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Listeriolysin O Is Essential for Virulence of *Listeria Monocytogenes*: Direct Evidence Obtained by Gene Complementation

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The role of listeriolysin O in the intracellular multiplication of *Listeria monocytogenes* and, therefore, its pathogenicity was questioned through a genetic complementation study. A nonhemolytic mutant was generated by inserting a single copy of transposon Tn*917* in the bacterial chromosome. This insertion was localized by DNA sequence analysis in *hlyA*, the gene coding for listeriolysin O. As was another mutant that we previously characterized, this mutant was avirulent in the mouse. It was transformed with a plasmid carrying only *hlyA*, able to replicate in *L. monocytogenes*, and stably maintained in vitro and in vivo. The complemented strain displayed a hemolytic phenotype identical to that of the wild-type strain and was fully virulent, therefore attributing a crucial role to listeriolysin O in virulence and excluding the hypothesis of a polar effect of the transposon insertion on genes adjacent to *hlyA* and possibly involved in virulence.

Listeria monocytogenes is a facultative intracellular gram-positive bacterium (37) responsible for severe infections in humans and other animal species (36). Humans at risk are pregnant women and immunocompromised individuals. Manifestations of the disease include abortion, septicemia, and meningoencephalitis; outbreaks have been traced to contaminated foods, suggesting that the gastrointestinal tract is the primary entry route. The pioneering studies of G. B. Mackaness (24) have shown that the natural susceptibility of mice to *L. monocytogenes* could be attributed to the survival and multiplication of the microorganism within host resident macrophages. It has been demonstrated that control of infection requires the emergence of a T cell-mediated response (6, 22, 25, 31) correlated with an influx of macrophages no more permissive to *Listeria* growth in liver (23) and that immunity to reinfection can be triggered only by live organisms (3, 16, 45). Although the macrophage is the main cell involved in the in vivo multiplication of *L. monocytogenes*, a number of other cell types such as fibroblasts and epithelial cells have been successfully infected in vitro (4, 12, 17, 21, 33), and it was demonstrated in the case of the human enterocyte-like Caco-2 cells that, after phagocytosis, bacteria leave the phagosome and multiply within the cytosolic compartment of these host cells (12). Therefore, identification of the factors which allow bacteria to escape phagosomes and to multiply intracellularly is essential to understand the virulence of the organism.

The observations that all strains of *L. monocytogenes* isolated from natural infections produce a zone of hemolysis on blood agar medium and that these strains are virulent in the mouse model, whereas nonhemolytic strains isolated after multiple subcultures (18) or from the environment are avirulent, first suggested that a hemolysin might be a relevant virulence factor (35). Recently, genetic and physiopathological studies with avirulent mutants obtained by transposon mutagenesis (13, 19, 33) gave strong support to the hypothesis that secretion of a hemolytic exotoxin is a crucial event promoting intracellular growth of *L. monocytogenes* in host tissues (12, 21, 33). This conclusion was based on the finding that nonhemolytic mutants were avirulent in the mouse, whereas virulence was restored in hemolytic revertant strains obtained by spontaneous loss of transposons. Moreover, nonhemolytic mutants invade different cell lines as efficiently as the wild type (12, 21, 33) but, in most cases, do not replicate intracellularly.

The hemolytic factor listeriolysin O has been purified (14) and shown to be a 58-kilodalton protein. It belongs to a family of cytolysins whose prototype is streptolysin O and which are produced by various gram-positive species, including *Streptococcus*, *Bacillus*, *Clostridium*, and *Listeria* spp. (39). These cytolysins share a number of common properties: similar molecular weight, immunological cross-reactivity, activation by thiol reducing agents (1), and binding to and inactivation by cholesterol (34). Among these cytolysins, listeriolysin O is the only one to be produced by an intracellular bacterium and to be active at acid pH (14). Since only hemolytic bacteria escape from the phagosome compartment into the cytosol of Caco-2 cells, it was proposed that listeriolysin O mediates virulence by damaging the phagosome membrane (12).

The gene coding for listeriolysin O, *hlyA*, has been cloned and sequenced (27, 28, 43). The deduced amino acid sequence of the protein shows that listeriolysin O is composed of 504 amino acids and contains a unique cysteine residue assumed to be essential for the cytolytic activity (39). Localization of transposon insertions within *hlyA* in several nonhemolytic, avirulent mutants (13, 19, 27, 33) strongly suggests that this region of the bacterial chromosome is crucial for virulence (8). However, these data do not rule out the possibility that disruption of the gene encoding listeriolysin O exerts a polar effect on genes adjacent to *hlyA* that could be involved in the expression of other virulence factors required for entry in the host and intracellular survival and multiplication.

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The aim of the present study was to evaluate the role of listeriolysin O in virulence and exclude the hypothesis of a polar effect, through a genetic complementation study. For this purpose, from an easily transformable virulent strain of *L. monocytogenes*, a new nonhemolytic mutant was constructed by means of transposon Tn*917* (47). This strain, as are other nonhemolytic mutants previously isolated (13, 19, 27, 33), was avirulent in the mouse. It was then transformed with a plasmid containing only the listeriolysin O gene. The transformed strain displayed a hemolytic phenotype and was virulent in the mouse, thus providing direct evidence that listeriolysin O is essential for the intracellular growth of *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture media. *L. monocytogenes* LO28, a virulent hemolytic strain from the Ramon Y Cajal Hospital Collection (43), was used in this study. Mutagenesis was performed with transposon Tn*917* present on plasmid pTV1 and encoding erythromycin resistance (47). The *Escherichia coli-Bacillus subtilis* shuttle vector pMK4 (40) used in this study to transform *L. monocytogenes* is a hybrid plasmid obtained by fusion of plasmid pUC9 and pC194. Plasmid pUC18 (46) and bacteriophage M13mp18 (29) were also used to clone DNA fragments. *E. coli* strains were grown at 37°C in LB medium (30). For strains containing pUC derivatives, ampicillin was added at final concentrations of 25 μg/ml in liquid medium and 100 μg/ml in solid medium. *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, Mich.) at 30 or 37°C, as required. Chloramphenicol (10 μg/ml) and erythromycin (1 μg/ml) were added to agar medium or to broth, when required. BHI agar plates supplemented with 5% horse blood were used to detect hemolytic activity.

Chemical and enzymes. Restriction enzymes and ligase were purchased from Amersham Corp. (Buckinghamshire, United Kingdom), Boehringer GmbH (Manheim, Federal Republic of Germany), or Genofit (Geneva, Switzerland) and were used as recommended by the manufacturer.

DNA techniques. *E. coli* plasmid DNA was purified by ultracentrifugation in cesium chloride gradients (26). Rapid preparation of *E. coli* or *Listeria* plasmid DNA was performed by the method of Birnboim and Doly (5). To facilitate restriction analysis, *Listeria* plasmid DNAs were transformed into and extracted from *E. coli*. DNA fragments were prepared by diffusion or electroelution from polyacrylamide gels or by use of the GeneClean kit (Bio 101, La Jolla, Calif.), after electrophoresis on agarose gels. *Listeria* chromosomal DNA was prepared as already described (28). Recombinant DNA techniques were performed as described previously (26). Southern blot DNA hybridization and colony hybridization were performed with Hybond-N nylon membranes (Amersham). DNA probes were labeled by the multiprime labeling system (kit RPN.160 1Y; Amersham). DNA sequence was determined with the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio).

Transformation procedure. *L. monocytogenes* was transformed by the protoplast transformation technique (44). Alternatively, protoplasts were electroporated using the Gene Pulser transfection apparatus (Bio-Rad Laboratories, Richmond, Calif.); in the latter, to 0.5 ml of protoplasts in suspension in SMMP buffer (7) were successively added 1.5 ml of polyethylene glycol 6000 (40% [wt/vol]) and plasmid DNA (0.5 μg in 50 ml of TE buffer). The mixture was subjected to a single pulse electroporation of 25 μF at 5,000 V/cm. Five milliliters of SMMP medium (7) was then added, and protoplasts were recovered by centrifugation for 10 min at 2,600 × *g*. Cells were suspended in 0.5 ml of SMMP buffer and incubated at 37°C for 2 to 3 h, and portions of 0.1 ml were plated on DM3 medium (7) supplemented with antibiotic for regeneration. Incubation for 4 days at 37°C was required. The electric pulse increased the transformation efficiency from 10 to 10^2 transformants per μg of plasmid DNA. Characterization of transformants was achieved by employing the classical tests described elsewhere (13), including Gram staining, catalase activity, motility, and fermentative capacity on 50 different substrates (API 50CH, API Systems, La Balme les Grottes, France), and by restriction analysis of plasmid DNA.

Subcloning of the *hlyA* gene in pUC18. Because of the presence of rearrangements on the original cosmid used to clone *hlyA* in *E. coli* (28), a 4-kilobase *Bam*HI chromosomal fragment from strain LO28 was recloned in plasmid pUC18, giving rise to a plasmid called pLis3. This plasmid was used to construct plasmid pLis4.

Isolation of Tn*917* insertion mutants of *L. monocytogenes*. Plasmid pTV1 was introduced in LO28 by the polyethylene glycol-protoplast transformation procedure (44). This plasmid contains the 5.3-kilobase transposon Tn*917* coding for erythromycin resistance, together with a thermosensitive replication origin and a chloramphenicol resistance gene (32, 47). It has been extensively used for transposon mutagenesis in another gram-positive bacterium, *B. subtilis* (42). Transformants were selected on DM3 plates (7) supplemented with chloramphenicol (5 μg/ml) and erythromycin (1 μg/ml) at 30°C. Plasmid DNA was then purified from transformants and tested for absence of rearrangements by restriction analysis. The results showed that plasmid pTV1 is stably maintained in strain LO28. The pTV1-harboring strain was then used as a source of Tn*917* insertion mutants. Transposition of Tn*917* from plasmid pTV1 onto the bacterial chromosome was obtained by growing bacteria to mid-log phase at 30°C in BHI broth containing chloramphenicol (5 μg/ml) and erythromycin (1 μg/ml). Tn*917* insertion mutants were obtained by incubating bacteria at 42°C on BHI agar supplemented with the same concentration of erythromycin, thus selecting for transposition events from the plasmid into the chromosome and for loss of plasmid pTV1. Among 2,000 erythromycin-resistant colonies, 10 colonies were identified as nonhemolytic on blood agar plates. They were further shown to be chloramphenicol sensitive and plasmid free. One mutant was further studied.

Assay for hemolytic activity. For titration of hemolytic activity in culture supernatants of the various strains, bacteria were grown in a charcoal-treated broth prepared as follows: a 10-fold concentrated proteose-peptone broth (proteose-peptone no. 3 [Difco], 20 g; yeast extract [Difco], 5 g; $Na_2HPO_4 \cdot 12H_2O$, 8.3 g; KH_2PO_4, 0.7 g; quartz-distilled water, 100 ml) supplemented with charcoal (activated charcoal; Prolabo, Paris, France) at a 0.2% final concentration was stirred (100 rpm) for 1 h at room temperature. The charcoal-treated concentrate was further diluted in quartz-distilled water (1:10), adjusted to pH 7.5, and autoclaved at 115°C for 20 min. Sterile glucose (1%, final concentration) was added before inoculation.

Bacteria were inoculated in 5 ml of this medium. Cultures were then diluted in 100 ml of the same medium and incubated with shaking for 10 h at 37°C until the end of log-phase growth. Cultures were then centrifuged for 10 min at 10,000 × *g* and 4°C. Supernatants were assayed for

hemolytic activity (14). Briefly, 0.5 ml of 2.25% sheep erythrocyte suspension (6×10^8/ml) in phosphate-buffered saline, pH 6.0, was added to 1.0-ml volumes of serially diluted supernatants supplemented with cysteine (20 mM, final concentration) in the same buffer supplemented with 0.1% bovine albumin (Sigma Chemical Co., St. Louis, Mo.). After 45 min of incubation at 37°C, tubes were centrifuged and the A_{541} was measured with a spectrophotometer (Beckman M25) as an indication of the hemoglobin released from erythrocytes. One hemolytic unit (HU) was determined as the amount of toxin needed to release half the hemoglobin of the erythrocytes and was estimated graphically by plotting percent lysis versus sample volume on a log-probit graph.

Virulence in the mouse. Specific-pathogen-free ICR female Swiss mice (Charles River, St. Aubin-les-Elboeuf, France), 6 to 8 weeks old, were used in this study. Bacteria described in this report were grown in BHI (Diagnostics Pasteur, Marnes-la-Coquette, France), harvested in log-phase growth (10^8 bacteria per ml), and stored in 1-ml portions at −80°C until required. For each experiment, a vial of the frozen stock was quickly thawed and diluted appropriately in 0.15 M NaCl for intravenous (i.v.) inoculations (0.5 ml per injection). Virulence was estimated by determining the 50% lethal dose (LD_{50}) by the probit method on groups of five mice or by monitoring bacterial growth in liver and spleen. Bacterial survival in these organs was determined after killing groups of five infected mice by cervical dislocation. The aseptically removed spleens and livers were separately ground, and samples (0.1 ml) of serial dilutions of the whole organ homogenates were plated on tryptic soy agar. Colonies were counted after 24 h of incubation at 37°C, and results were expressed as the $\log_{10}$ bacterial counts per organ.

RESULTS

To evaluate the role of listeriolysin O in the survival and intracellular growth of *L. monocytogenes* and the effect of transposon insertions within the structural gene encoding hemolysin, a genetic complementation approach was devised, involving three steps: (i) a DNA fragment carrying only *hlyA*, the gene coding for listeriolysin O, was cloned in a *E. coli-Listeria* shuttle vector; (ii) this plasmid was then introduced by transformation into a nonhemolytic, avirulent mutant of *L. monocytogenes* obtained by transposon mutagenesis with Tn*917* and whose insertion had been fully characterized; and (iii) the physiological properties and the virulence of the transformants were analyzed.

Subcloning of the listeriolysin O gene in a shuttle vector. The *hlyA* gene, recently cloned and sequenced (27, 28, 43), was subcloned in *E. coli* in the shuttle vector pMK4. Sequence analysis of the regions adjacent to *hlyA* (data not shown) revealed two *Sau*96A restriction sites which enabled us to clone the gene *hlyA* alone without any neighboring coding regions. The 2,436-base-pair (bp) *Sau*96A fragment was purified from pLis3 (described in Materials and Methods) and cloned in the *Sma*I site of the shuttle vector pMK4, giving rise to plasmid pLis4 (Fig. 1).

Isolation and characterization of a Tn*917* insertion nonhemolytic mutant and its transformation with shuttle vectors harboring or not harboring the *hlyA* gene. By means of transposon Tn*917* (47), we generated a bank of mutants from *L. monocytogenes* LO28 (described in Materials and Methods). Nonhemolytic mutants screened on blood agar plates were found at a frequency of 10^{-4}. Nonhemolytic colonies were verified to be gram positive, catalase positive, and motile and to display the characteristic fermentative pattern of *L. monocytogenes* towards 50 different substrates on API 50CH plates.

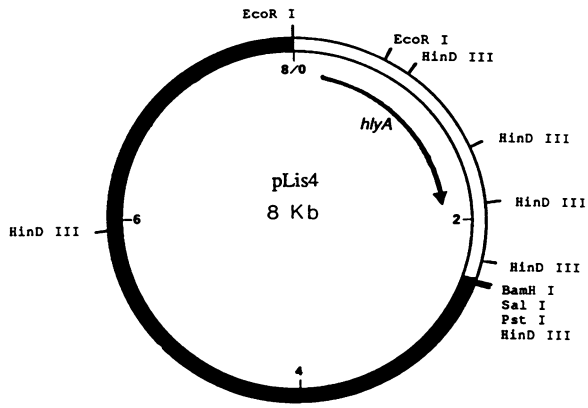

FIG. 1. Restriction map of plasmid pLis4. Plasmid pLis4 was obtained by cloning in plasmid pMK4 the *Sau*96A DNA fragment containing the *hlyA* gene of *L. monocytogenes*. Kb, Kilobases.

One of these nonhemolytic mutants was further characterized by Southern blotting analysis, with a 1.5-kilobase *Hin*dIII fragment internal to Tn*917* as a probe. Before electrophoresis, the chromosomal DNA was restricted by *Eco*RI, *Pst*I, and *Bam*HI, three endonucleases which do not cut Tn*917*, and by *Hin*dIII, which recognizes two sites in Tn*917* (32). A single chromosomal DNA fragment hybridized with the Tn*917* probe after digestion with *Eco*RI, *Bam*HI, or *Pst*I, thus providing evidence that a single copy of Tn*917* had inserted into the chromosome (Fig. 2, panel a). The precise localization of Tn*917* was achieved by rehybridizing the filter with two other probes corresponding to different parts of *hlyA*. The 651-bp *Hin*dIII fragment internal to this gene remained intact in the insertion mutant, hybridizing as a single band (Fig. 2, panel b). In contrast, two chromosomal DNA fragments were recognized by the *Hin*dIII 410-bp probe containing the 3′ end of gene *hlyA* (Fig. 2, panel c), demonstrating that this part of the *hlyA* gene was interrupted by the transposon in the nonhemolytic mutant. These results indicate that Tn*917* had inserted in the 3′ distal part of gene *hlyA*. This was further confirmed by sequencing one of the *Hin*dIII junction fragments after cloning in plasmid pUC18 and subcloning in M13mp18. Tn*917* had inserted after codon 503 (corresponding to amino acid 478 of the secreted protein) (Fig. 3), thus generating a fusion protein of 503 amino acids containing 25 amino acids derived from Tn*917* (data not shown). As will be shown later, the transposon insertion severely affected both the function of the toxin and the virulence of the mutant, designated LO28 (*hlyA*::Tn*917*).

The final step of this approach was to transform strain LO28 and its nonhemolytic derivative, LO28 (*hlyA*::Tn*917*), with plasmids pMK4 and pLis4, which were found able to replicate in *L. monocytogenes*. Transformants were characterized as described in Materials and Methods.

Production of listeriolysin O in culture supernatants. The hemolytic phenotype was first examined by plating bacteria for 24 h at 37°C on 5% horse blood agar. Strains LO28, LO28 (pMK4), and LO28 (pLis4) displayed a homogeneous and stable hemolytic phenotype. Strains LO28 (*hlyA*::Tn*917*) and LO28 (*hlyA*::Tn*917*, pMK4) were nonhemolytic under these conditions. In contrast, colonies of strain LO28 (*hlyA*::Tn*917*, pLis4) were homogeneously hemolytic on blood agar

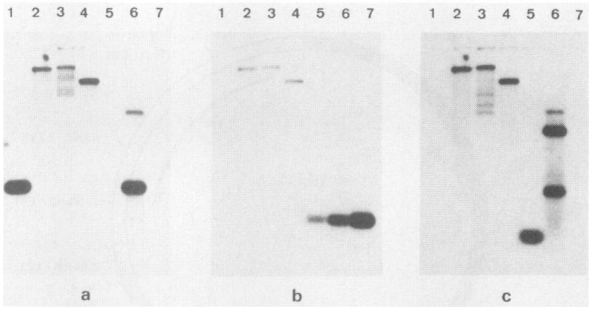

FIG. 2. Electrophoresis showing localization of Tn*917* in the chromosome of a Tn*917* insertion nonhemolytic mutant by Southern blot DNA hybridization. The same filter was successively hybridized with the following probes: (i) a 1.5-kilobase fragment internal to Tn*917* (a); (ii) a 651-bp *Hin*dIII fragment of *hlyA* (b); (iii) a 410-bp *Hin*dIII fragment of the 3′ end of the gene (c). Lanes: 1, the Tn*917* probe; 2 through 4, LO28 (*hlyA*::Tn*917*) DNA restricted by *Eco*RI, *Pst*I, and *Bam*HI, respectively; 5, LO28 DNA restricted by *Hin*dIII; 6, LO28 (*hlyA*::Tn*917*) DNA cut by *Hin*dIII; 7, the 651-bp *Hin*dIII probe. A partial digestion is detected in lanes 3 and 6, in both panels a and c. A single chromosomal DNA fragment hybridized with the Tn*917* probe after digestion with three restriction enzymes (panel a, lanes 2 through 4). The 651-bp *Hin*dIII probe for the *hlyA* gene hybridized with a single fragment of the digested chromosomal DNA (panel b, lane 5), meaning that this region of *hlyA* remained intact in the insertion mutant. In contrast, two main chromosomal DNA fragments were recognized by the *Hin*dIII 410-bp probe containing the end of gene *hlyA*, showing that this part of the *hlyA* gene was split in the mutant (c, lane 6). These results indicate that Tn*917* had inserted in the 3′ distal region of gene *hlyA*.

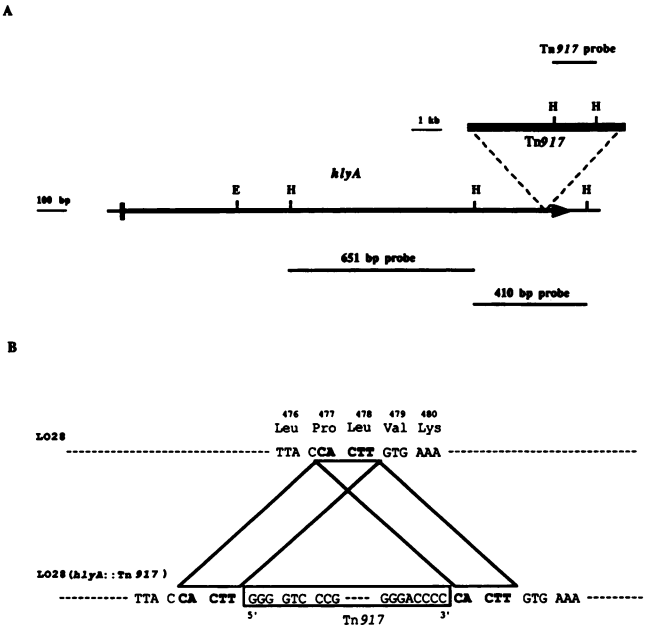

FIG. 3. (A) Schematic drawing of the Tn*917* insertion in *hlyA*. Tn*917* and *hlyA* are not drawn on the same scale. *Eco*RI (E) and *Hin*dIII (H) restriction sites are indicated. The three probes used in the Southern blot hybridization shown in Fig. 2 are represented. kb, Kilobase. (B) Tn*917* insertion in *hlyA*. The *Hin*dIII fragment containing the Tn*917-hlyA* 3′ end junction has been sequenced. Knowledge of *hlyA* (28) and Tn*917* (38) sequences and of a duplication event of 5 bp occurring during transposition of Tn*917* (32) allowed the precise localization of Tn*917* in *hlyA*. The insertion generated a fusion protein containing 25 amino acids derived from the Tn*917* left end sequence.

plates, indicating not only that pLis4 was stably maintained in vitro but also that *hlyA* was expressed.

The hemolytic activity was then titrated in the supernatants of these six strains grown for 10 h at 37°C in charcoal-containing broth, when hemolytic activity was maximal (15). Results are reported in Table 1. The hemolytic activity of the wild-type strain was 900 HU/ml and was similar to that of the strain harboring plasmid pMK4. Surprisingly, no significant change in the hemolytic titer was detected when LO28 harbored the recombinant plasmid pLis4. In the nonhemolytic mutant and its derivative carrying plasmid pMK4, the hemolytic activity was estimated to be less than 10 HU/ml, whereas in the complemented strain LO28 (*hlyA*::Tn*917*, pLis4), it reached a level of 1,200 HU/ml. This result provides evidence that the *hlyA* gene is fully expressed in the complemented mutant.

Virulence of nonhemolytic mutants transformed with a plasmid carrying the hemolysin gene. Groups of five Swiss mice were inoculated i.v. with increasing doses of LO28 or its derivatives, and mortality was monitored over the next 10 days. The LD_{50}s obtained are listed in Table 1. Introduction of plasmid pMK4 into strain LO28 significantly decreased the LD_{50} by more than 1.0 log unit (LD_{50}, $10^{6.5}$ bacteria per mouse versus $10^{5.2}$ bacteria per mouse for the parental strain). This indicates that expression of virulence was partially impaired by the presence of pMK4. A similar decrease in virulence was observed after introducing pLis4 into LO28. In contrast, the nonhemolytic strain LO28 (*hlyA*::Tn*917*) and its derivative LO28 (*hlyA*::Tn*917*, pMK4) were totally avirulent (LD_{50}, $>10^{9.5}$ bacteria per mouse), as were the nonhemolytic mutants previously obtained with Tn*1545* (13, 33) or Tn*916* (19, 33). Virulence was restored at a level identical to that of LO28 (pMK4) or LO28 (pLis4) when plasmid pLis4 carrying the *hlyA* gene was introduced into the nonhemolytic avirulent mutant. The LD_{50} was $10^{6.8}$ bacteria per mouse in the complemented strain, which represents a 3-log increase when the *hlyA* gene is present in this strain.

These results were further confirmed by monitoring bacterial growth in the spleens and livers of i.v. infected mice. When mice were inoculated with 3×10^5 to 8×10^5 bacteria of LO28, LO28 (pMK4), or LO28 (pLis4), bacteria rapidly multiplied in the organs over the next 2 days, although at a slower rate in the case of the two plasmid-containing strains (Fig. 4). The two latter strains did not induce early mortality, as opposed to LO28. The two nonhemolytic strains LO28 (*hlyA*::Tn*917*) and LO28 (*hlyA*::Tn*917*, pMK4) were rapidly destroyed within 48 h of infection (Fig. 5) and failed to create visible abscesses even with high infecting doses (~10^8 bacteria per mouse). In contrast, the complemented strain LO28 (*hlyA*::Tn*917*, pLis4) harboring the *hlyA* gene on plasmid pLis4 replicated in spleen and liver, inducing visible ab-

TABLE 1. Hemolysin production and virulence in the mouse

Strain	Hemolytic titer (HU/ml)	LD_{50}
LO28	900	$10^{5.2}$
LO28 (pMK4)	1,000	$10^{6.5}$
LO28 (pLis4)	900	$10^{6.8}$
LO28 (*hlyA*::Tn*917*)	<10	$>10^{9.5}$
LO28 (*hlyA*::Tn*917*, pMK4)	<10	$>10^{9.5}$
LO28 (*hlyA*::Tn*917*, pLis4)	1,200	$10^{6.8}$

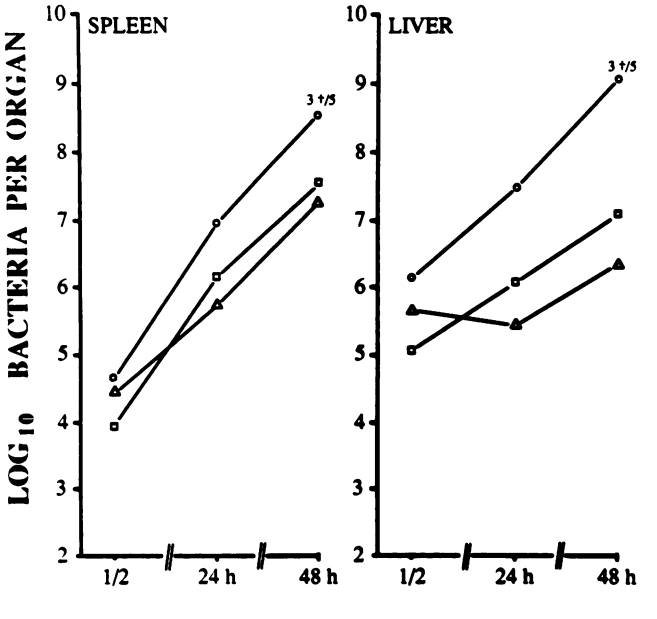

FIG. 4. Growth curves of the wild-type strain LO28 and its transformants. Mice were inoculated i.v. with strains LO28 (○), LO28 (pMK4) (□), or LO28 (pLis4) (△). Bacterial growth was monitored in the spleen and the liver over the next 2 days. LO28 induced early mortality by day 2 (three of five [3†/5] mice). The virulence of the two plasmid-containing strains was reduced, as shown by a lower rate of bacterial growth and absence of early mortality. Exact infecting i.v. doses were 3.6×10^5 for LO28, 8.5×10^5 for LO28 (pMK4), and 2.5×10^5 for LO28 (pLis4). Groups of five mice per time point.

scesses and early mortality in mice at high infecting doses (1.6×10^8 bacteria per mouse). The rate of in vivo bacterial multiplication was similar to that of the two plasmid-containing hemolytic strains.

Finally, it was important to verify that the parallel restoration of hemolytic phenotype and virulence was due to complementation and not to a recombination event taking place in vivo in strain LO28 (*hlyA*::Tn*917*, pLis4). For this purpose, the number of viable bacteria present in organs of mice at day 3 of infection (10^6 bacteria i.v.) was estimated by plating dilutions of ground tissues on BHI agar, BHI blood agar, and BHI agar supplemented with chloramphenicol, erythromycin, or both antibiotics. Bacteria were hemolytic on blood agar plates, and the number of colonies was similar on the five culture media, strongly suggesting that transposon Tn*917* and plasmid pLis4 were stably maintained in vivo. In addition, the plasmid DNA content of 12 colonies originating from host tissues and randomly taken from the different plates was analyzed. The restriction profiles of the plasmids were identical to that of the original pLis4, thus demonstrating the stable maintenance of this plasmid in vivo as well as in vitro.

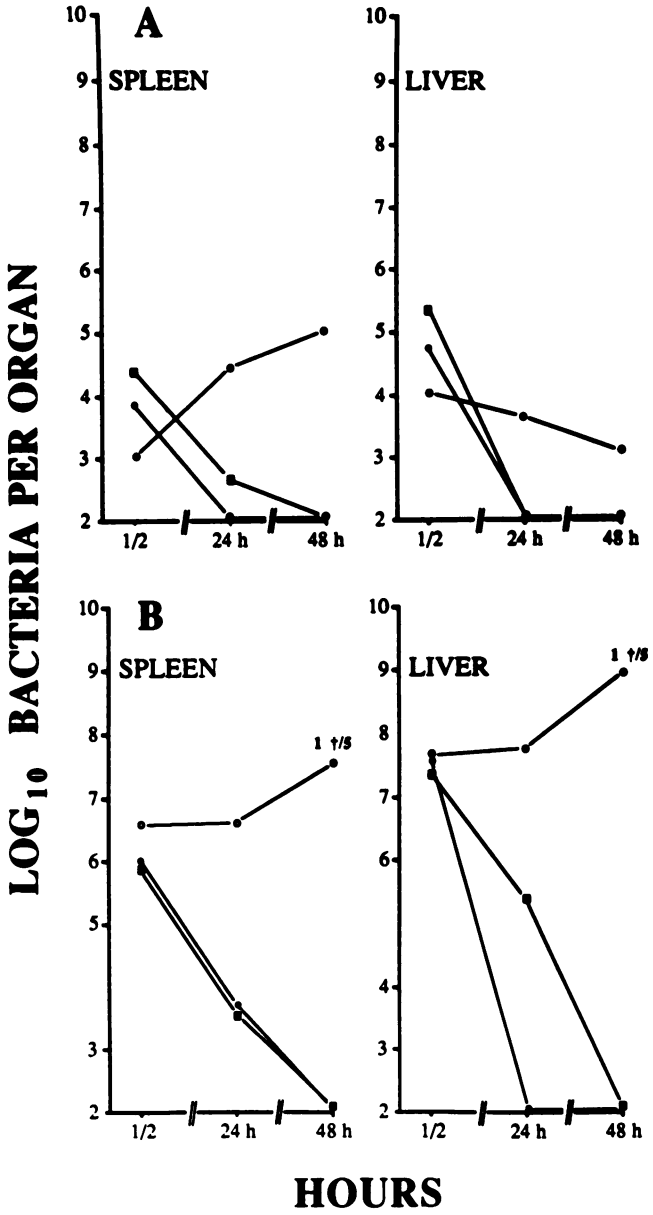

FIG. 5. Growth curves of the nonhemolytic mutant LO28 (*hlyA*::Tn*917*) and its transformants. Mice were inoculated i.v. with strain LO28 (*hlyA*::Tn*917*), LO28 (*hlyA*::Tn*917*, pMK4), or LO28 (*hlyA*::Tn*917*, pLis4). (A) Low infecting doses: LO28 (*hlyA*::Tn*917*), 2.6×10^5 (■); LO28 (*hlyA*::Tn*917*, pMK4), 6.2×10^5 (●); LO28 (*hlyA*::Tn*917*, pLis4), 5.5×10^4 (○). (B) High infecting doses: LO28 (*hlyA*::Tn*917*), 1.1×10^8 (■); LO28 (*hlyA*::Tn*917*, pMK4), 1.0×10^8 (●); LO28 (*hlyA*::Tn*917*, pLis4), 1.6×10^8 (○). The two nonhemolytic strains failed to grow in the livers and spleens of infected mice, even at high infecting doses, whereas the strain harboring pLis4 multiplied in host tissues, creating visible abscesses and inducing mortality when mice were infected with high doses of bacteria (~10^8 per mouse). 1†/5, One of five mice died. Groups of five mice per time point.

DISCUSSION

This report provides conclusive evidence obtained through a genetic complementation study that the production of listeriolysin O is necessary for the virulence of *L. monocytogenes*. Recent reports demonstrated that insertion of transposable elements in the structural gene coding for the hemolysin prevented the capacity of *L. monocytogenes* to multiply in host tissues (13, 19, 33). This strongly suggested that the hemolysin region of the chromosomal DNA is crucial for virulence (8, 27). However, insertional events can exert a polar effect on adjacent genes. Therefore, these genetic results did not eliminate the possibility that genes adjacent to the hemolysin gene might be involved in the

process of virulence. Moreover, several independent observations apparently question the role of hemolysin. First, a strict relationship between the in vitro level of hemolytic activity and the expression of virulence of *L. monocytogenes* in the mouse is not observed (2, 15). Second, some nonhemolytic insertional mutants of *L. monocytogenes* do not seem to be affected in their capacity to grow in the Henle 407 human epithelial cell line, although they are avirulent in the mouse (33). Finally, a closely related species, *Listeria seeligeri*, which produces, albeit at low levels, a sulfhydryl-activated toxin antigenically related to listeriolysin O (15), fails to induce natural infections and is avirulent in the mouse (35). Thus, it was important to directly demonstrate that the *hlyA* gene is involved in the process of intracellular growth of *L. monocytogenes*.

If the *hlyA* gene were the only gene affected by the transposon-induced gene disruption event, the original virulence should be restored by introducing into the mutant the *hlyA* gene carried on a plasmid. In order to test this hypothesis, we chose to insert the *hlyA* gene into a shuttle vector able to replicate in *L. monocytogenes* and to transform a well-characterized, transposon-induced, nonhemolytic avirulent mutant of *L. monocytogenes*. Several conditions had to be satisfied before conclusions could be drawn from such a complementation study: (i) the shuttle vector used to transform the recipient mutants should exclusively harbor the *hlyA* gene; (ii) the recipient mutant should imperatively result from a single insertional transposition event within the structural gene of the hemolysin; (iii) the complemented mutant should stably express the hemolytic phenotype both in vitro and in vivo; and (iv) no rearrangements should be detected between the plasmid and the chromosomal DNA in the complemented mutant. These requirements were fulfilled in the present study.

The first requirement was satisfied by taking advantage of two restriction sites located 240 and 560 bp upstream and downstream from the *hlyA* gene, respectively. The sequence of this fragment and transcriptional studies revealed that the plasmid contained the *hlyA* gene with its promoter, the beginning of a putative open reading frame starting 340 bp downstream from *hlyA*, and no open reading frame within the 240-bp upstream region (J. Mengaud, M. F. Vicente, and P. Cossart, Infect. Immun., in press). This indicated that pLis4 harbors exclusively the *hlyA* gene.

A nonhemolytic, avirulent mutant from strain EGD derived by insertion of a single copy of transposon Tn*1545* (13) was available. It was initially used as a recipient in the transformation studies, as its transposon insertion had been precisely localized (27). However, the transformants obtained by electroporation were unstable in vitro, with a high rate of spontaneous loss of plasmids. The reasons for this instability have not been completely elucidated but may be due to the nature of the vector used; other replicons are now able to replicate in this strain (unpublished results). We therefore used another nonhemolytic mutant as a recipient strain. This nonhemolytic derivative was obtained by mutagenesis of a virulent strain of *L. monocytogenes*, strain LO28, with transposon Tn*917*. This mutant, LO28 (*hlyA*::Tn*917*), harbored a single copy of Tn*917* inserted within the structural gene of listeriolysin O. The insertion was located after codon 503, thus producing an inactive secreted protein of 503 amino acids containing 25 amino acids derived from Tn*917*. It is to be noted that transposon Tn*917* had never been used in the genus *Listeria* and, as shown in this study, is a new tool for genetic studies in this species. Its main advantage is that it is much smaller than the two conjugative plasmids Tn*1545* (9) and Tn*916* (11), which facilitates DNA manipulations. In addition, a single bacterium transformed with plasmid pTV1 carrying transposon Tn*917* is per se a potential bank of mutants: to isolate mutants, one only needs to raise the temperature and screen for the desired phenotype. Interestingly, the insertion described here occurred at a location different from that observed in the Tn*1545* insertion nonhemolytic mutant previously characterized (27), where Tn*1545* had inserted upstream from the region coding for the highly conserved undecapeptide found in the four sequenced thiol-activated toxins (20, 28, 41). This undecapeptide may have an important role in the function of the protein, as it contains the unique cysteine of the protein known to be inactivated by thiol-alkylating agents (14). In the Tn*917* insertion nonhemolytic mutant, the insertional event occurred downstream from the region coding for this highly conserved region which was therefore unaffected by the mutation. This result emphasizes that integrity of the C-terminal part of the protein is required for the function of listeriolysin O. Mutant LO28 (*hlyA*::Tn*917*) and the original strain LO28 were transformed with plasmids pMK4 and pLis4. On the basis of the antibiotic resistance pattern of the transformed strains and the analysis of their DNA content, it was shown that pMK4 and pLis4 were stably maintained in vitro, allowing further use of the strains.

The first result of this study is that strain LO28 (*hlyA*::Tn*917*, pLis4) displayed a stable hemolytic phenotype identical to that of the wild type: its maximal hemolytic titer in the culture supernatant (1,200 HU/ml) did not significantly differ from those of strains LO28, LO28 (pMK4), or LO28 (pLis4), taken as controls. The result obtained in the last case as well as in the case of the mutant complemented with pLis4 was somewhat intriguing, since the presence of a multicopy plasmid carrying the hemolysin gene does not increase the hemolytic titer. It seems, at least in the case of strain LO28, that a maximum level of hemolytic activity is reached regardless of the number of copies of the *hlyA* gene, implying that hemolysin production must be precisely regulated. It is indeed already known that there is an inverse correlation between iron concentration and hemolysin production (10). Other factors are probably also involved.

Assays for virulence were performed by i.v. infection of Swiss mice. The hypothesis that the presence of a plasmid in strain LO28 might affect virulence was first investigated by analyzing the virulence of strain LO28 harboring pMK4 or pLis4. Indeed, introduction of either one of the two plasmids into LO28 significantly reduced the level of virulence: the LD_{50}s dropped by more than 1.0 log unit in both LO28 (pMK4) and LO28 (pLis4), with a parallel reduction of their replicating capacity in the spleens and livers of infected mice. It appears that the presence of a plasmid impairs bacterial multiplication. It is likely that under the stress conditions encountered in host tissues, bacterial survival and replication involve activation and repression of several genes, mechanisms which apparently are affected by the presence of a plasmid. As in the case of other mutants already described (13, 19, 33), the nonhemolytic mutant LO28 (*hlyA*::Tn*917*) and its transformant derivative LO28 (*hlyA*::Tn*917*, pMK4) were avirulent in the mouse. Even at high infecting doses (10^8 per mouse), bacteria were rapidly eliminated from the spleens and the livers within 48 h. In contrast, the capacity to grow in these host tissues was restored in the complemented strain LO28 (*hlyA*::Tn*917*, pLis4), in parallel with the production of listeriolysin O in the culture supernatant. Virulence estimated by LD_{50}s was expressed at the same level as that of the control strains

LO28 (pMK4) and LO28 (pLis4). At high infecting doses, strain LO28 (*hlyA*::Tn*917*, pLis4) induced visible abscesses in liver and spleen and ultimately killed mice.

In conclusion, our results show that expression of the hemolysin gene was necessary and sufficient to restore virulence in a nonhemolytic mutant. One can conclude that the transposon in the Tn*917* insertion nonhemolytic mutant did not exert any polar effect on adjacent genes and that *hlyA* was the only gene affected by the insertion. It is also probably the case for other nonhemolytic mutants previously isolated (13, 19, 33). This result is in agreement with recent transcriptional studies suggesting that *hlyA* is a monocistronic unit (Mengaud et al., in press). The implication of this genetic study is that the hemolysin gene product is absolutely required for intracellular multiplication of *L. monocytogenes*. This supports the hypothesis that production of a cytolysin inside the phagolysosomal compartment would allow bacteria to escape this hostile intracellular environment to freely multiply in the cytosol (12). Listeriolysin O now appears to be the first key factor for intracellular growth identified at the molecular level in *L. monocytogenes*. In addition, the genetic tools developed in this study (Tn*917* mutagenesis and gene complementation) should prove useful in the analysis of other virulence factors.

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