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Genetics

Intracellular methicillin selection of *Listeria monocytogenes* mutants unable to replicate in a macrophage cell line

(intracellular parasitism/transposon mutagenesis/hemolysin)

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ABSTRACT **To dissect the determinants of *Listeria monocytogenes* that are required for pathogenicity, we designed an intracellular selection protocol based on penicillin selection to isolate mutants defective for intracellular growth. Eight independent mutants obtained by insertion of Tn*916* were isolated that were resistant to methicillin treatment following internalization by the J774 macrophage-like cell line. Seven mutants were absolutely defective for intracellular growth, whereas one showed abortive intracellular growth. The majority of the mutants were nonhemolytic and lacked a secreted 58-kDa polypeptide thought to be the *L. monocytogenes* hemolysin, listeriolysin O. Southern blot analysis indicated that one mutant contained a Tn*916* insertion in *hlyA*, the listeriolysin O structural gene, which resulted in a truncated listeriolysin O polypeptide, whereas another mutant contained an insertion immediately upstream of *hlyA*, which resulted in reduced expression of listeriolysin O. The other mutants contained Tn*916* insertions in genes other than *hlyA*, although all but one were nonhemolytic. Revertants isolated by their ability to grow within tissue culture cells regained hemolytic activity. These data show that intracellular methicillin selection facilitates isolation of mutations in genes required for intracellular growth and strengthens the premise that listeriolysin O is essential for intracellular growth.**

Intracellular pathogens are responsible for a devastating amount of disease worldwide. For example, the bacterial pathogens *Mycobacteria leprae* and *Mycobacteria tuberculosis* infect ≈30 million individuals (1, 2), whereas *Chlamydia trachomatis*, an obligate intracellular bacterial pathogen, is one of the leading causes of blindness worldwide and is a major cause of sexually transmitted disease in the United States (3). The protozoal intracellular pathogens, *Trypanosoma cruzi, Leishmania*, and *Plasmodium*, together, infect >100 million people (4). Although all of these intracellular pathogens have been extensively studied, little is known about the molecular mechanisms by which they invade and proliferate within eukaryotic cells. Such an understanding will presumably aid in prevention and treatment of these diseases. Unfortunately, many of these pathogens are difficult or impossible to cultivate *in vitro* and, in the case of the protozoal pathogens, no genetic systems have yet been developed. Therefore, we have decided to focus on a model bacterial pathogen, *Listeria monocytogenes*.

L. monocytogenes is a facultative intracellular bacterial pathogen that is easily cultivated *in vitro* and for which an excellent intracellular growth assay has recently been developed (5). Furthermore, *L. monocytogenes* has been extensively utilized in a murine model for the study of cell-mediated immunity (6–8). However, although the immune response to *L. monocytogenes* has received a great deal of attention, bacterial determinants required for intracellular growth have yet to be fully evaluated. One characterized determinant of pathogenesis is the elaboration of a hemolysin referred to as listeriolysin O (9, 10). Listeriolysin O is a member of a family of sulfhydryl-activated hemolysins of which streptolysin O is the prototype (11–13). Mutants of *L. monocytogenes* lacking hemolytic activity are avirulent and fail to grow in tissue culture cells (5, 14–17).

To characterize molecular mechanisms required for intracellular growth of *L. monocytogenes* we designed a selection protocol based on penicillin selection (18, 19) for the isolation of *L. monocytogenes* mutants defective in their ability to replicate intracellularly. In the present study we describe the isolation and initial characterization of eight such mutants.

MATERIALS AND METHODS

Bacterial Strains, Growth Media, and Mutant Construction. All strains used in this study were stored at −70°C in brain/heart infusion broth (BHI, Difco) containing 50% (vol/vol) glycerol. *L. monocytogenes* 10403S (20), the primary strain used in this study, belongs to serotype 1, is resistant to streptomycin, and has an LD_{50} for mice of 3.3×10^4 (5). *Streptococcus faecalis* CG110 (21) was used as the donor of Tn*916* to introduce insertion mutations into the virulent *L. monocytogenes* 10403S. Bacterial conjugation was performed on 0.45-μm Millipore filters for 24 hr at 37°C on BHI agar. The filters were suspended in phosphate-buffered saline (PBS, pH 7.4), and an appropriate dilution resulting in ≈10,000 transconjugants was plated on M9 (22) minimal agar medium containing 2 mM $MgSO_4$, 0.2% glucose, 0.2 mM thioctic acid, 0.2 mM riboflavin, 0.5 mM thiamine, 0.1 mM biotin, 1 mg of streptomycin sulfate per ml, 12.5 μg of tetracycline per ml, and the following L-amino acids: 0.3 mM cysteine, 5 mM glutamine, 0.3 mM leucine, 0.3 mM valine, 0.6 mM arginine, 0.1 mM histidine, 0.3 mM methionine, and 0.1 mM tryptophan. After growth at 37°C for 4 days, the resultant bacterial colonies were suspended in sterile PBS containing 50% (vol/vol) glycerol and stored at −70°C.

Intracellular Methicillin Selection. The murine macrophage-like cell line J774 shares many properties with murine macrophages such as phagocytosis (23) but is apparently unable to kill ingested microorganisms such as *Escherichia coli* K-12 (unpublished observations). The J774 cells were maintained in a spinner flask at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 100 units of penicillin per ml, 10 μg of streptomycin per ml, 5% fetal bovine serum, and 2 mM L-glutamine. J774 cells (4×10^5) were seeded onto 35-mm Petri dishes the evening before use and incubated in 2 ml of medium without antibiotics. The *L. monocytogenes*:: Tn*916* library was grown overnight in BHI broth at 30°C to a density of 2×10^9 bacteria per ml. One milliliter of culture was sedimented in a microcentrifuge tube (12,000 × *g*) for 1

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min, the supernatant fluid was discarded, and the sedimented cells were washed once with 1 ml of PBS. Monolayers of J774 cells containing $\approx 8 \times 10^5$ cells were infected with 4×10^6 bacteria and, after a 30-min incubation, the monolayers were washed three times with 37°C PBS to remove nonadherent bacteria; this was followed by the addition of 2 ml of 37°C medium. After an additional 30-min incubation, gentamicin sulfate and methicillin were added to final concentrations of 0.01 and 1 mg/ml, respectively. After 24 hr, the monolayers were washed three times with PBS to remove the antibiotics and then lysed with 1 ml of sterile distilled water. The recovered bacteria were grown overnight in BHI broth and used to infect fresh J774 cell monolayers. After the third such enrichment, a population of *L. monocytogenes*::Tn*916* mutants resistant to intracellular methicillin killing was obtained. The recovered mutants were purified by single colony isolation on blood agar.

Growth of *L. monocytogenes* in J774 Cells. *L. monocytogenes* wild-type and mutant strains were analyzed for growth in J774 cells as described (5). Briefly, cell monolayers were grown on 12-mm round coverslips in 60-mm Petri dishes. Thirty minutes after the initial infection, monolayers were washed three times with 37°C PBS to remove nonadherent bacteria; this was followed by the addition of 5 ml of 37°C medium. After an additional 30 min, gentamicin sulfate was added to a final concentration of 10 μg/ml. The number of bacteria per coverslip was determined at each time point in triplicate by vigorously mixing each coverslip in 5 ml of sterile distilled water in a 15-ml conical tube and then deducing the number of colony-forming units by growth on BHI agar. Intracellular methicillin resistance of the *L. monocytogenes*::Tn*916* mutants was tested by growing the appropriate strain in J774 cells as described above but in the presence of methicillin at 1 mg/ml.

SDS/PAGE and Hemolytic Activity of Secreted Proteins. *L. monocytogenes* wild-type and mutant strains were grown in LB broth (22) (pH 7.5) at 37°C for 18 hr with aeration. LB broth culture supernatants were prepared for analysis by SDS/7% PAGE as described (5). The amount of extracellular protein loaded per lane was standardized with respect to the number of bacteria in each culture measured as the optical density at 520 nm. An amount approximately equivalent to 2 ml of culture supernatant was loaded per well. Protein molecular mass standards (Bio-Rad) were used to calibrate molecular masses.

BHI broth culture supernatants were assayed for hemolytic activity as described by Kingdon and Sword (24). Briefly, 2-fold serial dilutions of samples were made in PBS containing 6 mM cysteine (pH 5.6). After 30 min of incubation at 37°C an equal volume of sheep erythrocytes (1% suspension) was added. After an additional 60 min of incubation at 37°C the tubes were subjected to centrifugation (12,000 × *g*) for 1 min and were scored for visible hemolysis. Hemolytic titers were expressed as the reciprocal of the highest dilution at which complete lysis of erythrocytes was observed. Complete hemolysis was induced by adding 1/100th volume of 10% Triton X-100 to a sample.

Southern Blot Analysis. DNA was isolated as described by Flamm *et al.* (25). DNA restriction enzyme digests were fractionated on 1% agarose gels in TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.0). The DNA fragments were subjected to acid depurination (26) and then transferred to nitrocellulose filters by the method of Southern (27). The filters were hybridized with DNA probes that had been labeled *in vitro* with ^{32}P. Plasmid pAM118 (28) containing Tn*916* was digested with *Eco*RI and labeled with [α-^{32}P]dCTP using an oligolabeling kit (Pharmacia). Plasmid pDP102 contained a 3.2-kilobase (kb) *Bam*HI fragment insert containing the listeriolysin O structural gene, *hlyA*, and was isolated by screening a recombinant DNA library with an oligonucleotide hybridization probe derived from the published *hlyA* sequence (12). DNA probes 1 and 2, which represent internal restriction fragments of *hlyA* derived from plasmid pDP102, were gel purified using low-melting-temperature agarose and labeled with ^{32}P as above. Hybridizations were performed at 37°C under conditions of high stringency [50% formamide/5× SSC (1 SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS/1× Denhardt's solution (22)/5.6 mM Tris, pH 7.5/100 μg of sheared and denatured calf thymus DNA per ml]. After 18 hr, the filters were washed twice in 2× SSC/0.1% SDS at 24°C; this was followed by two washes in 0.1× SSC/0.1% SDS at 50°C. Hybridization was revealed by autoradiography with XAR-5 x-ray films (Eastman Kodak) in the presence of intensifying screens at −70°C.

Isolation of Revertants. Revertants were isolated by their ability to form macroscopic plaques in confluent J774 cell monolayers. Each *L. monocytogenes*::Tn*916* mutant strain was grown overnight in BHI broth at 30°C to a density of 2×10^9 bacteria per ml. The bacteria were sedimented in a microcentrifuge tube (12,000 × *g*) for 1 min and washed once in PBS. Confluent J774 cell monolayers in 35-mm Petri dishes were infected with 4×10^7 bacteria per dish, and after 1 hr the monolayers were washed three times with 37°C PBS to remove nonadherent bacteria; this was followed by the addition of a 3-ml overlay of 1% agarose in DMEM containing 5 μg of gentamicin sulfate per ml. After 2 days, bacterial plaques were visualized by addition of a second overlay of 1 ml of 1% agarose in DMEM containing 0.08% neutral red (Sigma). Several revertant plaques from each mutant strain were picked and the revertants were purified by colony isolation on blood agar. Revertants were analyzed for their sensitivity to tetracycline, hemolytic titer, and growth in J774 cells.

RESULTS

Isolation of *L. monocytogenes*::Tn*916* Mutants Defective for Intracellular Growth. In a previous study it was shown that virulent *L. monocytogenes* grew in the mouse macrophage-like cell line, J774, with an intracellular doubling time of ≈60 min (5). However, in the presence of methicillin, intracellular *L. monocytogenes* were effectively killed within the J774 cells (Fig. 1*A*). Therefore, since β-lactam antibiotics such as methicillin kill only growing bacteria, a protocol was designed to isolate intracellular growth mutants, which we refer to as intracellular methicillin selection (see *Materials and Methods*). Approximately 100,000 *L. monocytogenes*::Tn*916* isolates were collected in 10 pools of 10,000 and subjected to intracellular methicillin selection. From these, eight independent isolates that survived methicillin selection

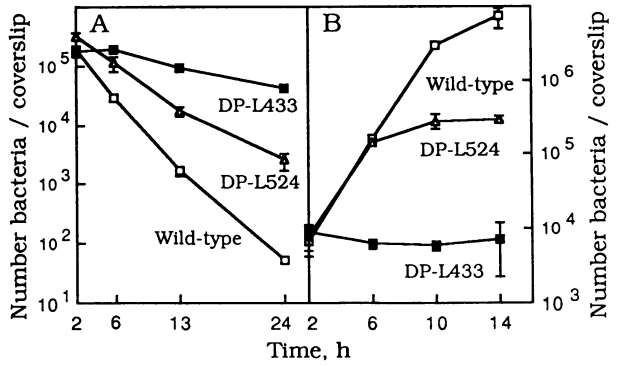

FIG. 1. Survival of wild-type and mutant *L. monocytogenes* in J774 cells in the presence of 1 mg of methicillin per ml (*A*) and growth in J774 cells in the absence of methicillin (*B*). Data points represent the mean of three coverslips (5).

were colony purified and chosen for further study (Table 1). The selection of these mutants was believed to have occurred due to their intracellular resistance to methicillin killing. To support this hypothesis we measured the intracellular methicillin resistance of the wild-type strain, 10403S, and mutant *L. monocytogenes* in J774 cells (Fig. 1*A*). All of the mutants were internalized by the J774 cell monolayers to a similar extent as the parental strain as determined by the number of gentamicin-protected bacteria per coverslip at $t = 2$ hr. However, although the parental strain showed a loss of >3 logarithms viability after 24 hr in the presence of methicillin, seven of the mutants showed almost complete resistance to methicillin after 24 hr as depicted for mutant DP-L433, whereas one mutant, DP-L524, showed an intermediate level of methicillin resistance compared to the parental strain (Fig. 1*A*). It was apparent that during 24 hr of intracellular methicillin selection, there was a 3-logarithmic enrichment of non-growing mutants over the wild type.

The intracellular growth rates of the wild-type and mutant *L. monocytogenes* were measured in the absence of methicillin. As previously reported (5), the wild-type strain, 10403S, grew in J774 cells with an intracellular doubling time of ≈1 hr. In contrast, seven mutants were completely defective for intracellular growth as depicted for mutant DP-L433, whereas mutant DP-L524 had an intermediate level of intracellular growth (Fig. 1*B*). This latter strain grew with an initial doubling time similar to that of the wild type, but after three doublings, growth ceased. This strain also formed small colonies on agar medium and failed to reach the same density as the wild type in liquid medium.

Hemolytic Expression of *L. monocytogenes*::Tn*916* Mutant Strains. The *L. monocytogenes* hemolysin, listeriolysin O, has previously been shown to be a required determinant for bacterial intracellular growth and pathogenicity (5, 14–17). Therefore, we analyzed the intracellular growth mutants for hemolytic activity (Table 1). The parental strain formed zones of hemolysis on blood agar and had a hemolytic titer in solution of 80 units/ml. Six of the eight mutants showed no detectable hemolytic activity in solution, whereas two mutants, DP-L524 and DP-L590, had 20 and <5 units/ml, respectively. Of these two mutant strains only DP-L524 formed zones of hemolysis on blood agar plates. Mutants DP-L434, DP-L534, and DP-L585 had no detectable hemolytic titer in solution but did form small zones of hemolysis on blood agar plates that were revealed by lightly scraping away single colonies.

To further characterize these mutant strains we analyzed their secreted protein profiles by SDS/PAGE. In previous studies (5, 16) all nonhemolytic mutants lacked a secreted polypeptide of 58 kDa. Similarly, in this study six of the eight mutant strains lacked a secreted 58-kDa polypeptide, whereas the remaining two mutants, DP-L434 and DP-L524, secreted decreased levels of this polypeptide (Fig. 2). Mutants DP-L433 and DP-L476, which lacked the 58-kDa polypeptide, secreted lower molecular mass polypeptides of ≈46 kDa. Three mutants, DP-L534, DP-L524, and DP-L585, which formed small colonies on LB agar and blood agar, showed very low amounts of all secreted polypeptides and may be general secretory mutants. Mutant DP-L524 could be distinguished from the others because it secreted significant amounts of the 58-kDa polypeptide and was hemolytic.

Southern Blot Analysis of Tn*916* Insertions. Recently, Mengaud *et al.* (12) reported the complete nucleotide sequence of *hlyA*, the structural gene for listeriolysin O. This gene sequence predicted a polypeptide of 58 kDa (12). To determine if the absence of the 58-kDa polypeptide in six of the mutant strains was due to insertional inactivation of *hlyA* by Tn*916*, we performed Southern blot analyses to map the sites of Tn*916* insertion. By using a hybridization probe specific for *hlyA* (see Fig. 3, probe 2), we showed that the 16.4-kb Tn*916* element had inserted into a 3.2-kb *Bam*HI restriction fragment, containing all of *hlyA*, in only two mutants, DP-L476 and DP-L590 (Fig. 4*A*). As expected, the high molecular mass bands for these two mutants also hybridized with the Tn*916* probe (data not shown). In the remaining six mutants, the 3.2-kb *Bam*HI fragment remained intact (Fig. 4*A*). Additional southern hybridization analyses were done to more precisely determine the sites of Tn*916* insertion in DP-L476 and DP-L590. A comparison of parental strain and DP-L476 DNA digested with *Hin*dIII and *Hpa* II and hybridized with probe 2 (Fig. 4*B*) revealed that Tn*916* had inserted into a 0.26-kb region between the 3′ *Hin*dIII and *Hpa* II sites within *hlyA* (Fig. 3). A comparison of parental strain and DP-L590 DNA digested with *Taq* I and hybridized with probe 1 (Fig. 4*C*) revealed that Tn*916* had inserted into a 0.74-kb *Taq* I fragment including the 5′ end of *hlyA* and sequence immediately upstream of *hlyA* (Fig. 3). Knowledge of the locations of two asymmetric *Taq* I sites present in the left and right termini of Tn*916* (29) allowed precise mapping of the Tn*916* insertion in DP-L590 to a position 0.33 kb upstream of *hlyA* (Fig. 3). Thus, we have shown that two mutant strains contained Tn*916* insertions in or adjacent to *hlyA*, whereas in

Table 1. Hemolytic activity and reversion frequency of wild-type and mutant *L. monocytogenes*

Strain	Hemolytic zones on blood agar	Hemolytic titer*	Reversion frequency†
10403S (wild-type)	++++	80	—
DP-L524	+++	20	10^{-3}
DP-L585	++	0	10^{-7}
DP-L534	+	0	$<10^{-7}$
DP-L434	+	0	10^{-6}
DP-L590	−	<5	10^{-5}
DP-L476	−	0	10^{-5}
DP-L433	−	0	10^{-6}
DP-L570	−	0	10^{-5}

*Hemolytic titer was expressed as the reciprocal of the highest dilution of culture supernatant at which complete lysis of erythrocytes was observed.

†Approximate reversion frequencies were calculated by comparing the number of bacteria required to form plaques in J774 monolayers versus the wild type.

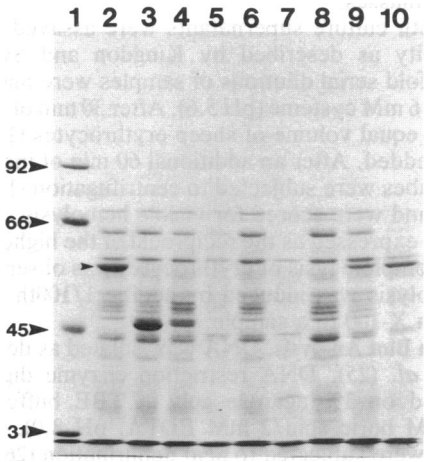

FIG. 2. SDS/PAGE of secreted polypeptides of mutant and wild-type *L. monocytogenes*. Proteins were precipitated from culture supernatant fluids with 10% trichloroacetic acid and separated on a 7% SDS/polyacrylamide gel. Lane 1, molecular mass standards (in kDa); lane 2, parental strain with 58-kDa polypeptide indicated by the arrowhead; lane 3, DP-L433; lane 4, DP-L476; lane 5, DP-L534; lane 6, DP-L570; lane 7, DP-L585; lane 8, DP-L590; lane 9, DP-L434; lane 10, DP-L524.

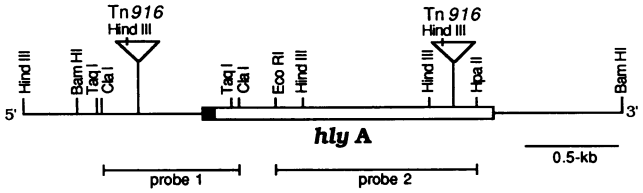

FIG. 3. Partial restriction endonuclease map of *L. monocytogenes* chromosomal region containing *hlyA*. For simplification, only restriction sites relevant to the Southern blot analyses are shown for each enzyme. The solid portion of *hlyA* represents the predicted signal sequence (12). Locations of hybridization probes 1 and 2 used for Southern blot analyses are indicated below *hlyA*. The sites of Tn*916* insertion in mutant strains DP-L590 and DP-L476 are indicated 0.33 kb upstream of *hlyA* and between the 3' *Hin*dIII and *Hpa* II sites within *hlyA*, respectively. Mapping was based on analysis of single and double digestions with the appropriate restriction enzymes, on the published *hlyA* sequence (12), and on the published sequences of the left and right termini of Tn*916* (29).

the five remaining mutant strains, all having greatly reduced or no hemolytic activity, the structural gene for listeriolysin O remained intact.

Isolation of Revertants. Tn*916* has been shown to excise precisely in several bacterial species (30, 31). Therefore, to evaluate if our mutants were caused by Tn*916* insertions, we attempted to isolate revertants. Revertants from seven of the eight mutant strains were selected by their ability to form visible plaques in confluent J774 cell monolayers, which was a direct correlate of intracellular growth. Revertants from these seven mutant strains also formed zones of hemolysis on blood agar identical to the wild type. Revertants from four of the mutant strains were sensitive to tetracycline, presumably due to excision and loss of Tn*916*, whereas three mutants, DP-L433, DP-L434, and DP-L590, repeatedly gave rise to tetracycline-resistant revertants. A revertant of mutant DP-L534 was not obtained. The reversion frequency of each mutant strain was estimated by comparing the number of bacteria required to form plaques compared to the wild type (Table 1).

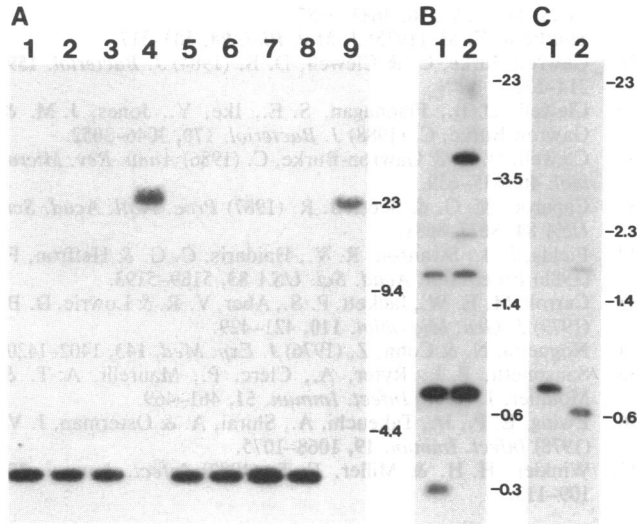

FIG. 4. Southern hybridization analysis of total DNA from *L. monocytogenes* parental and mutant strains. Numbers to the right represent the sizes in kilobases. (*A*) DNA digested with *Bam*HI and hybridized with probe 2. Lane 1, parental strain; lane 2, DP-L433; lane 3, DP-L434; lane 4, DP-L476; lane 5, DP-L524; lane 6, DP-L534; lane 7, DP-L570; lane 8, DP-L585; lane 9, DP-L590. (*B*) DNA digested with *Hin*dIII and *Hpa* II and hybridized with probe 2. Lane 1, parental strain; lane 2, DP-L476. (*C*) DNA digested with *Hin*dIII and *Nco* I and hybridized with probe 1. Lane 1, parental strain; lane 2, DP-L590.

DISCUSSION

Although intracellular pathogens are of serious clinical importance, little is known about the mechanisms they employ to grow intracellularly. To define such mechanisms we developed a selection scheme, which we refer to as intracellular methicillin selection, to isolate mutants defective for intracellular growth in the model intracellular pathogen, *L. monocytogenes*. This method exploited the property of β-lactam antibiotics—i.e., to kill only growing bacteria, by enriching the growth-defective mutants within the intracellular milieu of the macrophage-like cell line, J774. Since *L. monocytogenes* is a facultative intracellular pathogen, certain mutants defective for intracellular growth were expected to be cultivatable *in vitro*. In the present study, eight independently isolated mutants were characterized. Interestingly, all of the mutants either lacked hemolysin (hly$^-$) or had reduced secreted hemolytic activity. Furthermore, none of the hly$^-$ mutants was capable of any growth in the J774 macrophage-like cell line. This is consistent with previous reports that hly$^-$ mutants are avirulent and incapable of growth in J774 cells (5), murine bone marrow-derived macrophages (5), murine peritoneal macrophages (17), fibroblasts (5), or the human enterocyte-like cell line Caco-2 (14).

To confirm that the mutations characterized in this study were caused by Tn*916* insertions, we attempted to isolate revertants by their ability to form visible plaques in confluent J774 cell monolayers. Tetracycline-sensitive revertants, due to loss of Tn*916*, were isolated from four of the eight mutant strains, DP-L476, DP-L524, DP-L570, and DP-L585. This strongly suggested that Tn*916* insertions were responsible for the mutant phenotypes of these four strains. We also obtained revertants for mutants DP-L433, DP-L434, and DP-L590, which remained resistant to tetracycline, suggesting that these mutants may have contained multiple copies of Tn*916* or Tn*916* transposed to other sites. Alternatively, the mutant phenotypes of these three strains may have been due to single mutations not involving Tn*916*, such as point mutations. We were unable to obtain a revertant for DP-L534, suggesting that multiple Tn*916* insertions or mutations not involving Tn*916*, such as deletions or point mutations, may be responsible for the mutant phenotype of this strain. Thus, a variety of Tn*916* insertions (and possibly other spontaneous mutations) that led to an intracellular growth-deficient phenotype were enriched by our selection.

All but one of the hly$^-$ mutants isolated in this study lacked a secreted 58-kDa polypeptide thought to be listeriolysin O. However, by using hybridization probes specific for *hlyA*, it appeared that only two of the hly$^-$ mutants, DP-L476 and DP-L590, suffered a Tn*916* insertion in, or immediately adjacent to, *hlyA*. Thus, it appears that there are sequences other than *hlyA* that are necessary for hemolytic expression. In addition, our observation that mutant DP-L434 still secreted a 58-kDa polypeptide but is hly$^-$ suggested that there may be other components involved in generating hemolytic activity. However, because we obtained only tetracycline-resistant revertants of mutant DP-L434, it remains a distinct possibility that a point mutation within *hlyA* had resulted in the secretion of a nonfunctional listeriolysin O protein product.

The precise defect in mutant DP-L433 is not known. This hly$^-$ mutant had no detectable secreted 58-kDa polypeptide and secreted a lower molecular mass polypeptide species in amounts similar to the wild-type 58-kDa polypeptide. This suggested that the lower molecular mass polypeptide may represent a truncated form of the 58-kDa polypeptide. However, no Tn*916* insertion in *hlyA* was detected in mutant DP-L433. The isolation of only tetracycline-resistant revertants from this mutant strain suggested that a point mutation within *hlyA* may have occurred. Alternatively, a polar mu-

tation resulting in both the loss of secretion of the 58-kDa polypeptide and the truncation of another distinct polypeptide species may have been responsible.

It was not surprising that nutritional auxotrophs were not isolated after methicillin selection because the Tn*916* recipients were grown on a minimal medium prior to infection. However, one mutant, DP-L524, seemed to have a general growth defect and was unique in that it secreted substantial hemolytic activity and was capable of limited intracellular growth. The precise defect in this strain is unknown, but it was also somewhat defective for extracellular growth as well. This strain may represent a possible vaccine strain since it shows abortive intracellular growth.

Future studies are necessary for isolation of auxotrophic mutants of *L. monocytogenes* by intracellular methicillin selection to examine nutritional requirements for intracellular growth. Fields *et al.* (32) have isolated Tn*10* mutants of *Salmonella typhimurium* that were avirulent and could not survive within macrophages. Many of these mutants were auxotrophic mutants (32). However, unlike *L. monocytogenes*, which replicates intracytoplasmically (14), *S. typhimurium* resides in a macrophage phagolysosome (33). Therefore, it will be interesting to compare the nutritional requirements for intracellular growth of these two pathogens.

The results of our study demonstrated that intracellular methicillin selection can be used to isolate mutations in genes required for intracellular growth and strongly suggested that hemolysin is an essential determinant for intracellular growth and pathogenicity of *L. monocytogenes*. The role of hemolysin appears to be lysis of a phagosome containing *L. monocytogenes* allowing the bacteria free access to the eukaryotic cytoplasm (ref. 14; D.A.P. and L. Tilney, unpublished data). Thus, *L. monocytogenes* resembles other intracellular parasites that have adapted to an intracytoplasmic lifestyle such as *T. cruzi* (34), *Shigella flexneri* (35), *Rickettsia tsutsugamushi* (36), *Rickettsia prowazekii* (37), and viral pathogens. Moreover, it has been suggested that, like *L. monocytogenes*, hemolytic activity is also involved in lysis of the phagosomal membrane by *S. flexneri* and *R. prowazekii*, which express a contact hemolysin (35) and phospholipase A (37), respectively. Thus, a broad range of pathogens ranging from viruses to protozoa have evolved different mechanisms to escape from host phagosomal or endosomal compartments. Further analysis of the mutants isolated in this study and other mutants isolated by intracellular methicillin selection should facilitate a detailed molecular analysis of the regulation and precise requirements of *L. monocytogenes* necessary for intracellular parasitism.

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1. Daniel, T. M. (1984) in *Tropical and Geographical Medicine*, eds. Warren, K. S. & Mahmoud, A. F. (McGraw–Hill, New York), p. 793.
2. Nordeen, S. K. & Bravo, L. L. (1986) *World Health Stat. Q.* **39,** 122–128.
3. Schachter, J. (1985) *Rev. Infect. Dis.* **7,** 713–716.
4. Walsh, J. A. (1984) in *Tropical and Geographical Medicine*, eds. Warren, K. S. & Mahmoud, A. F. (McGraw–Hill, New York), p. 1078.
5. Portnoy, D. A., Jacks, P. S. & Hinrichs, D. J. (1988) *J. Exp. Med.* **167,** 1459–1471.
6. Hahn, H. & Kaufman, S. H. E. (1981) *Rev. Infect. Dis.* **3,** 1221–1250.
7. Mackaness, G. B. (1962) *J. Exp. Med.* **116,** 381–406.
8. North, R. J. (1970) *J. Exp. Med.* **132,** 521–534.
9. Jenkins, E. M., Njoku-Obi, A. N. & Adams, E. A. (1964) *J. Bacteriol.* **88,** 418–424.
10. Njoku-Obi, A. N., Jenkins, E. M., Njoku-Obi, J. C., Adams, J. & Covington, V. (1963) *J. Bacteriol.* **86,** 1–8.
11. Geoffroy, C., Gaillard, J. L., Alouf, J. E. & Berche, P. (1987) *Infect. Immun.* **55,** 1641–1646.
12. Mengaud, J., Vincente, M., Chenevert, J., Pereira, J. M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J. & Cossart, P. (1988) *Infect. Immun.* **56,** 766–772.
13. Smyth, C. J. & Duncan, J. L. (1978) in *Bacterial Toxins and Cell Membranes*, eds. Jelaszewicz, J. & Wadstrom, T. (Academic, New York), pp. 129–183.
14. Gaillard, J. L., Berche, P., Mounier, J., Richard, S. & Sansonetti, P. (1987) *Infect. Immun.* **55,** 2822–2829.
15. Gaillard, J. L., Berche, P. & Sansonetti, P. (1986) *Infect. Immun.* **52,** 50–55.
16. Kathariou, S., Metz, P., Hof, H. & Goebel, W. (1987) *J. Bacteriol.* **169,** 1291–1297.
17. Kuhn, M., Kathariou, S. & Goebel, W. (1988) *Infect. Immun.* **56,** 79–82.
18. Lederberg, J. & Zinder, N. (1948) *Am. Chem. Soc. J.* **70,** 4267.
19. Davis, B. D. (1948) *Am. Chem. Soc. J.* **70,** 4267.
20. Bishop, D. K. & Hinrichs, D. J. (1987) *J. Immunol.* **139,** 2005–2009.
21. Gawron-Burke, C. & Clewell, D. B. (1982) *Nature (London)* **300,** 281–284.
22. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
23. Ralph, P., Pritchard, J. & Cohn, M. (1975) *J. Immunol.* **114,** 898–905.
24. Kingdon, G. C. & Sword, C. P. (1970) *Infect. Immun.* **1,** 363–372.
25. Flamm, R. K., Hinrichs, D. J. & Tomashow, M. F. (1984) *Infect. Immun.* **44,** 157–161.
26. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3683–3687.
27. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
28. Gawron-Burke, C. & Clewell, D. B. (1984) *J. Bacteriol.* **159,** 214–221.
29. Clewell, D. B., Flannagan, S. E., Ike, Y., Jones, J. M. & Gawron-Burke, C. (1988) *J. Bacteriol.* **170,** 3046–3052.
30. Clewell, D. B. & Gawron-Burke, C. (1986) *Annu. Rev. Microbiol.* **40,** 635–659.
31. Caparon, M. G. & Scott, J. R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8677–8681.
32. Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5189–5193.
33. Carroll, M. E. W., Jackett, P. S., Aber, V. R. & Lowrie, D. B. (1979) *J. Gen. Microbiol.* **110,** 421–429.
34. Nogueira, N. & Cohn, Z. (1976) *J. Exp. Med.* **143,** 1402–1420.
35. Sansonetti, P. J., Ryter, A., Clerc, P., Maurelli, A. T. & Mounier, J. (1986) *Infect. Immun.* **51,** 461–469.
36. Ewing, E. P., Jr., Takeuchi, A., Shirai, A. & Osterman, J. V. (1978) *Infect. Immun.* **19,** 1068–1075.
37. Winkler, H. H. & Miller, E. T. (1982) *Infect. Immun.* **38,** 109–113.