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Isolation of *Listeria monocytogenes* Small-Plaque Mutants Defective for Intracellular Growth and Cell-to-Cell Spread

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To dissect the regulatory and structural requirements for *Listeria monocytogenes* intracellular growth and cell-to-cell spread, we designed a protocol based on transposon mutagenesis and the isolation of mutants which form small plaques in monolayers of mouse L2 cell fibroblasts. Two different transposable elements were used to generate libraries of insertion mutants: Tn*916* and a derivative of Tn*917-lac*, Tn*917*-LTV3. Ten classes of mutants were isolated and evaluated for growth and cell-to-cell spread in J774 mouse macrophagelike cells, Henle 407 human epithelial cells, and mouse bone marrow-derived macrophages. Mutants were also evaluated for secretion of hemolysin and phospholipase (assayed by egg yolk opacity) and association with F-actin in the cytoplasm of cells, using NBD-phallacidin staining. The ten classes of mutants included (i) mutants showing abortive intracellular and extracellular growth; (ii) mutants showing abortive intracellular growth; (iii) rough mutants; (iv) mutants showing greatly reduced hemolysin and phospholipase secretion but showing normal growth in cells and little or no association with F-actin; (v) mutants with mutations mapping to an open reading frame (ORF) adjacent to *hlyA* and referred to as ORF U, lacking phospholipase activity, and with 50% normal hemolysin activity; (vi) mutants with reduced secretion of both hemolysin and phospholipase; (vii) nonhemolytic mutants with mutations mapping to the structural gene, *hlyA*; (viii) mutants with 25% normal hemolysin secretion and absolutely no association with F-actin; (ix) mutants with mutations mapping to ORF U, lacking phospholipase activity, and with normal hemolysin activity; and (x) mutants showing a mixed-plaque morphology but normal for all other parameters.

Listeria monocytogenes is a gram-positive, rapidly growing food-borne human pathogen responsible for serious infections in immunocompromised individuals and pregnant women (13). In a murine model of infection, *L. monocytogenes* is a facultative intracellular pathogen and immunity is cell mediated (14). Although in vitro studies have for the most part focused on the interaction of *L. monocytogenes* with macrophages, it is clear from both in vivo and in vitro studies that *L. monocytogenes* invades and grows in a variety of mammalian cells, including macrophages, epithelial cells, and fibroblasts (11, 15, 21, 26, 35, 38–40, 42). In addition, like *Shigella flexneri* (2, 27, 36, 37), *L. monocytogenes* spreads from cell to cell (15, 33, 48), an observation which is reflected by the formation of plaques in monolayers of tissue culture cells.

The morphological stages observed during the spread of *L. monocytogenes* from cell to cell have recently been documented (33, 48) and are summarized in Fig. 1. Subsequent to internalization, the bacteria escape from a host vacuole, an event mediated, at least in part, by the action of a pore-forming hemolysin, listeriolysin O (3, 11, 48). Rapid cell division ensues, and the bacteria become encapsulated by short actin filaments and other actin-binding proteins (7a, 33, 48). The actin-based structure is rearranged to form a long tail behind the bacteria which appears to mediate movement of the bacteria through the cytoplasm to the cell periphery. Some of the bacteria are presented in pseudopod-like structures which are apparently recognized and internalized by neighboring cells. Thus, within the cytoplasm of the second cell, the bacteria are found surrounded by a double membrane. Both membranes are solubilized, and the cycle is repeated. In this model, the bacteria spread from one cell to another without ever leaving the cytoplasm, thereby providing a cell biological explanation for the absolute requirement of cell-mediated immunity and the observation that antibody is not protective (14).

To dissect the regulatory and structural requirements for *L. monocytogenes* intracellular growth and cell-to-cell spread, we designed a protocol based on transposon mutagenesis and the isolation of small-plaque mutants. In this communication, we report the isolation and characterization of 10 classes of mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* 10403S was the parental strain used in these studies (4). All of the mutants were derived from this strain and are listed in Table 1. Bacteria were grown on either brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) agar and broth or Luria-Bertani medium (32). All stock cultures were stored as suspensions of cells at −70°C in 50% glycerol. For routine use, bacteria were kept on BHI agar at room temperature. All strains harboring Tn*916* were kept on BHI agar containing tetracycline at 12.5 μg/ml. *Streptococcus faecalis* CG110 (provided by D. Clewell, University of Michigan) was used as a donor of Tn*916* (18). Minimal medium used to check for auxotrophs was as previously described (5). *Escherichia coli* DH5α-MCR (Bethesda Research Laboratories, Gaithersburg, Md.) was used as the host strain for transformation.

Tissue culture cells and growth medium. The mouse macrophagelike cell line J774, the human epithelial cell line Henle 407, and primary cultures of bone marrow-derived macrophages were propagated as previously described (38). The mouse L2 fibroblast cell line (43) was obtained from Susan Weiss (University of Pennsylvania School of Medi-

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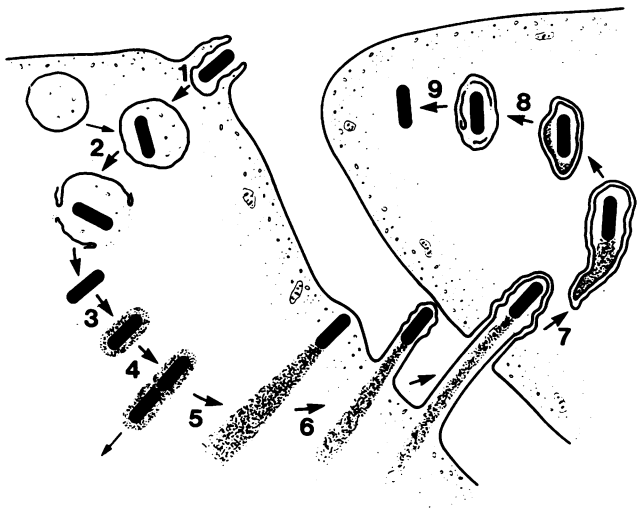

FIG. 1. Morphological stages in the entry, growth, movement, and spread of *L. monocytogenes* from one cell to another. 1, Internalization; 2, lysis of the vacuole; 3, nucleation of actin filaments; 4, growth; 5, reorganization of the actin from a cloud to a tail; 6, migration within the cytoplasm and association with the cell membrane; 7, recognition of the bacterium-containing pseudopod by a neighboring cell, followed by internalization; 8, solubilization of the inner membrane; 9, solubilization of the outer membrane. Reproduced from the *Journal of Cell Biology* by copyright permission of the Rockefeller University Press (48).

cine) and was maintained in Dulbecco modified Eagle medium (DME) supplemented with 5% fetal calf serum.

Isolation of transposon insertion mutants. Libraries of *L. monocytogenes* containing insertions of a derivative of Tn*917-lac*, Tn*917*-LTV3, were generated as recently described (6). Each library was shown to contain numerous insertions in many different genes, although hot spots were evident (6). Libraries were stored at −70°C in 50% glycerol. Libraries of *L. monocytogenes* containing Tn*916* were generated as previously described (5), except that bacterial conjugation was performed for 4 h rather than 24 h and the mating mixture was grown on 150-mm BHI agar plates containing streptomycin (0.5 mg/ml) and tetracycline (12.5 μg/ml). Approximately 10,000 transconjugants from individual libraries were removed from plates and stored at −70°C in 50% glycerol.

Plaque formation by *L. monocytogenes* in L2 cells. In all cases, L2 cells were plated so that they were confluent on the day of the infection. Bacteria were grown overnight at 30°C in BHI broth to a density of 2×10^9 CFU/ml, washed once in phosphate-buffered saline (PBS), and suspended in PBS to the original density. In the initial screening for small plaques, L2 cells grown in 10 ml of medium (DME plus 5% fetal bovine serum and without antibiotics) in 100-mm petri dishes were infected with 20 μl of the washed bacterial suspension. After 1 h, the monolayers were washed three times with 37°C PBS and overlaid with 10 ml of medium containing 1% agarose and 10 μg of gentamicin sulfate per ml. (A stock of 2× medium was prewarmed at 37°C and mixed with 56°C 2% agarose immediately before use.) As an alternative to gentamicin, ampicillin at a final concentration of 2 μg/ml was used. In this case, the monolayers were infected with 1.2 μl of the washed bacterial suspension. After 3 days, plaques were visualized by the addition of a 10-ml overlay of medium containing 1% agarose and 0.2% neutral red (Sigma) (neutral red was made as a 1% stock in water and filter sterilized). Plaques (about 700 per plate) were visualized after 4 to 8 h as clear zones in a lawn of red cells. Any plaque that was smaller than the others was picked with a sterile toothpick and grown overnight in BHI broth.

Rescreening of plaques was performed in six-well cluster dishes (Costar, Cambridge, Mass.). Cells were infected in 2 ml of medium with 2.5 μl of bacteria. After 3 days, plaques

TABLE 1. Bacterial strains and relevant characteristics

Strain	Class	Transposon	Hemolytic units[a]	Actin[b]	Egg yolk opacity	Cell-to-cell spread[c]	Additional comments	Growth rate[d] in:		Site of insertion[e]
								J774 cells	Henle cells	
10403S		None	80	+	+	8.70 ± 3.13	Parental	51	67	None
DP-L758	1	Tn*916*	40	+	+	2.10 ± 0.57	Abortive[f]	80	194	Unknown
DP-L793	2	Tn*916*	80	+	+	3.80 ± 1.40	Abortive[g]	79	88	Unknown
DP-L867	3	Tn*916*	80	+	+	6.50 ± 2.72	Rough[h]	69	69	Unknown
DP-L967		Tn*917*-LTV3	80	+	+	8.47 ± 3.36	Control[i]	60	59	Unknown
DP-L973	4	Tn*917*-LTV3	5	−	±	2.00 ± 0.94		51	65	Base 152
DP-L995	5	Tn*917*-LTV3	40	±	−	2.30 ± 1.77		54	86	Base 1194
DP-L1034	6	Tn*917*-LTV3	<5	−	−	1.00 ± 0.00		No growth	No growth	Unknown
DP-L1044	7	Tn*917*-LTV3	0	−	+	1.00 ± 0.00		No growth	244	Base 2733
DP-L1049	8	Tn*917*-LTV3	20	−	+	1.00 ± 0.00		64	79	Unknown
DP-L1054	9	Tn*917*-LTV3	80	±	−	1.30 ± 0.48		54	74	Base 841
DP-L1107	10	Tn*917*-LTV3	80	+	+	4.80 ± 1.87	Mixed[j]	59	74	Unknown
DP-L1154		Tn*917*-LTV3	80	+	+	7.79 ± 3.30	Control[k]	49	80	Unknown

[a] Expressed as the reprocal of the dilution at which 50% lysis of the erythrocytes was observed.
[b] Bacterial association with NBD-phallacidin after 4 h of infection of J774 cells.
[c] Average number ± standard deviation of bone marrow-derived macrophages infected per focus of infection after 48 h of infection.
[d] Expressed as the doubling time in minutes between 2 and 8 h of culturing.
[e] See Fig. 3.
[f] Abortive extracellular growth and abortive intracellular growth.
[g] Normal extracellular growth and abortive intracellular growth.
[h] Rough colony morphology.
[i] Control strain expressing constitutively high levels of β-galactosidase activity under all conditions tested.
[j] Mixed-plaque morphology consisting of small and medium plaques.
[k] Control strain expressing low background levels of β-galactosidase activity.

were visualized by the addition of 1 ml of the neutral red solution. After purification of a small-plaque mutant to homogeneity, single colonies were purified directly from the plaque by streaking on BHI agar and were stored as suspensions in 50% glycerol at −70°C.

Assay for hemolytic activity. Hemolytic activity was determined as previously described (38), except that hemolytic units were expressed as the reciprocal of the dilution of bacterial supernatant fluid showing 50% lysis of erythrocytes.

Assay for egg yolk opacity (phospholipase activity). Egg yolk plates were prepared as follows. Fresh egg yolk was diluted in half with PBS and mixed vigorously. Luria-Bertani agar plates were overlaid with 5 ml of soft agar (0.7% agar) containing 5% egg yolk. Individual colonies were patched in triplicate and incubated at 37°C for 2 days. A positive reaction was seen as a slight, but definitive, zone of opacity surrounding the patch (10).

Intracellular growth assay. Intracellular growth in J774 cells and Henle 407 cells was accomplished as previously described with minor modifications (38). Monolayers of tissue culture cells were grown on round cover slips (12 by 1 mm; Propper Manufacturing Co. Inc., Long Island City, N.Y.) in 60-mm petri dishes containing 5 ml of the appropriate medium without antibiotics. Bacteria were grown overnight in BHI broth at 30°C, washed once with PBS, and used to infect the monolayers. Monolayers of J774 cells were infected with 5×10^5 bacteria per petri plate for 30 min, and Henle 407 cells were infected with 2×10^7 bacteria per petri plate for 60 min. Both protocols resulted in the infection of approximately 1 of 10 cells with a single bacterium after 1 h. After the initial infection, monolayers were washed three times with 37°C PBS, followed by the addition of 5 ml of prewarmed medium. After 30 min, gentamicin sulfate was added to a final concentration of 5 μg/ml. After 1 h and at each subsequent time point, the number of bacteria per cover slip was determined by depositing cover slips, in triplicate, into 5 ml of sterile distilled water in a 15-ml conical tube. After vigorous mixing for 15 s, dilutions were plated onto BHI agar.

Cell-to-cell spread. Day-6 bone marrow-derived macrophages (2×10^6) were deposited onto cover slips in 60-mm petri dishes containing DME supplemented with 10% fetal bovine serum and 30% L-cell supernatant (source of colony-stimulating factor 1 [CSF-1]) the evening before use. Monolayers were infected with each of the mutants so that approximately 1 of 50 cells contained a single bacterium after 30 min of incubation. After three washes with 37°C PBS, 37°C medium was added; after 30 min of incubation, gentamicin sulfate was added to a final concentration of 5 μg/ml. After 8 h, cover slips were stained with Diff-Quik (Baxter Heathcare Corp., Edison, N.J.) and examined by light microscopy. Individual foci of infection were examined, and the numbers of cells containing five or more bacteria were counted.

Fluorescence labeling of F-actin. After 4 h of infection, J774 cells on cover slips were transferred to a solution of 3.7% formaldehyde in PBS and fixed for a minimum of 15 min at room temperature. The cover slips were incubated in a solution containing 1.7×10^{-7} M NBD-phallacidin (Molecular Probes, Eugene, Oreg.) and 0.4% Triton X-100 at 37°C (7a). Intracellular bacteria were stained as described previously (38) by indirect immunofluorescence with *Listeria* O rabbit antiserum (Difco), which was added to the NBD-phallacidin solution at a 1/320 dilution. After one wash in PBS, cover slips were incubated with a 1/40 dilution of rhodamine isothiocyanate (RITC)-conjugated goat $F(ab')_2$ anti-rabbit immunoglobulin G (Tago Inc., Burlingame, Calif.). Cover slips were mounted in 50% glycerol in PBS for fluorescence microscopy. Bacteria were identified as staining with RITC, while bacterium-associated F-actin was identified as staining with NBD.

Mapping sites of Tn*917*-LTV3 insertions. Initially, all of the mutations were mapped by Southern blotting to determine if the transposons were inserted into the *hlyA* region of the chromosome as previously described (5). The exact sites of insertion for some of these transposons within this region of the chromosome were determined by DNA sequencing of the transposon-chromosome junctions which had been cloned as described previously (6). Dideoxy sequencing of double-stranded plasmid DNA was performed as recommended by the manufacturer with a Sequenase 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio). Junction sequences were obtained with the following oligonucleotide sequencing primer, which was complementary to a sequence 83 bp from the *lacZ*-proximal end of Tn*917*-LTV3: 5′-CAATAGAGAGATGTCACCG-3′ (Genosys Biotechnologies, Inc., The Woodlands, Tex.).The precise sites of *L. monocytogenes* sequences flanking transposon insertions mapping within the *hlyA* region were identified with DNA Strider 1.0 software (28) by searching the previously published sequence of the *hlyA* region (29, 30).

The transposon insertion mutations not mapping to the *hlyA* region of the *L. monocytogenes* chromosome were grouped on the basis of their phenotypes and according to the sizes of the *Eco*RI fragments which resulted after insertion of the transposon. Since neither transposon (Tn*916* or Tn*917*-LTV3) contained an *Eco*RI site, a single hybridizing band was revealed for each mutant. To determine the relative sizes of these transposon-containing *Eco*RI fragments, Southern blotting of *Eco*RI-digested chromosomal DNA was performed as previously described (5), except that agarose gel electrophoresis was performed in 0.4% agarose.

RESULTS

Identification and isolation of mutants. Two fundamentally distinct transposable elements, Tn*916* (18) and a variant of Tn*917-lac*, Tn*917*-LTV3 (6), were used to construct libraries of *L. monocytogenes* insertion mutants as described in Materials and Methods. These insertion libraries were screened for mutants which formed small or abnormal plaques in mouse L2 fibroblast monolayers after 3 days in culture (Fig. 2). L2 cells were used because of all the cells tested, they produced the most homogeneous and distinct plaques. However, it should be noted that the actual uptake of *L. monocytogenes* by L2 cells is relatively inefficient.

Putative small-plaque mutants which appeared after 3 days were purified by isolating the bacteria from the plaque and repeating the plaquing procedure until a homogeneous plaque morphology was obtained (usually three cycles of plaque purification). Seventy small-plaque mutants were isolated from approximately 100,000 plaques screened from 19 libraries. Although this procedure facilitated screening large numbers of plaques, it is likely that some classes of mutants, such as those which failed to plaque, were missed. Nevertheless, a minimum of 10 different classes of mutants were isolated (characterization is described below). All of the classes formed plaques that were smaller than wild-type plaques and ranged from barely visible to approximately one-half the size of the parental plaque. One class, represented by strain DP-L1107, always showed a mixture of

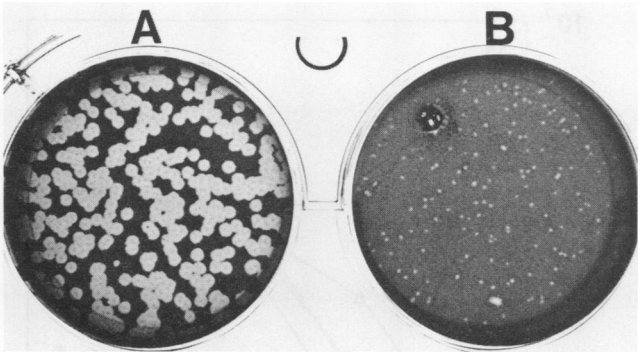

FIG. 2. Plaque formation by *L. monocytogenes* in L2 cells. (A) Wild-type strain. (B) Representative small-plaque mutant, DP-L793. Plaques were stained with neutral red after 3 days.

small and medium plaques, and isolation and purification of bacteria from either plaque type resulted in this mixed-plaque phenotype. All of the other mutants had a homogeneous plaque morphology. Prototype strains representing these 10 classes were subjected to further study and are listed in Table 1. Some of these mutant classes were isolated repeatedly and were clearly the result of independent insertion events, while other classes were only isolated from a single library and may represent siblings (Table 2). It is also noteworthy that there was little overlap in mutants isolated with the two different transposable elements, suggesting that neither element inserted randomly and that each has a different target specificity.

Extracellular growth of mutants. All of the mutants grew on minimal medium, indicating that auxotrophs had not been isolated. In addition, 8 of the 10 classes of mutants showed extracellular growth rates and colony size and morphology identical to those of the wild type. The exceptions included mutant DP-L758, which formed small colonies on all solid media and grew to one-half the density of the wild type in liquid medium, and mutant DP-L867, which formed rough

TABLE 2. Distribution of small-plaque mutants

Transposon library[a]	Class(es)[b] (no. of isolates)[c]
1	7 (1)
2	7 (8), 10 (1)
3	7 (2)
4	6 (4), 9 (2)
5	4 (3), 5 (5), 7 (2), 9 (4), 10 (2)
6	4 (1)
7	None
8	5 (1), 10 (1)
9	4 (1), 7 (1)
10	4 (1), 8 (4), 10 (3),
11	1 (2)
12	1 (1)
13	None
14	2 (4)
15	None
16	None
17	None
18	3 (2)
19	7 (2)

[a] Transposon libraries 1 to 10 consisted of *L. monocytogenes* 10403 S::Tn*917*-LTV3. Transposon libraries 11 to 19 consisted of *L. monocytogenes* 10403S::Tn*916*.
[b] See Table 1.
[c] Twelve isolates did not fall into any of the classes.

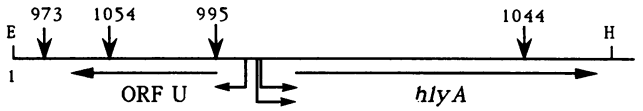

FIG. 3. Physical map of the *L. monocytogenes hlyA* chromosomal region and precise sites of Tn*917*-LTV3 insertions. This map, including the indicated promoters (bottom arrows), was adapted from that of Mengaud et al. (30). Base pair 1 begins at the *Eco*RI site (E). H, *Hin*dIII site at the end of *hlyA*. 1044, Site of insertion in DP-L1044; 1054, site of insertion in DP-L1054; 995, site of insertion in DP-L995; 973, site of insertion in DP-L973.

colonies. The latter mutant appeared to be identical to rough variants which arise spontaneously in *L. monocytogenes* (20). Like other rough strains, this strain formed filaments of nonseptating rods in liquid cultures. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of secreted proteins indicated that a single polypeptide of 60 kDa was secreted in greatly reduced amounts, as reported in the literature (20) (data not shown). Spontaneous smooth variants which were tetracycline sensitive could be isolated, indicating that the Tn*916* element had been excised and suggesting that the transposon insertion caused the rough phenotype.

Mapping of mutations. There are only two regions of the *L. monocytogenes* genome for which sequence data exist (19, 29, 30). One of these is a region of approximately 3 kb which contains the promoter and structural gene for listeriolysin O (*hlyA*) and one additional open reading frame (ORF), ORF U (Fig. 3) (30). Transposon insertion sites in all 70 mutants were mapped by Southern blotting to determine whether they were present in the *hlyA* region, and those mutants with insertions in this region were analyzed in more detail. Four of the 10 classes of mutants had mutations that mapped to the *hlyA* region, including multiple sites of insertion in the structural gene for *hlyA*, to ORF U, and to an uncharacterized sequence 5′ of ORF U (Fig. 3). The precise sites of insertion in representative mutants were determined by DNA sequence analysis (Table 1 and Fig. 3). The transposon insertions in the prototype strains representing the six remaining classes of mutants all mapped to unique *Eco*RI fragments of unknown location, indicating that these six classes represent separate insertions. It is likely that additional classes or subclasses will emerge after a more detailed analysis of mutants within a given class.

Hemolytic activity of mutants. Transposon insertions which result in reduced hemolytic activity or no hemolytic activity abolish the growth of *L. monocytogenes* in tissue culture cells of murine origin (21, 38). Hence, one might have predicted that mutants with insertions in the structural gene for *hlyA* would not grow or form plaques. Nevertheless, mutants with insertions in the structural gene for *hlyA* were isolated. These mutants formed very tiny plaques which may reflect delayed entry into the host cell cytoplasm or possibly growth within a vacuole.

Three other classes of mutants also had reduced levels of hemolytic activity, but the mutations mapped outside the *hlyA* gene. One of these mutants, represented by DP-L973, had only 5 U of hemolytic activity per ml, and the mutation mapped to a region just 3′ of ORF U (Fig. 3); the mutations of two other mutants mapped to unknown regions of the chromosome. These data suggest that other genes are necessary for the expression of hemolytic activity.

Egg yolk opacity (phospholipase activity) of mutants. *L. monocytogenes* produces a zone of opacity surrounding

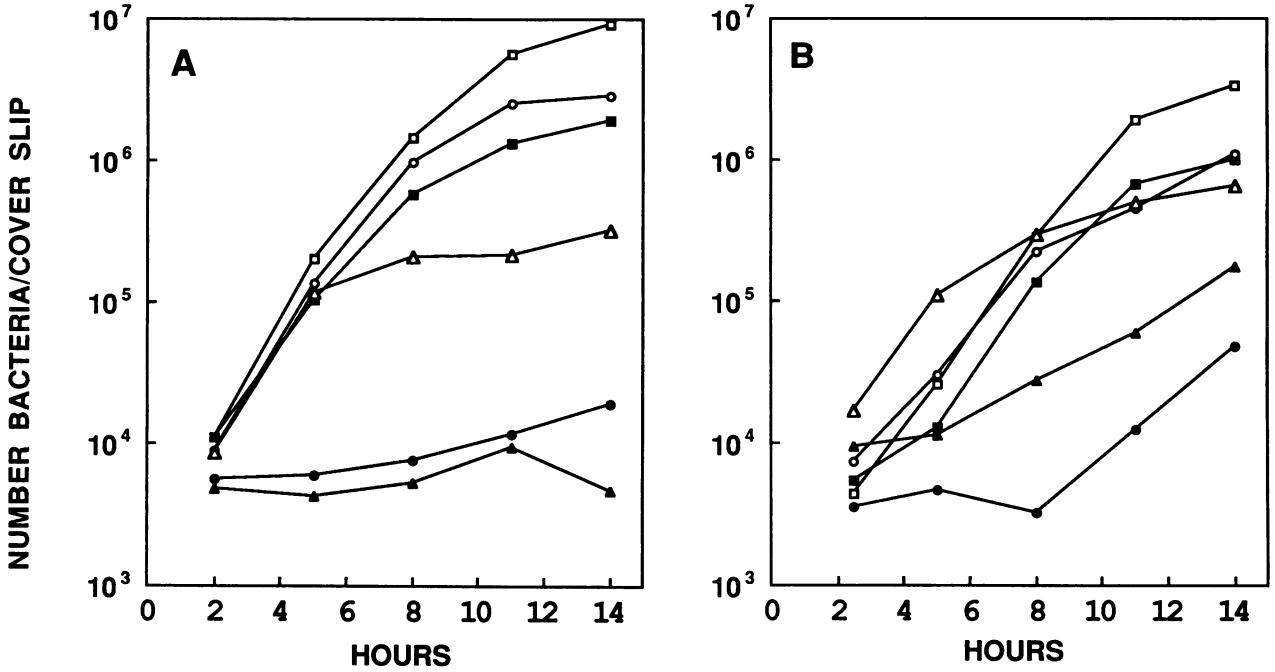

FIG. 4. Intracellular growth of representative *L. monocytogenes* strains. (A) Growth in the J774 mouse macrophagelike cell line. (B) Growth in the Henle 407 human epithelial cell line. Symbols: □, wild-type strain 10403S; ■, DP-L1049; ○, DP-L1054; ●, DP-L1034; △, DP-L793; ▲, DP-L1044. Data represent averages for three cover slips.

colonies grown on egg yolk medium; this zone has been shown to reflect the activity of phospholipase C (10, 22). Three classes of mutants failed to produce a zone of opacity on egg yolk agar. Although it may be premature to conclude that these mutants lacked phospholipase C, the data certainly suggest that these mutants were defective in the expression of this enzyme. Most interestingly, mutants with insertions in ORF U (Fig. 3) lacked phospholipase activity, suggesting that ORF U may encode phospholipase activity or alternatively regulate the expression of this activity. Another class of mutants, represented by DP-L1034, lacked egg yolk opacity and also had greatly reduced hemolytic activity. The insertion in this class apparently affected a gene product involved in the production of both hemolysin and phospholipase.

Growth of mutants in J774 cells and Henle 407 cells. In a previous report, we showed that hemolysin was necessary for the growth of *L. monocytogenes* in murine macrophages and fibroblasts but not in human epithelial cells or fibroblasts (38). In this study, all of the mutants were assayed for growth in the mouse macrophagelike cell line J774 and the human epithelial cell line Henle 407 (Table 1 and Fig. 4). In J774 cells, the mutants fell into four classes. (i) Mutants (the majority) which grew like the wild type, with intracellular doubling times of approximately 1 h; (ii) mutants which had less than 10% wild-type hemolytic activity and did not grow intracellularly; (iii) mutant DP-L973, which had a mutation that mapped to a region 3′ of ORF U, secreted only 5 U of hemolytic activity per ml, but grew normally; and (iv) two classes of mutants which initially grew like the wildtype but, after four or five doublings, almost ceased to grow. These mutants will be described as showing abortive intracellular growth. One class of these mutants, represented by DP-L793, grew like the wild type extracellularly, while another class of these mutants, represented by DP-L758, showed abortive extracellular growth as well.

The growth of the mutants in Henle 407 cells was similar to that in J774 cells, except that, as previously reported (38), mutations in *hlyA* did not abolish intracellular growth. However, mutant DP-L1034, which was defective for the expression of both hemolysin and phospholipase, failed to show any growth until 8 h of culturing in the Henle 407 cells. This result may reflect a very low level of escape into the host cytoplasm. Since hemolytic activity is not required for the growth of *L. monocytogenes* in Henle cells (38), these data suggest that phospholipase can substitute for hemolysin in Henle cell lines but that, in the absence of both hemolysin and phospholipase, there is almost no intracellular growth. Whether a phospholipase can mediate lysis of the bacterium-containing vacuole by itself remains to be demonstrated.

Cell-to-cell spread of mutants. With the exception of the hemolysin mutants and the abortive intracellular growth mutants, the other mutants grew quite well in both J774 cells and Henle 407 cells, yet formed small plaques. To begin characterization of the cell biological defect in these mutants, we observed them in stained monolayers of infected Henle cells, J774 cells, and primary cultures of bone marrow macrophages by light microscopy. To quantitate the cell-to-cell spread, we used bone marrow-derived macrophages, because they are best suited for visual inspection (Fig. 5). This analysis revealed that most of the mutants were indeed defective for cell-to-cell spread. The most striking example was DP-L1049, which seemed absolutely defective for intracellular spread (Fig. 5C). This mutant was unable to move within a cell or to adjacent cells. DP-L1054 and DP-L995, which had mutations that mapped to ORF U and which lacked phospholipase activity, were also quite defective for cell-to-cell spread, although some spreading was evident (Fig. 5B). These mutants seemed capable of limited movement to the periphery of an infected cell but were defective in spreading to adjacent cells. Occasionally, bacteria could be seen in pseudopods, and some bacteria could be detected

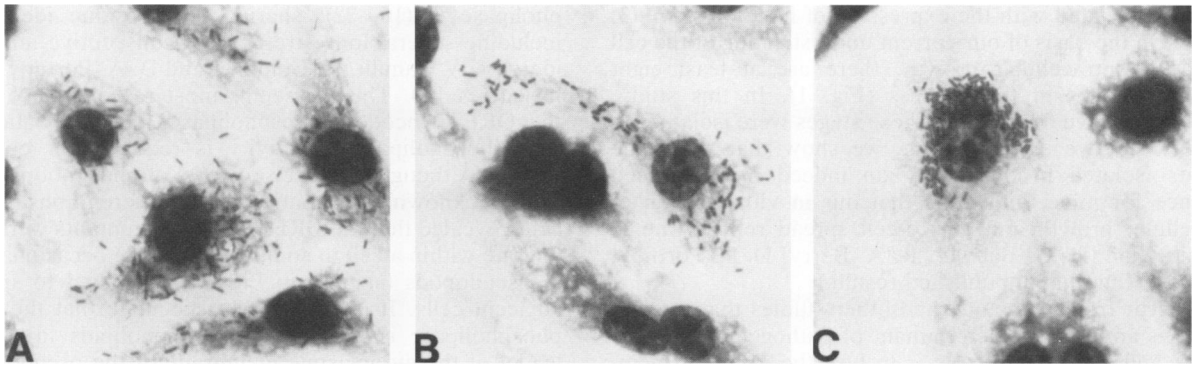

FIG. 5. Light micrographs of representative *L. monocytogenes* strains grown in bone marrow-derived macrophages for 8 h. (A) Wild-type strain 10403S. Bacteria have spread from cell to cell. (B) DP-L1054. Bacteria have migrated within the infected cell but have not spread to adjacent cells. (C) DP-L1049. Bacteria have not migrated within the infected cell or spread to adjacent cells.

in neighboring cells. The other *hly*$^+$ mutants showed extensive cell-to-cell spread, although not to the same extent as the parental strain.

Association with F-actin. As previously reported, intracytoplasmic *L. monocytogenes* organisms utilize host actin filaments to mediate their spread within a cell and from cell to cell (7a, 33, 48). Although electron microscopy is the only method which allows visualization of individual actin filaments (48), actin filaments associating with the bacteria can also be indirectly visualized by fluorescence microscopy with NBD-phallacidin, a reagent which binds to F-actin and not to G-actin (7a). Thus, J774 macrophages infected with each of the mutants were stained with NBD-phallacidin (Fig. 6). Hly$^-$ mutants failed to stain with NBD-phallacidin, as expected, because of their vacuolar compartmentalization. All of the other mutants were positive for actin polymerization, with four exceptions. The most striking example was seen with mutants DP-L1049 and DP-L973. In no case could we find any evidence for NBD-phallacidin staining surrounding the intracytoplasmic bacteria (Fig. 6). This result is consistent with the absolute inability of these mutants to spread from cell to cell. However, since these mutants were also defective for hemolysin expression, the transposon insertion likely occurred in genes which may regulate both actin polymerization and hemolysin expression. Mutants DP-L1054 and DP-L995, for the most part, did not stain with NBD-phallacidin, although an occasional bacterium which stained positively could be found (data not shown). We concluded that the latter mutants were affected in actin polymerization but not to the extent that DP-L1049 was. However, it should be noted that this analysis becomes difficult to interpret in mutants which fail to spread, as the bacteria tend to accumulate around the nucleus rather than spreading to the cell periphery (Fig. 6). Characterization of the precise actin defect in these mutants awaits a thorough electron microscopic study. Preliminary electron microscopic data were consistent with the NBD-phallacidin results; i.e., there were no actin filaments surrounding DP-L1049 and apparently reduced amounts of actin filaments surrounding DP-L1054, although both mutants were clearly in the cell cytoplasm (L. Tilney, P. Connelly, and D. A. Portnoy, unpublished data).

DISCUSSION

The capacity of *L. monocytogenes* to enter the mammalian cell cytoplasm, grow, and spread to adjacent cells is probably essential for the full expression of pathogenicity and certainly requires a number of structural and regulatory components. Until this study, only one stage had been defined at the molecular level, that being entry of the bacteria into the host cytoplasm, a stage which has been

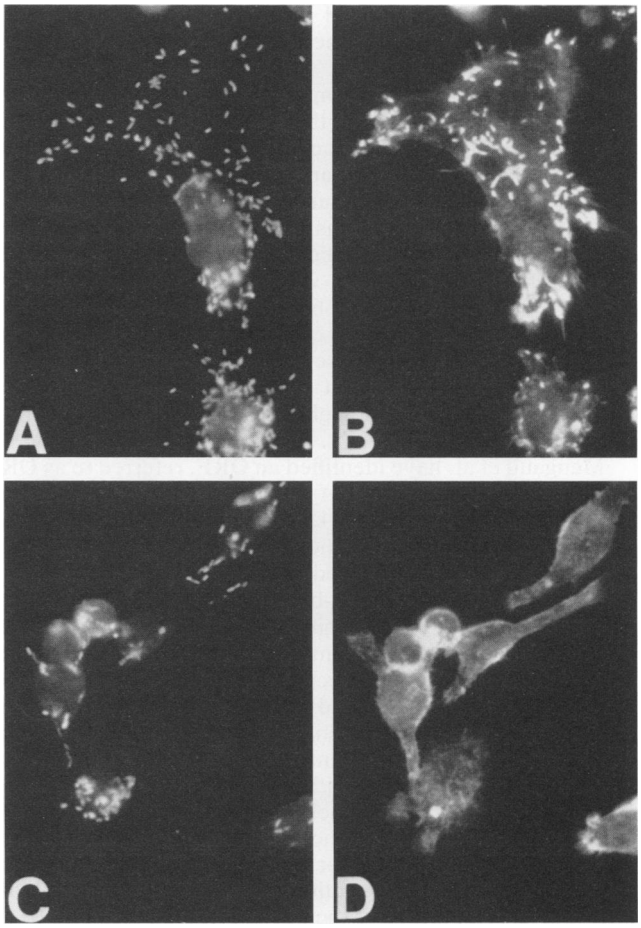

FIG. 6. Association with F-actin of *L. monocytogenes* in J774 cells. (A) Wild-type strain 10403S labeled with RITC (all of the bacteria). (B) Same as panel A but labeled with NBD-phallacidin (F-actin stain). (C) DP-L1049 labeled with RITC. (D) Same as panel C but labeled with NBD-phallacidin.

directly correlated with the expression of listeriolysin O (3, 11, 48). On the basis of our current understanding of the cell biology of intracellular growth, there are at least eight additional stages in the process (Fig. 1). In this study, mutants defective for many of these stages were isolated. In a report that we are preparing, we show that all of the mutants isolated in this study do indeed have reduced virulence for mice, indicating that our in vitro system of intracellular growth and cell-to-cell spread reflects the in vivo situation (H. G. Bauwer, R. A. Barry, D. A. Portnoy, and D. J. Hinrichs, unpublished results).

It is clear from this study and other studies that listeriolysin O is an essential determinant of pathogenicity (7, 12, 18, 38), and its apparent role is to lyse the vacuolar membrane (stage 2 in Fig. 1). It was therefore surprising that insertion mutants with insertions within *hlyA* produced plaques at all. The plaques produced by these mutants were exceedingly small and often difficult or impossible to visualize. On the basis of other work, it is now clear that these mutants are trapped in a vacuole and should be unable to grow. However, since these mutants formed tiny plaques, we suggest that (i) it takes an extended period of time for them to escape to the cell cytoplasm, possibly mediated by residual hemolytic activity or another activity; (ii) some growth can occur in a vacuole; or (iii) hemolysin is also required for cell-to-cell spread. A possible role for hemolysin in cell-to-cell spread would be to lyse one or both membranes after spreading of the bacteria to a neighboring cell (stages 8 and 9 in Fig. 1).

Although hly mutants formed tiny plaques in L2 cells and failed to grow in J774 cells, they did grow to some extent in Henle 407 human epithelial cells, indicating that there may be gene products other than hemolysin that can mediate escape to the cytoplasm. Our results suggest that phospholipase C activity, which was assayed by the production of egg yolk opacity, may indeed replace hemolysin activity in Henle 407 cells. Consistent with this idea, mutants lacking both hemolysin and phospholipase activities had greatly reduced growth in Henle cells. We speculate that both hemolysin and phospholipase normally act in concert to lyse the vacuolar membrane but that in some cell lines, either activity is sufficient, although the efficiency of escape may not be optimal.

Mengaud et al. have identified an ORF, referred to as ORF U, that is adjacent to *hlyA* and may encode a predicted protein of approximately 27 kDa (30). ORF U and *hlyA* have back-to-back divergent promoter elements and share a 14-bp palindrome. It was suggested that ORF U and *hlyA* may be regulated by the same DNA-binding protein and that ORF U may itself encode a DNA-binding protein with a predicted 18-amino-acid stretch resembling a helix-turn-helix motif (30). Interestingly, one class of small-plaque mutants isolated in the current study had Tn*917* insertions in ORF U but were normal for hemolysin production and intracellular growth. However, ORF U insertion mutants were defective for cell-to-cell spread and did not produce egg yolk opacity; therefore, they probably lacked phospholipase C (10, 23). Thus, ORF U regulates phospholipase C production, encodes the structural gene for phospholipase C, or performs some other accessory function required for phospholipase activity. A comparison of the published sequence of ORF U with those of other proteins in the National Biomedical Research Foundation data base by use of the computer program FASTP (25) revealed that the ORF U sequence has significant amino acid relatedness with the sequence for *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (16, 22), sharing 30% residue identity and including several long stretches of consecutive amino acid identity (A. Camilli, H. Goldfine, and D. A. Portnoy, unpublished results). Thus, it seems most reasonable to assume that ORF U encodes a phospholipase C, and the data suggest that phospholipase C activity is required for cell-to-cell spread. Although the precise role(s) of phospholipase activity is not known, examination of light micrographs of stained cells revealed that the ORF U insertion mutants were able to migrate within a cell to some extent, were occasionally seen in pseudopods (stage 6 in Fig. 1), but failed to spread to adjacent cells. It is tempting to speculate that the role for phospholipase is to hydrolyze phopholipids in the inner leaflet of the cytoplasmic membrane of the pseudopod and that this hydrolysis is required for successful cell-to-cell spread. Determination of the precise activity of phospholipase(s) both in vitro and in vivo will certainly occupy us in the near future.

The mobilization of actin filaments is an essential feature of the intracellular movement and cell-to-cell spread of *L. monocytogenes* (7a, 33, 48). We have shown that intracytoplasmic *L. monocytogenes* organisms grown in the presence of cytochalasin D have little or no actin on the bacterial surface, yet in permeabilized cells, the bacteria nucleate the assembly of actin filaments from exogenously added G-actin (L. G. Tilney, P. Connelly, and D. A. Portnoy, J. Cell Biol., in press). Thus, we believe that the bacteria possess a surface or secreted actin nucleator or a product which binds a host actin nucleator. Characterization of this gene product may be of general interest in cell biology, as the mechanism of actin nucleation in mammalian cells is not yet understood (46). In this study, two classes of mutants, in addition to the hly mutants, failed to associate with F-actin and accordingly were defective for cell-to-cell spread. However, the insertions probably occurred in regulatory genes, since these mutants were partially defective for hemolysin production as well. It is also possible that these classes of mutants were defective for a number of other important gene products. These mutants are the first isolated in *L. monocytogenes* which failed to associate with F-actin in the cell cytoplasm, although it appears that the transposon insertion was not in a gene(s) encoding the putative actin nucleator. In *S. flexneri*, however, mutants defective in cell-to-cell spread and association with F-actin have been isolated and shown to be lacking a cell surface antigen of 120 kDa encoded by VirG (2, 24, 37). Whether the *L. monocytogenes* mutants lack an analogous protein remains to be determined.

One class of mutants, represented by DP-L867, showed a rough colony morphology and grew as filaments. This strain appears to be identical to other rough isolates of *L. monocytogenes* which arise spontaneously at a relatively high frequency (20). Like other rough mutants (20), this mutant was defective for the secretion of a 60-kDa polypeptide and for invasion. Thus, it was somewhat surprising that we isolated a rough mutant on murine fibroblasts, since its plaque-forming efficiency was 10 times lower than normal (data not shown). Once internalized, however, this mutant grew quite well in both J774 cells and Henle 407 cells, and it is not clear why it formed a small plaque. It is premature to assign a specific role to the 60-kDa protein which it lacks, since the drastic alterations in cellular morphology, such as lack of septation, make it impossible to interpret its precise role.

Two classes of mutants isolated in this study showed abortive intracellular growth, in which growth was initially normal but ceased after four to five generations. Although

both classes of mutants were identical inside cells, one class (DP-L758) also showed abortive extracellular growth. This mutant showed a generalized defect in the secretion of a number of polypeptides (data not shown). We hypothesize that this strain was defective in response to nutrient limitation, which affected secretion as the bacteria approached the stationary phase. Perhaps this mutant is similar to the *agr* mutant observed in *Staphylococcus aureus*, in which there is defective secretion of a number of proteins normally secreted during the post-exponential phase of growth (41). The other class of mutant (DP-L793) was absolutely normal extracellularly but behaved like DP-L758 inside cells. Perhaps the two mutants have analogous mutations, but the latter mutant is defective in the appropriate response to nutritional stress only present in the intracellular environment.

It was somewhat surprising that additional mutants defective for intracellular growth, such as auxotrophs, were not isolated. This was also our previous experience with the method of intracellular methicillin selection, in which mutants unable to grow intracellularly were directly selected for (5). These results may be consistent with recent data which indicate that *Bacillus subtilis* expressing listeriolysin O can grow extensively in the J774 cytoplasm (3). Thus, the mammalian cytoplasm may be a very permissive environment for bacterial growth, and auxotrophs may have no difficulty obtaining nutrients. However, since *L. monocytogenes* is unable to replicate extensively in primary cultures of peritoneal macrophages (39), our results may reflect the use of tissue culture cell lines. In vivo, the host may have mechanisms to prevent intracytoplasmic growth, perhaps by limiting essential nutrients.

Some of the mutants isolated in this study have pleiotropic effects that may be regulatory in nature. These results, however, are consistent with those of other bacterial systems in which there is coordinate regulation of multiple virulence determinants (8, 31). Thus, we have isolated mutants which secrete reduced hemolysin and phospholipase activities (DP-L1034), reduced hemolysin and actin nucleation (DP-L1049) and, most probably, numerous as-yet-uncharacterized gene products. We hypothesize that the mutations are in genes which coordinately regulate multiple *L. monocytogenes* virulence determinants. Clearly, one approach to take in the future would be to devise a method for identifying those genes which are regulated by the regulatory genes, an approach used successfully in *B. subtilis* and *Vibrio cholerae* (17, 47). Such an approach should lead to the cloning and characterization of both known and novel gene products involved in pathogenicity.

There are other pathogens in addition to *L. monocytogenes* which have adapted to an intracytoplasmic life-style, including shigellae, rickettsiae, and *Trypanosoma cruzi* (34, 44, 49). Interestingly, all of these organisms have hemolytic activity associated with their ability to gain access to the cytoplasm (1, 44, 49). Also, it is clear that *S. flexneri* uses host actin filaments to mediate its cell-to-cell spread (2), and there is evidence in the literature that *Rickettsia tsutsugamushi* may use a similar mechanism of cell-to-cell spread (9, 45). Although these three bacterial species are not related taxonomically, it is interesting that they may share a common pathway of cell-to-cell spread. It will be interesting to determine if this is an example of convergent or divergent evolution.

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