INFECTION AND IMMUNITY, July 1993, p. 3073–3075
0019-9567/93/073073-03$02.00/0

Vol. 61, No. 7

Detection of a *prfA*-Independent Promoter Responsible for Listeriolysin Gene Expression in Mutant *Listeria monocytogenes* Strains Lacking the PrfA Regulator

EUGEN DOMANN,[1] JÜRGEN WEHLAND,[2] KIRSTEN NIEBUHR,[2] CHRISTOF HAFFNER,[3] MICHAELA LEIMEISTER-WÄCHTER,[4] AND TRINAD CHAKRABORTY[1]*

Institut für Medizinische Mikrobiologie, Klinikum der Justus-Liebig Universität Giessen, 6300 Giessen,[1] Gesellschaft für Biotechnologische Forschung, Abteilung Mikrobiologie, 3300 Braunschweig,[2] and Institut für Klinische Chemie und Pathobiochemie, Medizinische-Universitäts Klinik, Universität Würzburg,[3] and Institut Virion GmbH,[4] 8700 Würzburg, Germany

Received 5 February 1993/Accepted 13 April 1993

Expression of listeriolysin, a major virulence factor of pathogenic *Listeria monocytogenes*, is positively regulated by the pleiotropic virulence regulator PrfA. In this study, we demonstrate that *L. monocytogenes* strains lacking the *prfA* regulator gene produce listeriolysin in small, albeit detectable, amounts when analyzed in a hemolysin assay and by immunoblots with listeriolysin-specific monoclonal antibodies. Transcriptional analysis revealed the existence of a PrfA-independent promoter that was responsible for the hemolytic activity expressed by these strains.

Listeria monocytogenes is a facultative intracellular bacterium that is capable of intracytoplasmic growth in infected host cells following escape from a phagolysosomal compartment (10, 13). Escape from this compartment of the infected host cell has been correlated to the expression of a secreted hemolysin, designated listeriolysin (12). *L. monocytogenes* strains harboring mutations within the listeriolysin (*hly*) gene are trapped within the phagolysosome, where bacterial growth is inhibited and where these bacteria are finally destroyed. A second class of mutants defective in the production of listeriolysin has also been recognized recently. Such strains are mutated at the *prfA* locus, which encodes a gene product that is a positive regulator of listeriolysin expression (6). The *prfA* locus was subsequently shown to be a pleiotropic regulator of expression of virulence genes in *L. monocytogenes* (2, 8). Mutations in either locus, *hly* or *prfA*, lead to the abrogation of virulence of these bacteria in a mouse infection model.

While using a gentamicin-based survival assay to examine the ability of various *L. monocytogenes* mutant strains to grow intracellularly in infected tissue culture cell lines, we observed growth of *prfA* mutants in infected host cells following overnight incubation. Light-microscopic observation confirmed the presence of bacteria in the host cytoplasm (data not shown). This result was obtained irrespective of the *prfA* mutant allele used and prompted us to reassess the ability of several *prfA* mutant strains to express listeriolysin.

The *L. monocytogenes* strains used in this study are described in Table 1. Listerial cultures were grown in brain heart infusion broth at the various temperatures indicated. The hemolysin assay was performed as described previously (5), except that human erythrocytes were used at a final concentration of 0.5%. Samples for Western immunoblots were obtained from bacterial cultures grown to an optical density at 600 nm of 0.6, which represented cultures in the exponential growth phase. The blots were developed by using a chemiluminiscence-based immunoassay (Amerscham Buchler, Braunschweig, Germany) as recommended by the manufacturer. For primer extension studies, end-labeled primer (5′-CATGGGTTTCACTCTCCTTCTAC-3′) was annealed to total bacterial mRNA and extended by using avian myeloblastosis virus reverse transcriptase (Pharmacia) in reaction mixtures as described previously (6). Dideoxy sequencing reactions, with the same primer and an appropriate DNA template, were run in parallel to allow determination of the endpoints of the extension products.

The *L. monocytogenes* strains SLCC 53 and EGD *prfA1*, which were mutated in the *prfA* gene, showed contact hemolysis below the colonies following overnight incubation at 37°C on blood agar plates with 1% human erythrocytes (2, 4, 6). Under standard assay concentrations of washed human erythrocytes, i.e., at end concentrations of 1 to 2%, we were unable to detect any hemolytic activity in the supernatant fluids of *prfA* mutant bacteria. However, when assays were performed with an erythrocyte concentration of 0.5%, the *prfA* mutant strains SLCC 53 and EGD *prfA1* produced detectable amounts of hemolytic activities, which were between 32- and 64-fold lower than those for the wild-type strains NCTC 7973 and EGD, respectively (Table 2). When we assayed the hemolytic activity of these strains at three different growth temperatures, we found that although the

TABLE 1. *Listeria* strains used in this study.

Strain	Serotype	Relevant genotype	Hemolytic phenotype[a]
L. monocytogenes			
EGD	1/2a	Wild type	+
NCTC 7973	1/2a	Wild type	++
EGD *prfA1*	1/2a	*prfA1*	−[b]
EGD *hly-1*	1/2a	*hly-1*	−
SLCC 53	1/2a	Δ*prfA*	−[b]
L. innocua			
NCTC 11288	6a	Wild type	−

[a] Hemolytic phenotypes observed on sheep blood agar plates were scored as follows: ++, strongly hemolytic; +, weakly hemolytic; −, nonhemolytic.
[b] Contact hemolysis as described in the text.

* Corresponding author.

TABLE 2. Hemolytic titer of supernatants of *L. monocytogenes* strains grown at different growth temperatures

Growth temp (°C)	Hemolytic titer (HU)[a] of strain:			
	EGD	EGD *prfA1*	NCTC 7973	SLCC 53
20	4	4	32	32
30	8	4	256	16
37	64	2	256	4

[a] Hemolytic titer is expressed in hemolytic units (HU), which is defined as the reciprocal of the highest dilution at which complete hemolysis was detected. Hemolysin assays were performed with 0.5% washed human erythrocytes in phosphate-buffered saline (pH 5.5) buffer.

detectable hemolytic activity decreased at lower growth temperatures for the wild-type strains, there was a two- to eightfold increase in hemolytic activity at 20°C depending on the *prfA* mutant used (Table 2).

To determine whether the hemolytic activity actually correlated with levels of production of listeriolysin, we performed immunoblotting experiments with a listeriolysin-specific monoclonal antibody (14). Supernatant fluids from the various bacteria grown as described above were concentrated by trichloroacetic acid precipitation and loaded onto gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer to nitrocellulose filters, listeriolysin was detected by a sensitive chemiluminiscence-based immunoblot assay. From the results in Fig. 1 it is clear that strains harboring either a deletion or insertion within the *prfA* gene clearly produced small amounts of the 60-kDa listeriolysin polypeptide. The specificity of the monoclonal antibody for listeriolysin was demonstrated by the detection of a truncated listeriolysin in a *hly* mutant (1)

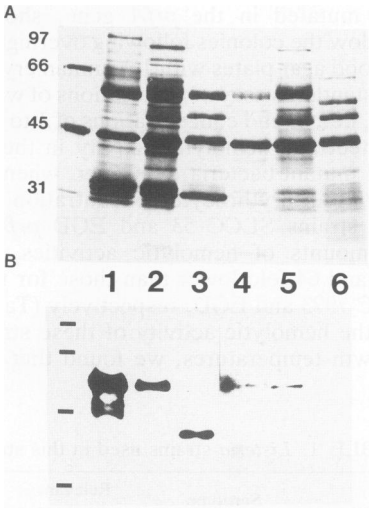

FIG. 1. Detection of the listeriolysin polypeptide produced in the supernatants of different listerial strains. Exponentially growing cultures were harvested by centrifugation, and supernatants were precipitated by overnight precipitation with 10% (wt/vol) trichloroacetic acid at 4°C and analyzed by SDS-PAGE. (A) Silver-stained SDS–10% polyacrylamide gel with culture supernatants from *L. monocytogenes* NCTC 7973 (lane 1), EGD (lane 2), EGD *hly-1* (lane 3), SLCC 53 (lane 4), and EGD *prfA1* (lane 5) and *L. innocua* NCTC 11288 (lane 6). (B) Corresponding immunoblot reacted with a listeriolysin-specific monoclonal antibody and developed with a chemiluminescent substrate. Molecular mass standards indicated at the left are 92, 68, 46, 29, and 14 kDa (top to bottom).

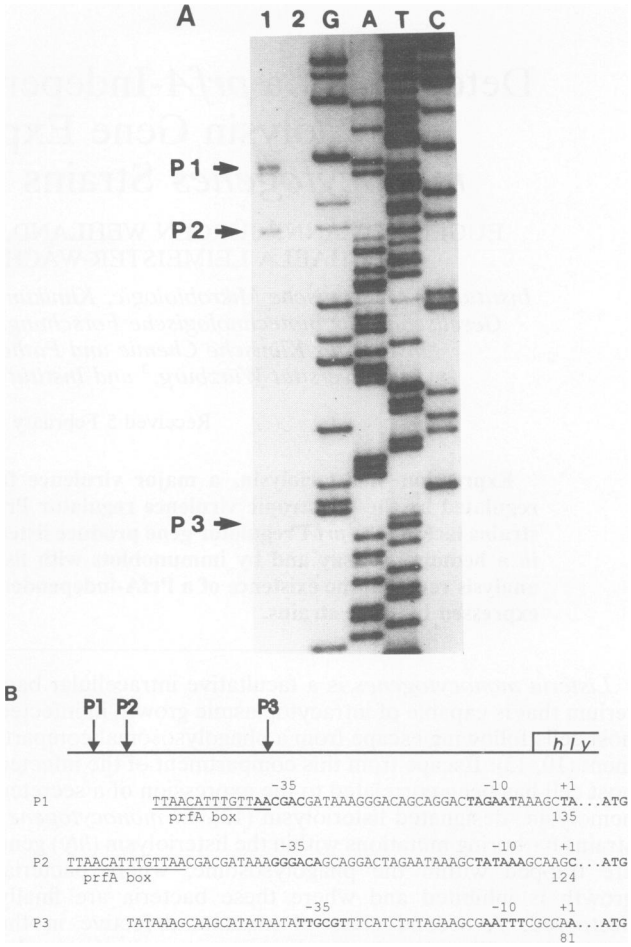

FIG. 2. (A) Mapping and detection of *hly* transcripts in various *L. monocytogenes* strains by primer extension analysis. An α-^{32}P-end-labeled oligonucleotide primer was used for reverse transcription of the transcript. The DNA products were separated on an 8% polyacrylamide gel simultaneously with a dideoxy sequencing ladder, with the same DNA primer and plasmid pLM47 as template to allow determination of the extension product. Lane 1 contains NCTC 7973; lane 2 contains SLCC 53. Lanes denoted G, A, T, and C are from the sequencing reaction. (B) Schematic representation of transcripts originating in the region 5′ to the *hly* gene. Alignment of nucleotide sequences corresponding to the P1, P2, and P3 transcription initiation start sites is shown. The numbering is taken from the sequence as reported in reference 3. The spatial regions corresponding to the −35 and −10 regions preceding the respective transcription start site (+1) are indicated. The sequence corresponding to the palindromic *prfA* box is underlined.

(lane 3) as well as lack of reaction to supernatant fluids of an *L. innocua* strain, a nonpathogenic species that does not produce listeriolysin (5) (lane 6).

We next examined the basis of listeriolysin production in the SLCC 53 *prfA* mutant strain and its parental strain, NCTC 7973 (1). Previous data had revealed the presence of two transcriptional start sites, P1 and P2, located 135 and 124 nucleotides, respectively, away from the ATG start codon of listeriolysin (3, 9). Transcription initiation at these promoters is dependent on the presence of a palindromic sequence that has been designated as a PrfA box. Hence it was of interest to determine whether production of *hly* transcripts in strains harboring a mutated *prfA* allele was actually due to

leaky, low-level transcription from either or both of these two previously determined promoters. Primer extension experiments with total-cell RNA isolated from the SLCC 53 *prfA* deletion mutant indicated that *hly* transcription in this strain actually initiates 43 nucleotides downstream of the P2 transcription start site (Fig. 2A). Although initiation of transcription at P1 and P2 is clearly visible in the wild-type strains, it is entirely abrogated in the mutant strain, indicating an absolute dependence of their respective promoter regions on the *prfA* gene product (Fig. 2A). We designated this third PrfA-independent promoter P3. The spatial regions corresponding to the −10 and −35 regions of all three promoters are depicted in Fig. 2B.

The detection of a *prfA*-independent promoter for listeriolysin expression now provides a rational basis for understanding some discrepant observations in the literature regarding hemolysin production by *prfA* mutant *Listeria* bacteria. First, it indicates that the weak contact hemolysis that has been repeatedly reported in the literature for several "nonhemolytic" *L. monocytogenes* strains is due to low-level production of listeriolysin (4, 11). Second, it explains the ability of small numbers of *prfA* mutant bacteria to escape from the phagolysosome following invasion of the eucaryotic cells (data not shown). Third, it is now clear why infection of mice with large numbers of listeriolysin-negative *prfA* mutants induce patterns of host Ia^+ macrophage and listeriolysin-specific T-cell expression similar to those induced by wild-type listeriolysin-producing *L. monocytogenes* bacteria (7). Hence, caution must be used in interpreting host cell responses to listeriolysin when such mutants are used. Finally, all studies on the regulated response of listeriolysin expression must now consider the contribution of the low-level *prfA*-independent constitutive response of the P3 promoter.

We thank Petra Köllner, Bianka Jansen, and Maren Bock for excellent technical assistance and Kenneth Timmis for his generous support and encouragement.

The work reported herein was supported by grant SFB 249 from the Deutsche Forschungsgemeinschaft (through project A13).

REFERENCES

1. **Chakraborty, T., and E. Domann.** Unpublished data.
2. **Chakraborty, T., M. Leimeister-Wächter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174**:568–574.
3. **Domann, E., and T. Chakraborty.** 1988. Nucleotide sequence of the listeriolysin gene from a *Listeria monocytogenes* serotype 1/2a strain. Nucleic Acids Res. **17**:6406.
4. **Hof, H.** 1984. Virulence of different strains of *Listeria monocytogenes* serovar 1/2a. Med. Microbiol. Immunol. **173**:207–218.
5. **Leimeister-Wächter, M., and T. Chakraborty.** 1989. Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri*. Infect. Immun. **57**:2350–2357.
6. **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.
7. **Marshall, N., and H. K. Ziegler.** 1991. Role of bacterial hemolysin production in induction of macrophage Ia expression during infection with *Listeria monocytogenes*. J. Immunol. **147**:2324–2332.
8. **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.
9. **Mengaud, J., M. F. Vicente, and P. Cossart.** 1989. Transcriptional mapping and nucleotide sequence of the *Listeria monocytogenes hlyA* region reveal structural features that may be involved in regulation. Infect. Immun. **57**:3695–3701.
10. **Mounier, J., A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocyte-like cell Caco-2. Infect. Immun. **58**:1048–1058.
11. **Pine, L., P. A. Pienta, J. Rocourt, W. Goebel, S. Kathariou, W. F. Bibb, R. E. Weaver, G. M. Carlone, and G. B. Malcolm.** 1987. *Listeria monocytogenes* ATCC 35152 and NCTC 7973 contain a nonhemolytic, nonvirulent variant. J. Clin. Microbiol. **25**:2247–2251.
12. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167**:1459–1471.
13. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell Biol. **109**:1597–1608.
14. **Wehland, J., T. Chakraborty, and K. Niebuhr.** Unpublished data.