

Identification of the Structural Gene Encoding the SH-Activated Hemolysin of *Listeria monocytogenes*: Listeriolysin O Is Homologous to Streptolysin O and Pneumolysin

JÉRÔME MENGAUD,¹ JANET CHENEVERT,¹ CHRISTIANE GEOFFROY,² JEAN-LOUIS GAILLARD,³
AND PASCALE COSSART^{1*}

Unité de Génie Microbiologique¹ and Unité des Antigènes Bactériens,² Institut Pasteur, 75724 Paris Cedex 15, and
Laboratoire de Microbiologie, Faculté de Médecine Necker-Enfants Malades, 75730 Paris Cedex 15,³ France

Received 30 April 1987/Accepted 14 September 1987

By immunoblotting with an antiserum raised against purified listeriolysin O, we have detected the presence of a truncated protein of 52 kilodaltons in culture supernatants of a Tn1545-induced nonhemolytic mutant of *Listeria monocytogenes* (J. L. Gaillard, P. Berche, and P. Sansonetti, *Infect. Immun.* 52:50-55, 1986). The region of insertion of the transposon has been cloned and sequenced. The transposon had inserted in an open reading frame the listeriolysin O gene. The deduced amino acid sequence of this open reading frame revealed that listeriolysin O is homologous to streptolysin O and pneumolysin, although homologies were not detectable at the DNA level.

Listeria monocytogenes is able to survive and even grow within macrophages (8), and it is generally assumed that this characteristic is mainly responsible for the virulence of this human and animal pathogen. Among various factors hypothesized as responsible for the virulence, the hemolysin(s) secreted by *L. monocytogenes* is suspected on the basis of two types of data. (i) All clinical isolates of *L. monocytogenes* are hemolytic; nonhemolytic species belonging to the genus *Listeria*, which are isolated only from asymptomatic humans or the environment, are avirulent when tested in the murine model (5, 11). (ii) A nonhemolytic (Hly⁻) mutant obtained by chromosomal insertion of a single copy of the conjugative transposon Tn1545 was avirulent (3). Virulence was restored in a hemolytic revertant strain obtained by the spontaneous loss of Tn1545. Identical results have been recently obtained with transposon Tn916 (6). The exact nature of the hemolytic factor(s) was unclear (10), but recently, the purification of a hemolytic factor from a culture supernatant of *L. monocytogenes* (strain EGD) has been achieved (4). The secreted listeriolysin O is a 60-kilodalton protein belonging to the group of SH-activated cytolysins, as evidenced by inhibition of the lytic activity with low amounts of cholesterol, activation by reducing agents, and immunological cross-reactivity with streptolysin O.

We used a rabbit antiserum raised against this highly purified protein to detect, by immunoblotting, the presence of listeriolysin O in the culture supernatant of the Tn1545-induced Hly⁻ mutant. The Hly⁻ mutant secreted a shorter polypeptide of 52 kilodaltons which reacted with the antiserum (Fig. 1). This result demonstrates that Tn1545 had inserted in the structural gene of listeriolysin O, excluding the possibility of an insertion in a regulatory gene, causing the Hly⁻ phenotype. These findings prompted us to clone the region in which Tn1545 had inserted. Taking advantage of the presence of a kanamycin resistance gene at the left end of Tn1545 (2), we cloned, in pBR322, a 7-kilobase *Hind*III fragment containing the listerial chromosomal DNA region flanking the transposon and the left part of Tn1545 (Fig. 2). A 400-base-pair *Hind*III-*Acc*I fragment was identified to

contain the listerial component of the insert (see the legend to Fig. 2). It was then subcloned in M13mp20 and M13mp21, and both strands were sequenced by the Sanger technique (Fig. 3) (12). By comparing the sequence with other known junction sequences of Tn1545 (F. Caillaud and P. Courvalin, *Mol. Gen. Genet.*, in press), we identified the left end of the transposon in the middle part of the insert (Fig. 3). The transposon had inserted in an open reading frame. The deduced protein sequence was compared with the recently determined sequences of two other SH-activated cytolysins, pneumolysin (13) and streptolysin O (M. Kehoe, personal communication). Striking homologies were detected at the protein level. These homologies lie in the carboxy-terminal end of pneumolysin and streptolysin O (M. Kehoe, personal

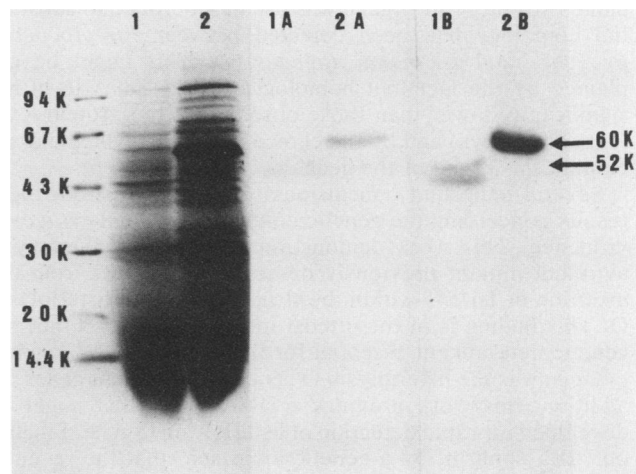


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the culture supernatants of wild-type *L. monocytogenes* and Hly⁻ mutant. Lanes: 1 and 2, Coomassie blue staining of Hly⁺ (lane 1) and Hly⁻ (lane 2); 1A, 2A, 1B, 2B, immunoblot with two different rabbit antisera: Hly⁻ (lanes 1A and 1B) and Hly⁺ (lanes 2A and 2B). The double band detected in 1A and 1B is due to spontaneous proteolysis, after successive thawings of the supernatants. Proteins of known molecular weight (in kilodaltons [K]) are indicated.

* Corresponding author.

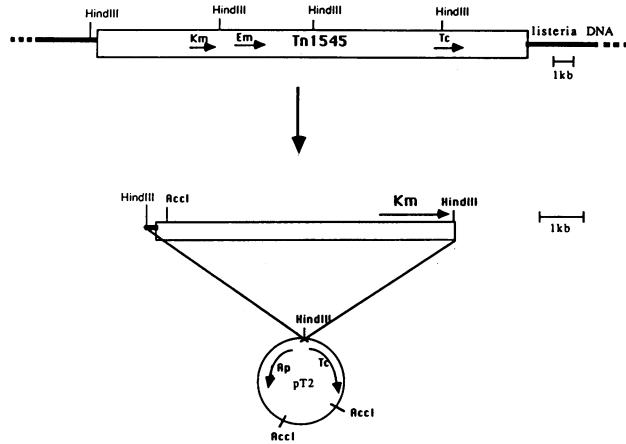


FIG. 2. Cloning of the *Hind*III fragment containing the left part of *Tn1545* and the adjacent *Listeria* region, in pBR322. Clones were identified as kanamycin resistant and tobramycin sensitive (to ensure the presence of the *Tn1545* kanamycin resistance gene in the insert). Restriction analysis of the recombinant plasmid and sequence determination of the two extremities of the insert by the Maxam-Gilbert technique (9) were then performed. Knowledge of the sequence around the *Hind*III site in *Tn1545* (P. Trieu-Cuot and F. Caillaud, personal communication) allowed to localize the *Listeria* part of the insert in the *Hind*III-*Acc*I 400-base-pair fragment, which was further subcloned and sequenced. Abbreviations: Ap, ampicillin; Km, kanamycin; Em, erythromycin; Tc, tetracycline; kb, kilobase.

communication). For these two toxins, this region corresponds to the region of strongest homology. In addition, the unique cysteine thought to be essential for activity is located in this part. For listeriolysin O, this region is also located towards the carboxy-terminal end of the protein, as the truncated protein detected by immunoblotting is 8 kilodaltons smaller than the native protein. These homologies are in perfect agreement with the well-established cross-reactivity between SH-activated hemolysins (1). However, when the cloned streptolysin O gene was used as a probe, no substantial homology had been detected between *Streptococcus pyogenes* and *L. monocytogenes* (7). This might be explained by the fact that homologies at the DNA level are significantly lower than those observed at the protein level (data not shown) and by the choice of a probe which did not contain the 3' end of the gene.

Several important conclusions can be drawn from these results concerning the genetic control of *L. monocytogenes* virulence. This work demonstrates that the nonhemolytic avirulent mutant previously described (3) results from the insertion of *Tn1545* within the structural gene of listeriolysin O. This finding is of the utmost interest. It reveals that the genetic determinant essential for the virulence of *L. monocytogenes* is the listeriolysin O gene or its adjacent genes. In addition, this work provides a DNA probe that might be developed for rapid detection of listeriolysin O gene. Finally, our data confirm, by a genetic approach, that the recently characterized listeriolysin O shares homologies with streptolysin O and pneumolysin, two cytolytic toxins that might also play a role in the pathogenesis of human infections. It should be noted that the hemolytic activity of listeriolysin O is optimal at pH 5.5 and undetectable at pH 7.0, which is not the case for streptolysin O or pneumolysin (optimum pH ≈ 7) (4). The total sequence of the listeriolysin O gene might clarify these differences which might be relevant in terms of

a

```

AA GCT TAT ACA GAT GGA AAA ATT AAC ATC GAT CAC TCT GGA GGA TAC
A Y T D G K I N I D H S G G Y
GTT GCT CAA TTC AAC ATT TCT TGG GAT GAA GTA AAT TAT GAT CCT GAA
V A Q F N I S W D E V N Y D P E
GGT AAC GAA ATT GTT CAA CAT AAA AAC TGG AGC GAA AAC AAT AAA AGC
G N E I V Q H K N W S E N N K S
AAG CTA GCT CAT TTC ACA TCG TCC ATC TAT TTG CCA GGT AAC GCG AGA
K L A H F T S S I Y L P G N A R
AAT ATT AAT GTT TAC G C T A A A A A T A G C A T A A A A A T C T A G T T A T C C G C A T A A A A A C T G
N I N V Y T n1545
GACTTATCACACTTTATCAAGGTCAAACCACTCAATTTACTACTAATTTACTACTTATGAAAT
GAGCTTTGATACGACGATTTATCCTTGAAAGTGAAGATATAAAGATACTTCCAATAAAATTT
GAATATTTAATAGGTA

```

b

		<i>Tn1545</i>
1	CTTTTCCCAACATTTCCCTTTTATT	T TAAAAAATAGCATAAAAATCTAGTT...
2	ATTGAAAATGCTGATTTCTTTATAG	A TAAAAA-TAGCATAAAAATCTAGTT...
3	TGTTGGATTTTCATTTCTCTTCTCCA	T TAAAAA-TAGCATAAAAATCTAGTT...
4	CACCTCATACATTTGATGTTTATCAG	C TAAAAA-TAGCATAAAAATCTAGTT...
5	TAACCGGAGAAATATTAATGTTTACG	C TAAAAA-TAGCATAAAAATCTAGTT...

c

```

LLO A Y T D G K I N I D H S G G Y V A Q F N I S W D E V N Y D P E G N E
SLO E Y T S G K I N L S H Q G A Y V A Q Y E I L W D E I N Y D D K G K E
PLY A Y R N G D L L D H S G A Y V A Q Y Y I T W D E L S Y D H Q G K E
LLO I V Q H R N W S E N N K S K L A H F T S S I Y L P G N A R N I N V Y
SLO V I T K R R W D N N W Y S K T S F F S T V I P L G A N S R N I R I M
PLY V L T P K A W D R N G Q D L T A H F T S I P L K G N V R N L S V K

```

FIG. 3. (a) DNA sequence of the *Listeria-Tn1545* junction. The position of the junction point in the sequence of the *Hind*III-*Acc*I fragment is indicated. The deduced amino acid sequence in the one-letter code is indicated under the DNA sequence. (b) Comparison of the *Listeria-Tn1545* junction (line 5) with four other known junctions (lines 1, 2, 3, and 4) (F. Caillaud and P. Courvalin, *Mol. Gen. Genet.*, in press). The variable bases at the end of the transposon in each junction are boxed. (c) Comparison of the deduced amino acid sequence of listeriolysin O (LLO) with streptolysin O (SLO) and pneumolysin (PLY). Boxes denote common residues. Numbers refer to amino acid positions in the protein sequence for SLO and PLY.

pathogenicity, since only listeriolysin O is produced by an intracellularly replicating bacterium (8).

We are greatly indebted to G. Boulnois and M. Kehoe for communication of the sequences of pneumolysin and streptolysin O before publication. We thank P. Trieu-Cuot and F. Caillaud for communication of unpublished sequence data concerning *Tn1545*. We greatly acknowledge Patrick Berche, Julian Davies, and Brigitte Gicquel-Sanzey for interest in this work and helpful criticism in the manuscript preparation. We thank J. Alouf for his enthusiastic interest in this project.

This work was supported by the Pasteur Institute, the CEE (contrat n ST2J-0319-C) and Institut National de la Santé et de la Recherche Médicale (grant CRE to the Laboratoire de Microbiologie, Faculté de Médecine Necker-Enfants Malades).

LITERATURE CITED

- Alouf, J. E. 1980. Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol. Ther.* 11:661-717.
- Caillaud, F., C. Carlier, and P. Courvalin. 1987. Physical analysis of the conjugative shuttle transposon *Tn1545*. *Plasmid* 17:58-60.
- 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.
4. Geoffroy, C., J.-L. Gaillard, J. E. Alouf, and P. Berche. 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. Infect. Immun. 55:1641-1646.
 5. Groves, R. D., and H. J. Welshimer. 1977. Separation of pathogenic from apathogenic *Listeria monocytogenes* by three in vitro reactions. J. Clin. Microbiol. 5:559-563.
 6. Kathariou, S., P. Metz, H. Hof, and W. Goebel. 1987. Tn916-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bacteriol. 169:1291-1297.
 7. Kehoe, M., and K. N. Timmis. 1984. Cloning and expression in *Escherichia coli* of the streptolysin O determinant from *Streptococcus pyogenes*: characterization of the cloned streptolysin O determinant and demonstration of the absence of substantial homology with determinants of other thiol-activated toxins. Infect. Immun. 43:804-810.
 8. Mackaness, G. B. 1962. Cellular resistance to infection. J. Exp. Med. 116:381-406.
 9. Maxam, A., and W. Gilbert. 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA 74:560-564.
 10. Parrisius, J., S. Bhakdi, M. Roth, J. Tranum-Jensen, W. Goebel, and H. P. R. Seeliger. 1986. Production of listeriolysin by beta-hemolytic strains of *Listeria monocytogenes*. Infect. Immun. 51:314-319.
 11. Rocourt, J., J. M. Alonso, and H. Seeliger. 1983. Virulence comparée des cinq groupes génomiques de *Listeria monocytogenes* (*sensu lato*) Ann. Microbiol. (Paris) 134A:354-364.
 12. Sanger, F., S. Nicklen, and R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
 13. Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois. 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. 55:1184-1189.