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Transposon Mutagenesis as a Tool to Study the Role of Hemolysin in the Virulence of *Listeria monocytogenes*

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The role of hemolysin in the virulence of *Listeria monocytogenes* was studied by using transposon mutagenesis. The 26-kilobase conjugative transposon Tn*1545*, originally found in *Streptococcus pneumoniae*, was transferred to a hemolytic virulent strain of *L. monocytogenes*. The frequency of transfer was estimated to be about 10^{-8} per recipient. This allowed us to isolate a nonhemolytic mutant which most likely harbors a single copy of Tn*1545*. Loss of hemolysin production was associated with loss of virulence. The 50% lethal dose of the mutant was assessed to about $10^{9.6}$ bacteria per mouse after intravenous challenge. Nonhemolytic bacteria were unable to grow in host tissues and were rapidly eliminated from the spleen and liver of infected mice. Virulence was restored in hemolysin-producing revertant obtained by spontaneous loss of transposon Tn*1545*. These results strongly suggest that hemolysin is a major virulence factor implicated in the intracellular growth of *L. monocytogenes*.

Listeria monocytogenes is a ubiquitous gram-positive microorganism responsible for severe infections in humans and animals (12). As with many other intracellular parasites, the virulence of this bacterium is due to its capacity to grow within macrophages (20). However, mechanisms allowing intracellular multiplication in host tissues and ultimately macrophage destruction remain unknown. Among various hypotheses, the secretion of a hemolysin has been proposed as an important mechanism promoting *L. monocytogenes* virulence. This extracellular protein is a sulfhydryl-dependent cytotoxin which is antigenically related to streptolysin O (16, 24, 30, 33). Purified hemolysin specifically binds to cholesterol and is responsible for in vitro lysis of many eucaryotic cells such as macrophages and hepatocytes (17). In vivo, it causes damage to the reticuloendothelial system and is lethal for experimental animals (17–19). The role of this cytotoxin is further supported by the observation that all strains isolated from natural infections produce hemolysin and are virulent in experimental models (28), whereas nonhemolytic strains isolated after multiple subcultures or from the environment are totally avirulent (3, 13, 28). However, the interpretation of these findings remains controversial for several reasons. The use of relatively high doses of purified hemolysin does not really mimic conditions at the early phase of acute infection when a few bacteria are multiplying within macrophages (20). Moreover, nonhemolytic avirulent mutants isolated after several in vitro passages may differ from virulent bacteria by mutations in genes related to virulence in addition to those responsible for hemolysin production.

The aim of the present work was to investigate the role of hemolysin by testing the virulence in mice of various strains of *L. monocytogenes* obtained by transposon mutagenesis. The conjugative transposon Tn*1545* used for this purpose is a 26-kilobase (kb) DNA molecule encoding resistance to kanamycin, tetracycline, and erythromycin (5, 7). The hemolysin-negative phenotype was associated with a complete loss of virulence in mice, and virulence was restored in the hemolysin-producing revertant obtained by spontaneous elimination of the transposon. These results strongly support the hypothesis that hemolysin is a major component of *L. monocytogenes* pathogenicity, which most likely acts at the crucial step of intracellular growth. These findings also demonstrate that transposon mutagenesis is feasible in this species, thus representing a powerful tool for future analysis of virulence.

MATERIALS AND METHODS

Bacterial strains. *L. monocytogenes* EGD (NCTC 7973) was originated from the Trudeau Institute and belongs to the serovar 1/2a (20). This hemolytic strain was isolated by E. G. D. Murray from a natural infection in a guinea pig (22). Agarose gel electrophoresis of plasmid DNA from strain EGD, prepared by procedure of Flamm et al. (9), did not reveal the presence of plasmid (data not shown). A spontaneous mutant resistant to streptomycin (minimum inhibitory concentration, >1,000 μg/ml) was isolated from strain EGD by plating the bacterial suspension onto tryptic soy agar (Institut Pasteur Production, Marnes la Coquette, France) supplemented with 1,000 μg of streptomycin per ml. This mutant, designated EGD-SmR, was used as the recipient for transposon Tn*1545*.

L. monocytogenes BM4140 (serovar 7), kindly supplied by P. Courvalin and C. Carlier (Institut Pasteur, Paris), was used as the donor of Tn*1545* for transposon mutagenesis. The 26-kb conjugative transposon Tn*1545* was originally found in *Streptococcus pneumoniae* (5) and was successfully transferred to *L. monocytogenes* strain LO17, which is devoid of plasmid (26). The transposon-bearing strain was designated strain BM4140. Tn*1545* exhibits three *Hin*dIII restriction sites and one *Pst*I restriction site, but no site for endonuclease *Eco*RI, and the kanamycin resistance gene is located at one end of the transposon (F. Caillaud, Institut Pasteur, Paris; personal communication).

Escherichia coli BM2962, kindly supplied by P. Courvalin and P. Trieu-Cuot (Institut Pasteur, Paris), harbors the 4.9-kb recombinant plasmid pAT93. This plasmid results from the cloning into plasmid pBR322 of a 0.53-kb *Hpa*II

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fragment which contains an intragenic sequence of the kanamycin phosphotransferase type III gene (19a). This fragment originates from the streptococcal plasmid pJH1 (15) and displays a high homology with the kanamycin phosphotransferase gene of Tn*1545* (F. Caillaud, personal communication). This strain was used as the source of DNA to probe for Tn*1545* in mutants.

Transposon mutagenesis and screening. Transposon mutagenesis was achieved by transferring Tn*1545* from *L. monocytogenes* BM4140 to *L. monocytogenes* EGD-SmR by the method of Perez-Diaz et al. (26). Briefly, donor and recipient bacteria were grown overnight in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.), mixed at a ratio of 1:10 on a membrane filter (HAWP, 0.45 μm; Millipore Corp., Bedford, Mass.), and incubated for 18 h at 37°C on BHI agar. Insertional mutants were selected on tryptic soy agar supplemented with streptomycin (100 μg/ml) and tetracycline (10 μg/ml).

Hemolysin production was tested by spotting each mutant on 5% horse blood–BHI agar and incubating for 48 h at 37°C. The presence of catalase was tested by mixing colonies into hydrogen peroxide. Motility was observed microscopically during exponential growth at 25°C in tryptic soy broth and after subculture on soft tryptic soy agar (1%). Mutants were then subcultured once in BHI broth, harvested in log-phase growth (10^8/ml), distributed into vials in 1-ml lots, and stored at −70°C until required for in vivo testing.

Hemolytic revertant clones were obtained as follows. One nonhemolytic colony was suspended in distilled water (5 ml), and serial dilutions of this suspension were plated on 5% horse blood–BHI agar, allowing us to estimate the relative number of nonhemolytic and hemolytic colonies. Hemolytic colonies, which appeared as hemolytic plaques among nonhemolytic bacteria, were subsequently isolated on the same culture medium, and their antibiotic susceptibility was tested.

Phenotypic analysis. Characterization of strains was achieved by employing the classical tests defined elsewhere (29) and by testing the fermentative capacity on 50 different substrates (API 50CH; API-System, La Balme les Grottes, France). Serotyping was performed by E. P. Espaze (Centre National des *Listeria*, Nantes, France). Lecithinase activity was tested on tryptic soy agar supplemented with 5% egg yolk. Antibiotic susceptibility was tested by the agar diffusion method on Mueller-Hinton medium (Institut Pasteur Production).

Hemolysin titration. Hemolysin titration was done on supernatants of bacterial cultures grown in BHI broth by the method of Alouf et al. (1). Briefly, supernatants supplemented with cysteine (20 mM final concentration) were serially diluted in phosphate-buffered saline, and sheep erythrocytes were added to a final concentration of 2.5%. After 45 min of incubation at 37°C, tubes were centrifuged, and absorbance at 541 nm was measured with a spectrophotometer (Stasas III; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). One hemolytic unit was defined as the minimum amount of hemolysin inducing 50% lysis of erythrocytes and was expressed as the inverse of the dilution titer.

Preparation of DNA and digestion with restriction enzymes. Chromosomal DNA was obtained from *L. monocytogenes* by the method of Flamm et al. (9), except that ethanol nucleic acid precipitates were finally solubilized in E buffer (40 mM Tris base, 2 mM disodium EDTA [pH 7.9]). Digestion of DNA by endonuclease *Eco*RI, *Hin*dIII, or *Pst*I was performed as indicated by the manufacturer (Boehringer Mannheim, Tutzing, Federal Republic of Germany). Electrophoretic separation of the fragments was then performed on 0.7% horizontal agarose gels as previously described (21).

Detection of transposon Tn*1545* by hybridization. Plasmid pAT93 was prepared as described by Birnboim and Doly (4). Plasmid DNA was subsequently purified on a $CsCl_2$ gradient centrifuged for 40 h at 145,000 × *g*. The DNA probe was obtained by double cleavage of the plasmid DNA by the endonucleases *Hin*dIII and *Eco*RI. The relevant fragment was then separated from the DNA of the vector by electrophoresis in a 0.7% low-melting-point agarose gel (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Probe DNA was then extracted and purified from contaminant agar as previously described by Maniatis et al. (21). This purified 0.53-kb DNA fragment could then be labeled by nick translation with ^{32}P-labeled dCTP and TTP (Amersham Corp., Buckinghamshire, England) by the method of Rigby et al. (27).

After separation of the restricted chromosomal DNA of the parental and mutant strains on agarose gels, DNA fragments were blotted onto a nitrocellulose filter (Schleicher & Schuell Inc., Dassel, Federal Republic of Germany) and hybridized with the radiolabeled probe by the method of Southern (31). Hybridization was made under stringent conditions: prehybridization in 50% formamide for 4 h at 42°C; hybridization in the same solution for 18 h at 42°C; three washings for 5 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7)–0.1% sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, Mo.); and two washings for 30 min at 50°C in 0.1× SSC–0.1% sodium dodecyl sulfate. Probed filters were then exposed to a Kodak X-Omat film (Eastman Kodak Co., Rochester, N.Y.). Autoradiograms were developed after 24 h of incubation at −70°C.

Virulence assays. Specific-pathogen-free ICR female Swiss mice (Charles Rivers, France) 6 to 8 weeks old were used in this study. 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 or by following bacterial growth in organs. The bacterial survival in organs was determined by killing groups of five infected mice by cervical dislocation. The spleens and livers were aseptically removed and ground as previously described (20). Samples (0.1 ml) of serial dilutions of whole organ homogenates were surface plated on tryptic soy agar. Colonies were counted after 24 h of incubation at 37°C, and the results were expressed as the $\log_{10}$ bacterial counts per organ.

RESULTS

Isolation of transposon-induced mutants. Insertional mutants were obtained by transferring the transposon Tn*1545* from donor strain BM4140 to recipient strain EGD-SmR. The frequency of transfer was 10^{-8} per recipient. More than 2,500 transconjugants selected on tryptic soy agar supplemented with streptomycin and tetracycline were screened for presence of hemolysin, catalase, and motility. This allowed us to isolate one hemolysin-negative mutant (Hly^-), two catalase-negative mutants (Cat-160, Cat-147), and one nonmotile mutant (Mot-58). Hemolytic clones were obtained from mutant Hly^- at a low rate (10^{-6}) by spontaneous loss of transposon Tn*1545*, as attested by the antibiotic susceptibility to kanamycin, tetracycline, and erythromycin. A hemolysin-producing revertant clone (Hly^+) was thus isolated and used for further characterization and virulence testing. The

mutant Hly$^-$ and its revertant Hly$^+$ were deposited in the collection of the Centre National des *Listeria* (Nantes) and designated CNL85/162 and CNL85/163, respectively. In vivo, it was not possible to isolate hemolysin-producing revertants from organs of mice inoculated with 10^5 to 10^6 bacteria i.v. during the first 48 h of infection.

Virulence of mutants in mice. The virulence of mutants was estimated by determining the LD_{50} in mice infected i.v. with increasing doses of bacteria (Table 1). Strain EGD-SmR exhibited lower virulence than the original strain EGD, since its LD_{50} was assessed at $10^{6.2}$ versus $10^{4.3}$ bacteria per mouse for strain EGD. The decrease in virulence of the streptomycin-resistant mutant is probably due to misreadings at the translation step of protein synthesis, as already described (11). However, strain EGD-SmR was still capable of killing mice, producing multiple abscesses in the spleen and the liver within 5 to 6 days when mice were infected with 10^6 to 10^7 bacteria. This was also true for mutants Cat-147, Cat-160, and Mot-58 and for revertant Hly$^+$. No significant difference in virulence was found between strain EGD-SmR and these mutant and revertant strains. On the other hand, the mutant Hly$^-$ appeared to be totally avirulent since mice survived challenges as high as 10^9 bacteria i.v. and did not exhibit abscesses in organs when infected with such high doses. The LD_{50} of mutant Hly$^-$ was estimated to about $10^{9.6}$ bacteria per mouse. These results indicate that loss of hemolysin production was associated with loss of virulence and that virulence was restored in the hemolysin-producing revertant obtained by loss of transposon Tn*1545*. Moreover, the presence of transposon Tn*1545* inserted in the bacterial chromosome did not influence per se the expression of virulence, since several hemolysin-producing transconjugants were still virulent (Table 1).

Inability of hemolysin-negative mutant to grow within host tissues. Bacterial growth in organs of infected mice was followed for 2 days. Mice were inoculated i.v. (2×10^6 bacteria) with mutant Hly$^-$ or revertant Hly$^+$. Surviving bacteria in the spleen and liver were counted (Fig. 1). After an initial reduction in bacterial counts in organs, hemolysin-producing bacteria increased in number during the next 48 h. Conversely, hemolysin-negative bacteria were rapidly eliminated in organs. This inability of mutant Hly$^-$ to multiply in host tissues was found with challenges as high as 10^7 or 10^8 bacteria i.v. (data not shown). This observation favors the view that hemolysin may play a major role in inducing bacterial growth in host tissues.

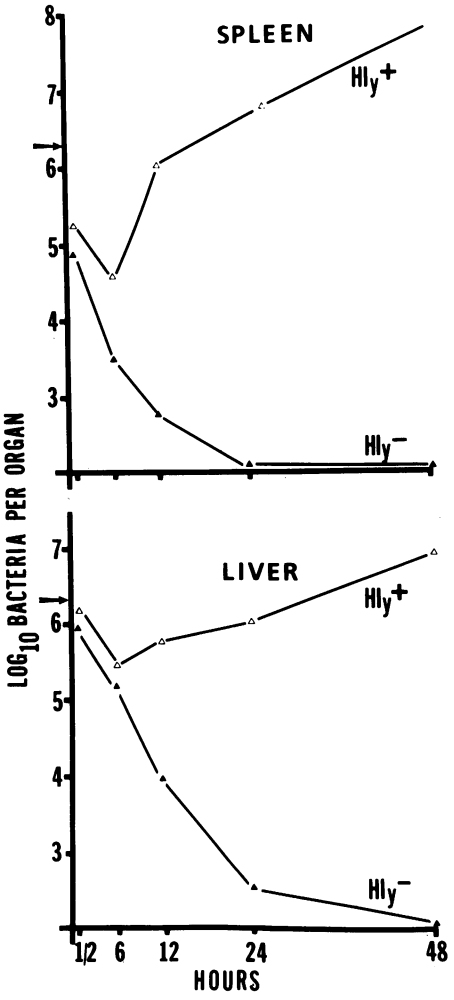

FIG. 1. Inability of hemolysin-negative mutant from *L. monocytogenes* to grow in host tissues. Mice were inoculated i.v. with 2×10^6 bacteria of the mutant Hly$^-$ or its revertant Hly$^+$. Bacterial growth was followed in the spleen and liver at various times. Results are expressed as the mean $\log_{10}$ bacteria per organ (mean of five organs; standard deviations, ≤ 0.30). Hemolysin-negative bacteria were rapidly destroyed in organs. The capacity of growing in host tissues was restored in the revertant Hly$^+$, which lost Tn*1545* and produced hemolysin.

TABLE 1. Virulence of mutants from *L. monocytogenes* in mice[a]

Bacteria	Presence of Tn*1545*[b]	Hemolysin production[c]	LD_{50}[d]
EGD	−	+	$10^{4.3}$
EGD-SmR	−	+	$10^{6.2}$
Cat-160	+	+	$10^{6.1}$
Cat-147	+	+	$10^{5.9}$
Mot-58	+	+	$10^{6.4}$
Hly$^-$	+	−	$10^{9.6}$
Hly$^+$	−	+	$10^{6.1}$

[a] Mutant Hly$^-$ was totally avirulent, as opposed to the others strains harboring or not transposon Tn*1545*. Loss of hemolysin was associated with that of virulence.

[b] The presence of Tn*1545* is attested by antibiotic resistance to kanamycin, tetracycline, and erythromycin.

[c] Tested on 5% horse blood–BHI agar.

[d] Groups of 8 mice were inoculated i.v. with progressive doses of bacteria, and mortality was followed for 3 weeks. LD_{50}s are given as bacterial cells per mouse.

Phenotypic analysis of hemolysin-negative mutant. Hemolysin production of strains EGD, EGD-SmR, Hly$^-$, and Hly$^+$ was determined at various times during bacterial growth at 37°C in BHI broth (Fig. 2). Strain Hly$^-$ did not produce detectable hemolytic activity, whereas no significant difference was observed in the levels of hemolytic activity among the three other strains.

Phenotypic analysis revealed that the mutant Hly$^-$ did not differ from strain EGD and EGD-SmR or from the revertant Hly$^+$ with respect to microscopic morphology, colonial aspect, motility, and classical biochemical tests of identification. These four strains belonged to serovar 1/2a. The mutant Hly$^-$ displayed the same pattern of fermentation toward 50 different carbohydrates on API 50CH as that of the three other strains tested and was resistant to kanamycin, tetracycline, and erythromycin. This mutant produced an opaque halo on egg yolk medium like the three other

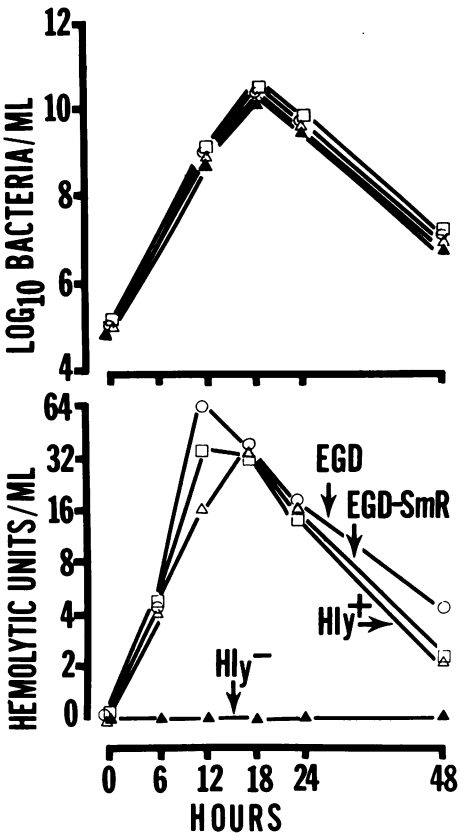

FIG. 2. In vitro production of hemolysin by various strains of *L. monocytogenes*. Bacteria of strains EGD (○), EGD-SmR (□), Hly$^-$ (▲), and Hly$^+$ (△) were cultured in BHI broth for 48 h at 37°C. Bacteria were counted at various times by plating progressive dilutions on BHI agar. At the same time titers of hemolysin were determined from supernatants. Bacterial growth followed the same kinetics for the four strains. Production of hemolysin was not detectable with the mutant Hly$^-$, whereas no significant difference in the level of hemolysin titers was observed among the three other hemolytic strains.

strains, indicating that hemolysin activity was not associated with that of lecithinase, as opposed to a previous report (10).

Evidence suggesting that hemolysin-negative mutant harbors a single copy of Tn*1545*. That a single copy of Tn*1545* has inserted into the chromosomal DNA of the mutant Hly$^-$ is suggested by the following observation. The reversion to the Hly$^+$ phenotype, which occurred at a low rate (10^{-6}), was always associated with the loss of the transposon-encoded antibiotic resistance (about 50 mutants tested). If more than one copy had inserted, one might expect to isolate hemolysin-producing revertants exhibiting transposon-encoded antibiotic resistance, which was not the case.

This opinion was furthermore supported by the result of the next experiment with a radioactive probe that specifically recognized the kanamycin phosphotransferase gene of Tn*1545*. After digestion by the restriction endonuclease *Eco*RI, *Hin*dIII, or *Pst*I, DNA samples from strains EGD, EGD-SmR, mutant Hly$^-$, and revertant Hly$^+$ were separated by agarose gel electrophoresis and transferred onto a nitrocellulose filter by Southern blotting. These restriction fragments were hybridized with the probe as shown on the autoradiogram (Fig. 3). The radioactive probe is visualized as a 0.53-kb *Hin*dIII-*Eco*RI fragment of the plasmid pAT93 (Fig. 3B, lane 2, arrow). This probe did not recognize DNA fragments from strains EGD, EGD-SmR, and revertant Hly$^+$ (Fig. 3B, lanes 3, 4, 6, 8, and 10). On the other hand, a single radioactive band was obtained from mutant Hly$^-$ DNA digested with *Eco*RI, *Hin*dIII, or *Pst*I, even when the autoradiogram was exposed for 3 days.

These results are interpreted as evidence that a single copy of Tn*1545* has inserted into the chromosome of mutant Hly$^-$ for the following reasons. The newly generated sequence obtained after *Eco*RI digestion (Fig. 3A, lane 5, arrow) and recognized by the probe (Fig. 3B, lane 5) migrates around 25 kb. This is not compatible with the hypothesis of the insertion of two copies on a single *Eco*RI chromosomal fragment, which would generate a much larger fragment (~50 kb). In addition, insertion of copies of Tn*1545* into different *Eco*RI chromosomal fragments of similar molecular size is very unlikely. Considering the distal location of the probe in Tn*1545*, the presence of two copies of the transposon in the chromosomal DNA of the mutant Hly$^-$ would result in the production of *Hin*dIII and *Pst*I DNA fragments of different sizes, due to the differences in size of the flanking *L. monocytogenes* chromosomal sequences.

DISCUSSION

During acute infection by *L. monocytogenes*, bacteria are easily phagocytized by fixed macrophages lining capillaries and multiply within the cytoplasm of these cells (20). Intracellular growth of this virulent microorganism might be due either to the intrinsic resistance of the bacterial cell wall components to the bactericidal mechanisms of macrophages or to the secretion of exotoxin(s) capable of inhibiting and ultimately destroying macrophages (or to both). Very little is known about virulence factors associated with the cell wall. There exists a periplasmic superoxide dismutase that renders bacteria more resistant to the oxidative burst of macrophages (34). Furthermore, a rough mutant appears avirulent despite its ability to produce active hemolysin (13). However, these data are based on the use of genetically uncharacterized strains. With respect to the role of hemolysin in virulence, this reservation is also true for the results reported with hemolysin-negative strains isolated from the environment (3, 28) or after multiple subcultures (13). After a tremendous development in genetic analysis on the virulence of gram-negative bacteria, studies on gram-positive bacteria appear more difficult because genetic systems are insufficiently developed. However, conjugative transposons have been already successfully used to isolate mutants in gram-positive bacteria, i.e., *Streptococcus faecalis* and *Streptococcus pyogenes* (6, 14, 23). Similarly, this work demonstrates that transposon Tn*1545* is also a reliable tool for the generation of a bank of well-characterized mutants in *L. monocytogenes* since it has a good efficiency of transposition.

Favoring the suggestion that secretion of exotoxin is an important mechanism of pathogenecity, in this work a hemolysin-negative mutant obtained by inserting transposon Tn*1545* into a virulent strain of *L. monocytogenes* was totally avirulent. Similar data showing that hemolysin inactivation results in loss of virulence have already been reported by testing a hemolysin-negative mutant from *S. faecalis* (14). The mutant Hly$^-$ isolated from *L. monocytogenes* was stable and unable to grow within host tissues. Virulence was restored by spontaneous loss of the transposon (Fig. 1). The insertion of Tn*1545* on other sites of chromosomal DNA, which resulted in loss of motility or catalase production, did not restrict virulence (Table 1),

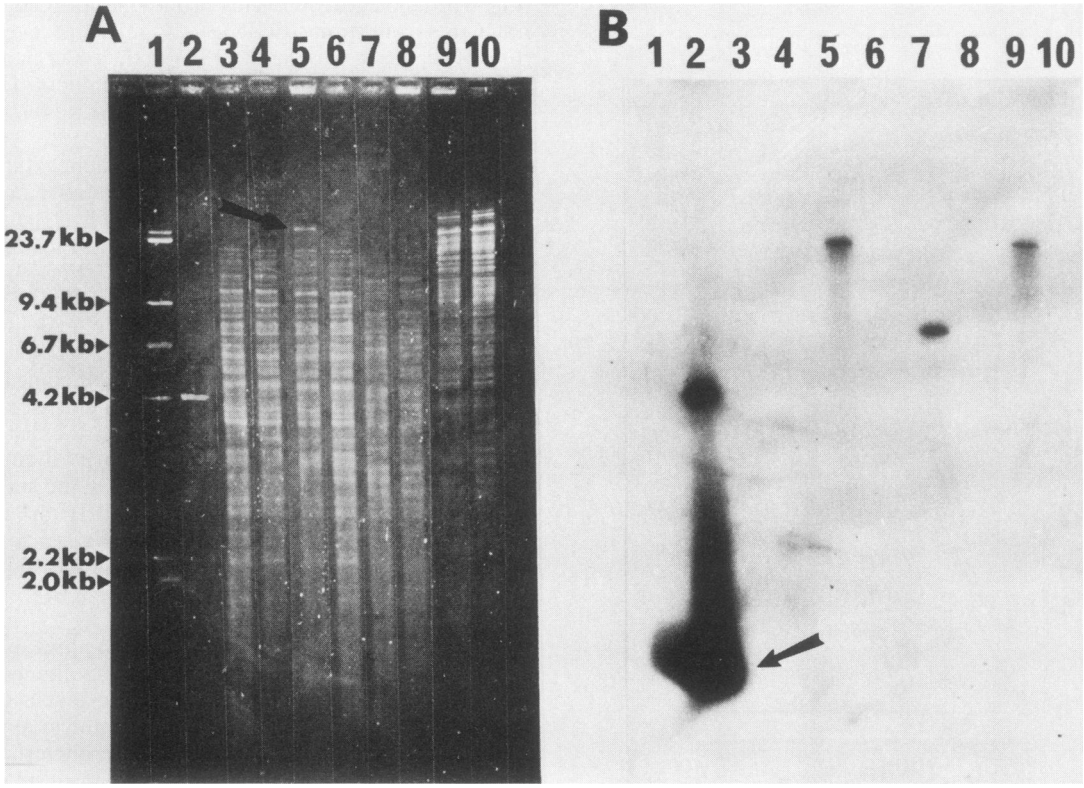

FIG. 3. (A) Agarose gel electrophoresis of chromosomal DNA from *L. monocytogenes*. DNA was digested with endonucleases *Eco*RI (lanes: 3, EGD; 4, EGD-SmR; 5, Hly$^-$; 6, Hly$^+$), *Hin*dIII (lanes: 7, Hly$^-$; 8, Hly$^+$), or *Pst*I (lanes: 9, Hly$^-$; 10, Hly$^+$). Other lanes: 1, *Hin*dIII-digested lambda phage DNA as molecular size standards; 2, *Hin*dIII-*Eco*RI-digested plasmid pAT93 DNA. Arrow indicates the *Eco*RI DNA fragment from mutant Hly$^-$, in which Tn*1545* has inserted. (B) Autoradiogram of Southern blot prepared from the gel shown in A and hybridized with ^{32}P-labeled Tn*1545* probe. The probe is the 0.53-kb *Hin*dIII-*Eco*RI fragment of the plasmid pAT93 (lane 2 arrow). The small dot (~5 kb) on lane 2 corresponds to a small amount of nonrestricted DNA of the recombinant plasmid pAT93 (4.9 kb) recognized by the probe. After restriction of Hly$^-$ mutant DNA with *Eco*RI (lane 5), *Hin*dIII (lane 7), or *Pst*I (lane 9) a single fragment is recognized by the Tn*1545* probe. This picture was obtained after 24 h of exposure at −70°C to Kodak X-Omat film.

indicating that the presence of inserted copies of Tn*1545* had no major effect on virulence, as long as the gene(s) encoding hemolysin production was not involved. Moreover, the hemolysin-negative mutant most likely harbored a single copy of Tn*1545*, suggesting that loss of virulence might result from an insertional event implicating a very limited number of genes. However, it is as yet unclear whether the transposon had inserted within a structural or a regulatory gene.

If hemolysin is of importance in the pathogenicity of *L. monocytogenes*, one might expect that this toxin is produced in vivo during acute infection and induces a specific immune response. This assumption is strongly supported by the observation that mice immunized with low doses of living bacteria are highly resistant to the lethal effect of purified hemolysin (18). Moreover, protective immunity against living bacteria can be induced by inoculating mice with high doses of bacteria (10^6, i.v.) from a hemolysin-producing rough mutant unable to multiply in host tissues (13). Another consequence expected from the assumption that hemolysin plays a major role in virulence is that virulence must always be associated with hemolysin production. Indeed, there is no report that any strain of *L. monocytogenes* (stricto sensu) isolated from natural infections does not produce hemolysin.

Our results are fully in agreement with the opinion presented in 1966 (2) supporting the view that hemolysin is a key mechanism of *L. monocytogenes* pathogenicity. As suggested by in vitro results with purified hemolysin (17–19) and in vivo electron microscopic studies (2), bacteria inside phagocytic vacuoles would produce hemolysin, disrupting cytoplasmic membranes, including lysosomes and ferritin vesicles. It has been shown that breakdown of vacuolar membranes is accompanied by damage of host cell cytoplasm and by liberation of intracellular iron (32), which strongly stimulates *L. monocytogenes* growth (8, 32). This process ultimately results in macrophage destruction.

Finally, the intracellular multiplication of *L. monocytogenes* might be considered as a competition between the hemolysin secretion inside the cellular microenvironment of macrophages and the bactericidal activity of these cells. Indeed, there is evidence that *L. monocytogenes* is not resistant per se to macrophages, based on the in vivo finding that most bacteria inoculated in the mouse are killed by fixed macrophages during the early phase of infection (20, 25). However, a limited number of bacteria regularly escape killing by the macrophages and initiate intracellular growth. It can be hypothesized that these bacteria are capable of producing enough cytotoxin inside macrophages to allow resistance to killing mechanisms and intracellular survival. Consequently, the number of bacteria phagocytized per macrophage is probably very critical in triggering intracellular multiplication. However, this concerns only the role of hemolysin as a virulence factor, and further information is needed on the role of hemolysin in the in vivo generation of T cell-mediated immunity against *L. monocytogenes*.

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