. Leimeister-Wächter, W. Goebel, and T. Chakraborty.** 1991. Molecular cloning, sequencing, and identification of a metalloprotease gene from *Listeria monocytogenes* that is species specific and physically linked to the listeriolysin gene. Infect. Immun. **59**:65–72.
5. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction fragments to high specificity. Anal. Biochem. **132**:6–13.
6. **Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikyatis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reingold.** 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. **312**:404–407.
7. **Gaillard, J. L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55**:2822–2829.
8. **Gaillard, J. L., P. Berche, and P. Sansonetti.** 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **52**:50–55.
9. **Göhmann, S., M. Leimeister-Wächter, E. Schiltz, W. Goebel, and T. Chakraborty.** 1990. Identification, cloning and nucleotide sequence of a *Listeria monocytogenes* specific protein capable of inducing delayed-hypersensitivity in *Listeria*-immune mice. Mol. Microbiol. **4**:1091–1099.
10. **Gryczan, T. J., J. Hahn, S. Contente, and D. Dubnau.** 1982. Replication and incompatibility properties of plasmid pE194 in *Bacillus subtilis*. J. Bacteriol. **152**:722–735.
11. **Husslein, V., B. Huhle, T. Jarchau, R. Lurz, W. Goebel, and T. Chakraborty.** 1988. Nucleotide sequence and transcriptional analysis of the *aer*Caer A region of the *Aeromonas sobria* encoding aerolysin and its regulatory region. Mol. Microbiol. **2**:507–517.
12. **Kathariou, S., P. Metz, H. Hof, and W. Goebel.** 1987. Tn*916*-induced mutation in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bacteriol. **163**:1291–1297.
13. **Kathariou, S., L. Pine, G. Carlone, and B. P. Holloway.** 1990. Nonhemolytic *Listeria monocytogenes* mutants that are also noninvasive for mammalian cells in culture: evidence for coordinate regulation of virulence. Infect. Immun. **58**:3988–3995.
14. **Kaufmann, S. H. E.** 1984. Acquired resistance to facultative intracellular bacteria and relationship between persistence, cross-reactivity at the T-cell level, and capacity to stimulate cellular immunity of different *Listeria* serovars. Infect. Immun. **45**:234–241.
15. **Köhler, S., M. Leimeister-Wächter, T. Chakraborty, F. Lottspeich, and W. Goebel.** 1990. The gene encoding the protein p60 of *Listeria monocytogenes* and its use as a specific probe for *L. monocytogenes*. Infect. Immun. **58**:1943–1950.
16. **Kuhn, M., S. Kathariou, and W. Goebel.** 1988. Hemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes*. Infect. Immun. **56**:79–82.
17. **Kuhn, M., M.-L. Prevost, J. Mounier, and P. J. Sansonetti.** 1990. A nonvirulent mutant of *Listeria monocytogenes* that does not move intracellularly but still induces polymerization of actin. Infect. Immun. **58**:3477–3486.
18. **Leimeister-Wächter, M., and T. Chakraborty.** 1989. Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes*, *Listeria ivanovii*, and *L. seeligeri*. Infect. Immun. **57**:2350–2357.
19. **Leimeister-Wächter, M., E. Domann, and T. Chakraborty.** 1991. Detection of a gene encoding a phosphatidylinositol-specific phospholipase C that is coordinately expressed with listeriolysin in *Listeria monocytogenes*. Mol. Microbiol. **5**:361–366.
20. **Leimeister-Wächter, M., W. Goebel, and T. Chakraborty.** 1989. Mutations affecting hemolysin production in *Listeria monocytogenes* located outside of the listeriolysin gene. FEMS Microbiol. Lett. **65**:23–30.
21. **Leimeister-Wächter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty.** 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. Proc. Natl. Acad. Sci. USA **87**:8336–8340.
22. **Linnan, M. J., L. Mascola, Y. D. Lon, V. Goulet, S. May, C. Salminen, W. Hird, M. L. Yonekawa, P. Hayes, R. Weaver, A. Audurier, B. D. Plikyatis, S. L. Famin, A. Kleks, and C. V. Broome.** 1988. Epidemic listeriosis associated with Mexican-style cheese. N. Engl. J. Med. **319**:823–828.
23. **Malinverni, R., J. Bille, C. Perret, I. Regli, F. Tanner, and B. Glauser.** 1985. Listeriose epidemique. Schweiz. Med. Wochenschr. **115**:2–10.
24. **Mengaud, J., C. Braun-Breton, and P. Cossart.** 1991. Identification of a phosphatidylinositol-specific phospholipase C activity in *Listeria monocytogenes*: a novel type of virulence factor. Mol. Microbiol. **5**:367–372.

24a. **Mengaud, J., S. Dramsi, E. Gouin, J. A. Vazquez-Boland, G. Milon, and P. Cossart.** 1991. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. Mol. Microbiol. **5**:2273–2283.

25. **Mengaud, J., C. Goeffroy, and P. Cossart.** 1991. Identification of a new operon involved in *Listeria monocytogenes* virulence: its first gene encodes a protein homologous to bacterial metalloproteases. Infect. Immun. **59**:1043–1049.
26. **Mengaud, J., M. Vicente, J. Chenevert, J. Moniz-Pereira, C. Goeffroy, B. Gicquel-Sanzey, F. Baquero, J. C. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant in *Listeria monocytogenes*. Infect. Immun. **56**:766–772.
27. **Mengaud, J., M. F. Vincente, and P. Cossart.** 1989. Transcriptional mapping and nucleotide sequence of the *Listeria mono-*

*cytogenes hlyA* region reveal structural features that may be involved in regulation. Infect. Immun. **57**:3695–3701.

28. **Mounier, J., A. Ryter, M. Rodon-Coquis, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocyte-like cell line Caco-2. Infect. Immun. **58**:1048–1058.
29. **Portnoy, D., S. P. Jacks, and D. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167**:1459–1471.
30. **Schlech, W. F., III, P. M. Lavigne, R. A. Bortolussi, A. C. Allen, S. H. King, E. S. Nichols, and C. V. Broome.** 1983. Epidemic listeriosis— evidence for transmission by food. N. Engl. J. Med. **308**:203–206.
31. **Seeliger, H. P. R., and D. Jones.** 1986. *Listeria*, p. 1235–1245. *In* P. H. Sneath et al. (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
32. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated in gel electrophoresis. J. Mol. Biol. **98**:503–517.
33. **Sun, A., A. Camilli, and D. A. Portnoy.** 1990. Isolation of *Listeria monocytogenes* small-plaque mutants defective in intracellular growth and cell-to-cell spread. Infect. Immun. **58**: 3770–3778.
34. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement and spread of the intracellular parasite, *Listeria monocytogenes*. J. Cell Biol. **109**:1597–1607.
35. **Wuenscher, M., S. Köhler, W. Goebel, and T. Chakraborty.** Gene disruption by plasmid integration in *Listeria monocytogenes*: disruption of the listeriolysin gene. Mol. Gen. Genet. in press.
36. **Youngman, P. J., J. B. Perkins, and R. J. Losick.** 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptoccocus faecalis* transposon Tn*917*. Proc. Natl. Acad. Sci. USA **80**:2305–2309.