

## Internalin B Promotes the Replication of *Listeria monocytogenes* in Mouse Hepatocytes

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**The uptake of *Listeria monocytogenes* by a variety of cell types in vitro is facilitated by the protein products of the *inlAB* (internalin) operon expressed by the organism. In the case of mouse hepatocytes, the extent to which *inlAB* expression influenced the uptake of *Listeria* in vitro was markedly dependent upon the ratio of bacteria to cells. At a ratio of 100:1, greater than 40-fold fewer transposon-induced *inlAB* mutant listeriae entered hepatocytes compared to the isogenic wild-type control; the difference was only fourfold, however, in cultures inoculated at a 1:1 ratio. Similarly, the uptake of in-frame *inlB* or *inlAB* deletion mutants differed only fourfold from the uptake of wild-type or *inlA* mutant *Listeria* at a 1:1 multiplicity of infection. Mutations affecting *inlB* or *inlAB*, on the other hand, resulted in a marked decrease in the capacity of *Listeria* to proliferate within mouse hepatocytes in vivo and in vitro. Electron micrographs of *Listeria*-infected hepatocytes demonstrated the impaired capacity of *inlB* mutants to escape from endocytic vacuoles and to enter the cytoplasm where proliferation occurs. These findings indicate that the protein product of *inlB* exerts a significant effect on the intracellular replication of *Listeria*.**

*Listeria monocytogenes* is a gram-positive bacterium capable of replicating intracellularly and causing severe, sometimes fatal infections in humans (14, 21). Listeriosis in mice is an experimental model used widely to study infections by intracellular pathogens and the factors that effect host resistance (15). Virulence of the organism is dependent upon a cluster of six genes: *hly*, *plcA*, *plcB*, *actA*, *mpl*, and *prfA* (18). The products of these genes mediate escape from phagolysosomes and the intracellular and intercellular movement of *Listeria*. In addition to these virulence factors, two cell-wall-associated proteins (internalin A [InlA]) and internalin B [InlB]) encoded by genes located within the *inlAB* (internalin) operon facilitate the uptake of *Listeria* by nonprofessional phagocytes. *Listeria* mutants unable to produce functional InlA and InlB exhibit a reduced capacity to infect a variety of cell types in vitro (3, 5, 13, 16). Recently, several new members of the internalin multigene family were identified and characterized: *irpA* (also called *inlC*), *inlC2*, *inlD*, *inlE*, and *inlF* (2, 4). The protein products of these genes bear strong amino acid sequence homology to InlA and InlB. Surprisingly, in-frame deletion mutations affecting these genes had no demonstrable effect on the capacity of *Listeria* to infect a number of cell lines. *inlC* mutants did exhibit a decrease in virulence, leading others to suggest that InlC may play a role in the dissemination of infection rather than the uptake of *Listeria* by nonprofessional phagocytes (2).

Most listeriae injected intravenously (i.v.) into mice are cleared rapidly from the bloodstream and taken up in the liver, where the parenchymal cells, i.e., hepatocytes, serve as the principal site of intracellular replication (8, 9, 12, 19). Recently, we reported that expression of the internalin operon and production of InlB were required for maximum entry of *L. monocytogenes* into primary cultures of mouse hepatocytes (10). *inlAB* expression, however, was not required for uptake of *Listeria* by hepatic cells in vivo. Rather, wild-type and *inlAB*

mutant strains of *Listeria* exhibited equivalent capacities to infect the parenchymal and nonparenchymal cells of the liver. While expression of the *inlAB* operon does not affect the uptake of *L. monocytogenes* by hepatic cells in vivo, we report here that *inlB* expression and the production of InlB promote escape from endocytic vacuoles and replication of *Listeria* within the cytoplasm of mouse hepatocytes.

### MATERIALS AND METHODS

**Bacteria.** Stock cultures of the following strains of *L. monocytogenes* (obtained from Pascale Cossart, Institut Pasteur, Paris, France) were prepared, stored, and used in accordance with methods previously reported (3, 10): BUG5, an isogenic transposon Tn1545-induced internalin (*inlAB*) mutant; BUG947, BUG1047, and BUG949, in-frame deletion mutants of *inlA*, *inlB*, and *inlAB*, respectively; and EGD-Sm<sup>r</sup> and BUG600, wild-type controls. Bacteria derived from broth cultures growing exponentially were used in the experiments described.

**Mice.** Specific-pathogen-free female C57BL/6J mice, purchased from the Jackson Laboratories (Bar Harbor, Maine), were cared for in accordance with the guidelines set forth by the Institute of Laboratory Animals Resources, National Research Council. Mice 8 to 12 weeks of age were used in the experiments reported. The number of listeriae recovered in the livers of infected animals was estimated from the colonies that grew on Trypticase soy agar plates inoculated with an aliquot of organ homogenate (23).

**Hepatocyte culture.** Purified hepatocytes were obtained from the livers of mice following perfusion with collagenase by the two-step method we reported previously (10). Purified hepatocytes were cultured in microtiter plates ( $2 \times 10^4$  cells/well) containing HEPES-buffered RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (Sterile Systems, Inc., Logan, Utah), and  $10^{-7}$  M recombinant human insulin (Humulin R; Eli Lilly Co., Indianapolis, Ind.).

The infection and replication of *Listeria* within hepatocytes in vitro were assessed by methods also reported previously (5, 10, 11). The number of intracellular listeriae that survive culture for  $\geq 1$  h in the presence of 5  $\mu$ g of gentamicin per ml (final concentration) was determined by lysing infected hepatocytes with 0.05% Triton X-100. Aliquots of the cell lysate were transferred to Trypticase soy agar plates, and viable listeriae were calculated from the number of colonies that grew.

**Electron microscopy.** Mouse hepatocytes ( $10^6$ ) were seeded into 10-cm tissue culture dishes, and the cells were incubated overnight. On the following day, the plates were inoculated with  $10^7$  listeriae and incubated for an additional 4 h. The cell monolayers were then washed four times with Hanks' balanced salt solution, and the cells were fixed in situ with 2.0% paraformaldehyde–2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The fixed cells were scraped into suspension, postfixed with 1% osmium tetroxide, and embedded in epoxy resin. Thin sections were cut, stained with lead citrate and uranyl acetate, and examined by electron microscopy (Philips Model 201).

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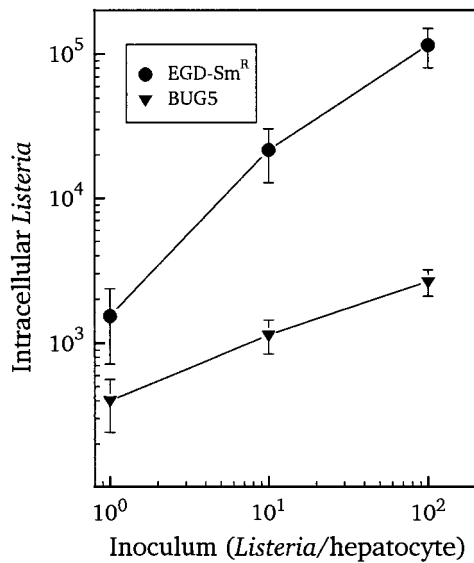


FIG. 1. The role of internalin in the uptake of *Listeria* by cultured mouse hepatocytes is diminished at lower bacterium-to-cell ratios. Cultures of mouse hepatocytes were inoculated with the wild-type (EGD-Sm<sup>R</sup>) or *inlAB* mutant (BUG5) strain of *Listeria*, centrifuged, and incubated for 1 h; gentamicin was added, and the cells were incubated for 1 additional h. Data are the means  $\pm$  standard deviations of numbers of viable intracellular listeriae in quadruplicate wells obtained in a single experiment; a second experiment yielded comparable results. Bacterial concentration exerted a significant effect on the role of *inlAB* expression in the uptake of *Listeria* by hepatocytes ( $P < 0.001$  [two-way analysis of variance]).

**Statistical analysis.** The results were analyzed by the SigmaStat statistics program (Jandel Scientific, San Rafael, Calif.).

## RESULTS

**Concentration-dependent effect of internalin on the uptake of *Listeria* by mouse hepatocytes.** Expression of the *inlAB* operon and the production of InlA and InlB affect the capacity of *Listeria* to enter a variety of cell types in vitro (3, 5, 10, 13, 16). Indeed, in agreement with results reported previously (10), primary mouse hepatocyte cultures inoculated at a high (100:1 ratio of bacteria to cells) multiplicity of infection (MOI) internalized >40-fold more wild-type (EGD-Sm<sup>R</sup>) than *inlAB* mutant (BUG5) listeriae (Fig. 1). At a lower MOI, however, internalin exerted far less of an effect on the uptake of *Listeria*. The capacities of the EGD-Sm<sup>R</sup> and BUG5 strains to infect hepatocytes differed only fourfold when cell cultures were inoculated with bacteria at a 1:1 ratio.

***inlAB* promotes the proliferation of *Listeria* in the liver.** The parenchymal cells serve as the principal site of listerial replication in the livers of mice. At  $\geq 6$  h postinfection i.v., essentially all of the listeriae recovered in the liver are located within the hepatocyte population (8, 9). As we reported previously (10), *inlAB* expression did not influence the recovery of *Listeria* in the liver at 6 h postinfection; the numbers of wild-type (EGD-Sm<sup>R</sup>) and *inlAB* mutant (BUG5) organisms recovered were virtually identical (Fig. 2). This finding is compatible with the diminished role of internalin in mediating the uptake of *Listeria* by hepatocytes at low MOI demonstrated above and the relatively low ratio of bacteria inoculated in this particular experiment ( $\approx 10^3$ ) to hepatocytes in the liver (estimated at  $10^8$ ). At 72 h postinfection, however, 1.1 log<sub>10</sub> fewer organisms were recovered in the livers of mice infected with BUG5. This latter finding demonstrates the decreased capacity of the *inlAB*

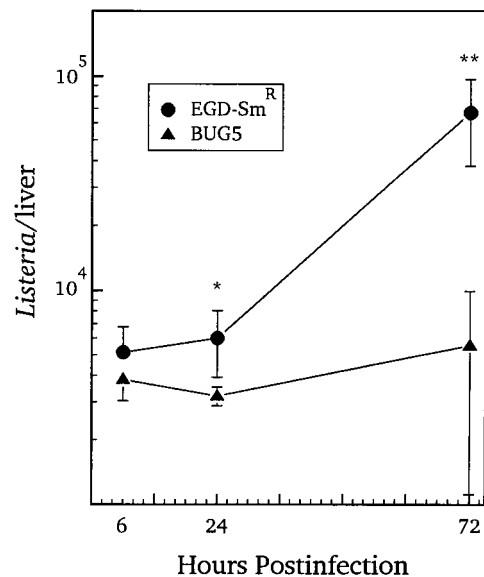


FIG. 2. *inlAB* expression promotes the replication of *Listeria* in vivo. Mice were infected i.v. with  $4.7 \log_{10}$  cells of BUG5 or  $4.8 \log_{10}$  cells of EGD-Sm<sup>R</sup>. Data are the means  $\pm$  standard deviations of the numbers of listeriae per liver obtained for four mice in a single experiment; comparable results were obtained in two similar experiments. Livers obtained from mice at 24 and 72 h postinfection with the EGD-Sm<sup>R</sup> strain of *Listeria* contained significantly more bacteria: \*,  $P = 0.02$ ; \*\*,  $P < 0.001$  (nonpaired Student's *t* test).

mutant strain of *Listeria* to proliferate in the liver, presumably within the hepatocyte population.

**InlB promotes the proliferation of *Listeria* within hepatocytes.** The diminished ability of BUG5 to proliferate within murine hepatocytes is documented by the in vitro experiment shown in Fig. 3. During a 10-h interval, the number of *inlAB*

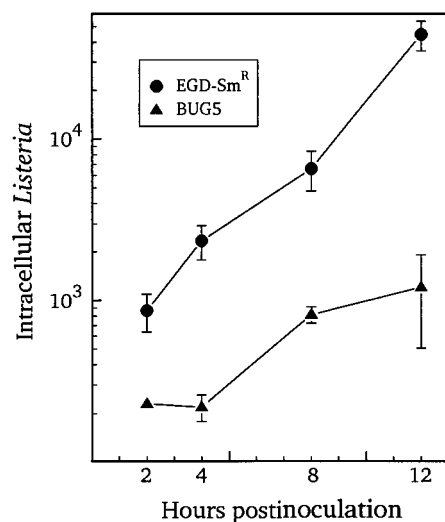


FIG. 3. *inlAB* expression promotes the replication of *Listeria* within mouse hepatocytes in vitro. Primary cultures of mouse hepatocytes were inoculated with the wild-type (EGD-Sm<sup>R</sup>) or *inlAB* mutant (BUG5) strain of *Listeria* (1:1 ratio of bacteria to cells), centrifuged, and incubated for 1 h prior to the addition of gentamicin. Values are the means  $\pm$  standard deviations of numbers of viable intracellular listeriae in quadruplicate wells at the times indicated. Data are derived from a single experiment; comparable results were obtained in two additional experiments. The EGD-Sm<sup>R</sup> strain grew intracellularly at a significantly greater rate ( $P < 0.001$  [two-way analysis of variance]).

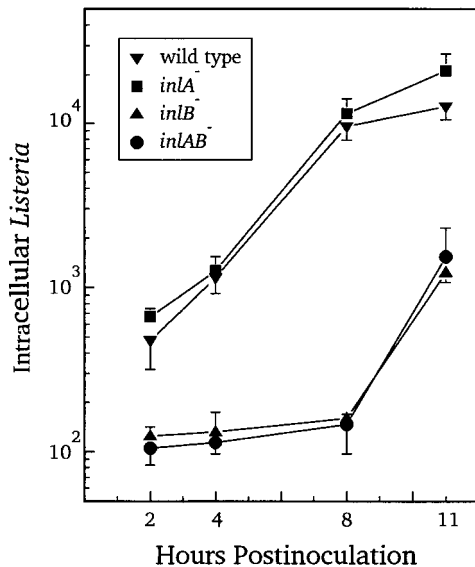


FIG. 4. *inIB* expression promotes the replication of *Listeria* in primary cultures of mouse hepatocytes. Overnight cultures of mouse hepatocytes were inoculated with wild-type (BUG600) and *inLA* (BUG1047), *inLB* (BUG947), and *inLAB* (BUG949) mutant strains of *Listeria* (1:1 ratio of bacteria to cells); centrifuged; and incubated for 1 h prior to the addition of gentamicin. Values are the means  $\pm$  standard deviations of numbers of viable intracellular listeriae in quadruplicate wells at the times indicated. Data are derived from a single experiment; comparable results were obtained in a second experiment. Both BUG600 and BUG1047 grew significantly better within hepatocytes than either BUG947 or BUG949 ( $P < 0.001$  [two-way analysis of variance]).

mutant listeriae increased only sixfold. The number of wild-type listeriae, on the other hand, increased approximately 50-fold within the same time frame. Similar findings were obtained in comparing the replication of mutants expressing in-frame deletions in *inLB* (BUG1047) or *inLAB* (BUG949), but not *inLA* (BUG947), to that of wild-type *Listeria* (BUG600). Between 2 and 8 h postinoculation, the numbers of intracellular BUG947 and BUG600 cells increased approximately 20-fold while the numbers of BUG1047 and BUG949 cells were unchanged (Fig. 4). BUG1047 and BUG949 began to replicate after 8 h; growth of BUG947 and BUG600, however, had already reached a plateau. The diminished capacity of BUG1047, relative to BUG947, to replicate within hepatocytes in vitro reflected the capacity of these organisms to replicate in vivo. While comparable numbers were recovered in the livers of mice at 6 h postinoculation i.v., the *inLA* mutant exhibited an approximate 1 log<sub>10</sub> increase during the subsequent 42-h period (Fig. 5). The *inLB* mutant, on the other hand, failed to proliferate during the same time interval.

#### InIB facilitates escape of *Listeria* from endocytic vacuoles.

An examination of thin sections derived from primary mouse hepatocyte cultures infected with wild-type *Listeria* revealed bacteria located free within the cytoplasm, i.e., unrestricted by a lipid membrane (Fig. 6A). These organisms were surrounded by a halo characterized by others as polymerized actin (22). In contrast, bacteria contained within membrane limiting vacuoles were evident in sections prepared from hepatocyte cultures infected with *inLB* mutant *Listeria* under comparable conditions (Fig. 6B). These findings demonstrate the impaired capacity of *inLB* mutants to escape from endocytic vacuoles and to enter the cytoplasm where proliferation occurs.

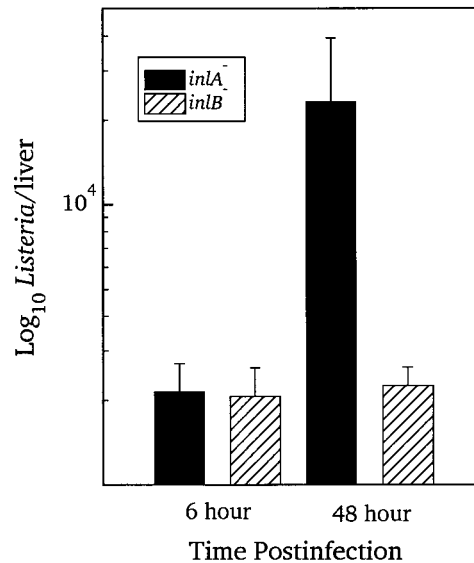


FIG. 5. An in-frame deletion in *inIB* diminishes the capacity of *Listeria* to replicate in vivo. Mice were infected i.v. with 4.1 log<sub>10</sub> cells of BUG947 (*inLB* mutant) or BUG1047 (*inLA* mutant). Data are the means  $\pm$  standard deviations of numbers of listeriae per liver obtained for four mice in a single experiment; comparable results were obtained in two additional experiments. Livers dissected from mice at 48 h postinfection with the *inLB* mutant strain of *Listeria* contained significantly fewer bacteria:  $P = 0.029$  (Mann-Whitney rank sum test).

## DISCUSSION

The cell-wall-associated protein products of the *inLAB* operon facilitate the entrance of *Listeria* into a variety of cell types in vitro (3, 5, 10, 13, 16). In the case of mouse hepatocyte cultures, the role of internalin was influenced by the concentration of bacteria added and was diminished markedly at lower bacteria-to-hepatocyte ratios. Thus, at a 1:1 ratio, wild-type and *inLAB* mutant strains of *Listeria* differed only fourfold in their capacities to infect hepatocytes; in the case of the mutants, e.g., BUG5,  $\approx 0.5\%$  of the inoculum penetrated the cells during a 1-h incubation period. This latter finding demonstrates the ability of *Listeria* to enter mouse hepatocytes in the absence of internalin and supports the proposed existence of internalin-independent pathways for the uptake of *Listeria* by eukaryotic cells (1, 3, 5, 6, 13).

Rather than affecting entry, *inLAB* expression appeared to exert its greatest effect on the replication of *Listeria* within hepatocytes both in vivo and in vitro. Relative to the wild-type control (EGD-Sm<sup>r</sup>), proliferation of the transposon-induced *inLAB* mutant (BUG5) in primary cultures of mouse hepatocytes was reduced substantially. Similar findings were obtained with a mutant (BUG949) that expressed an in-frame deletion in *inLAB*. This negates the possibility existing in the case of BUG5 that the reduced capacity to proliferate was due to a polar mutation affecting some gene product other than internalin. Experiments comparing the *inLB* and *inLA* mutant strains of *Listeria* demonstrate the positive influence of InIB, but not InLA, on listerial replication in mouse hepatocytes. This correlates with the specific role of InIB in mediating the uptake of *Listeria* by mouse hepatocytes in cultures inoculated at a high MOI (10).

Our results demonstrating the decreased capacities of *inLAB* and *inLB* mutants to replicate within mouse hepatocytes contradict a previous study reporting the comparable growth rates of *inLAB* mutant and wild-type *Listeria* cells in a purported mouse hepatocyte cell line, TIB73 (characterized originally in



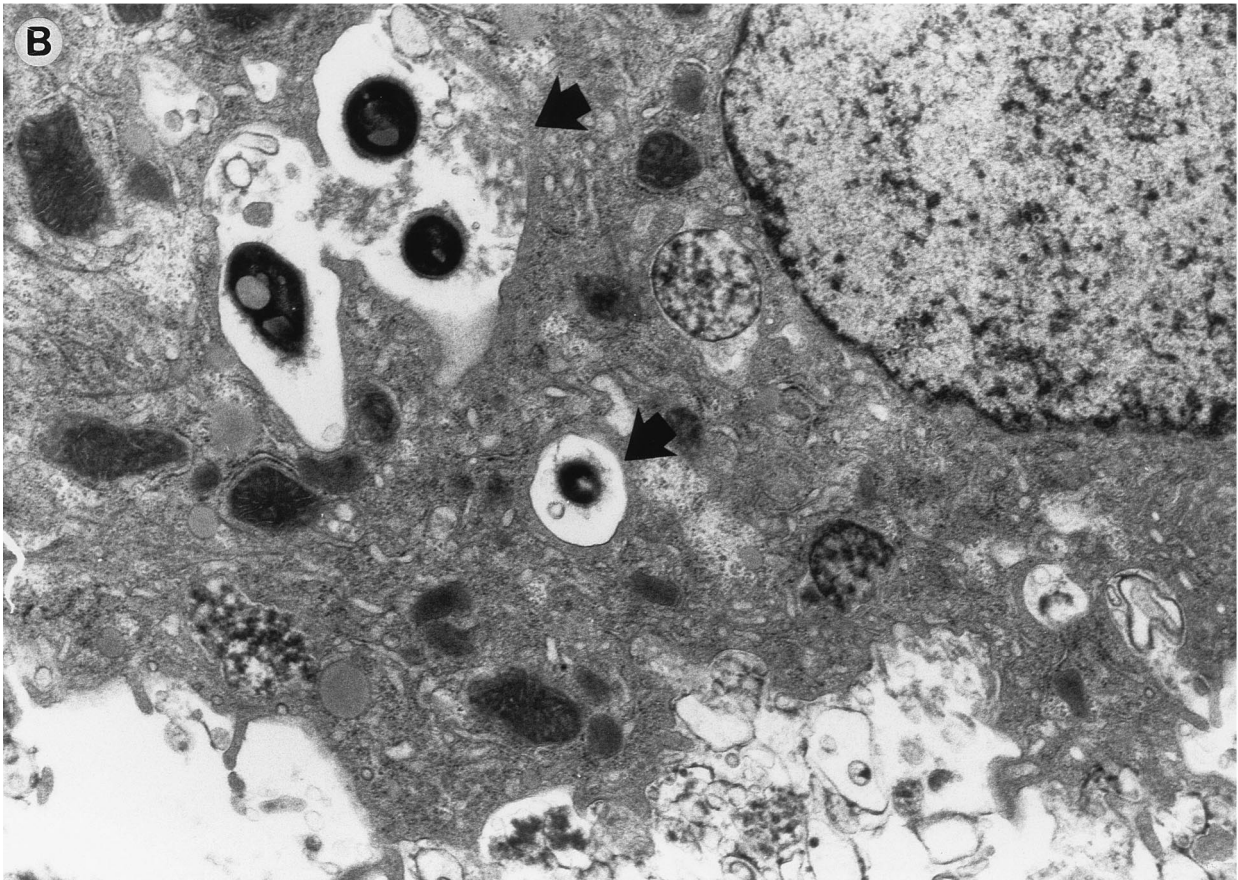
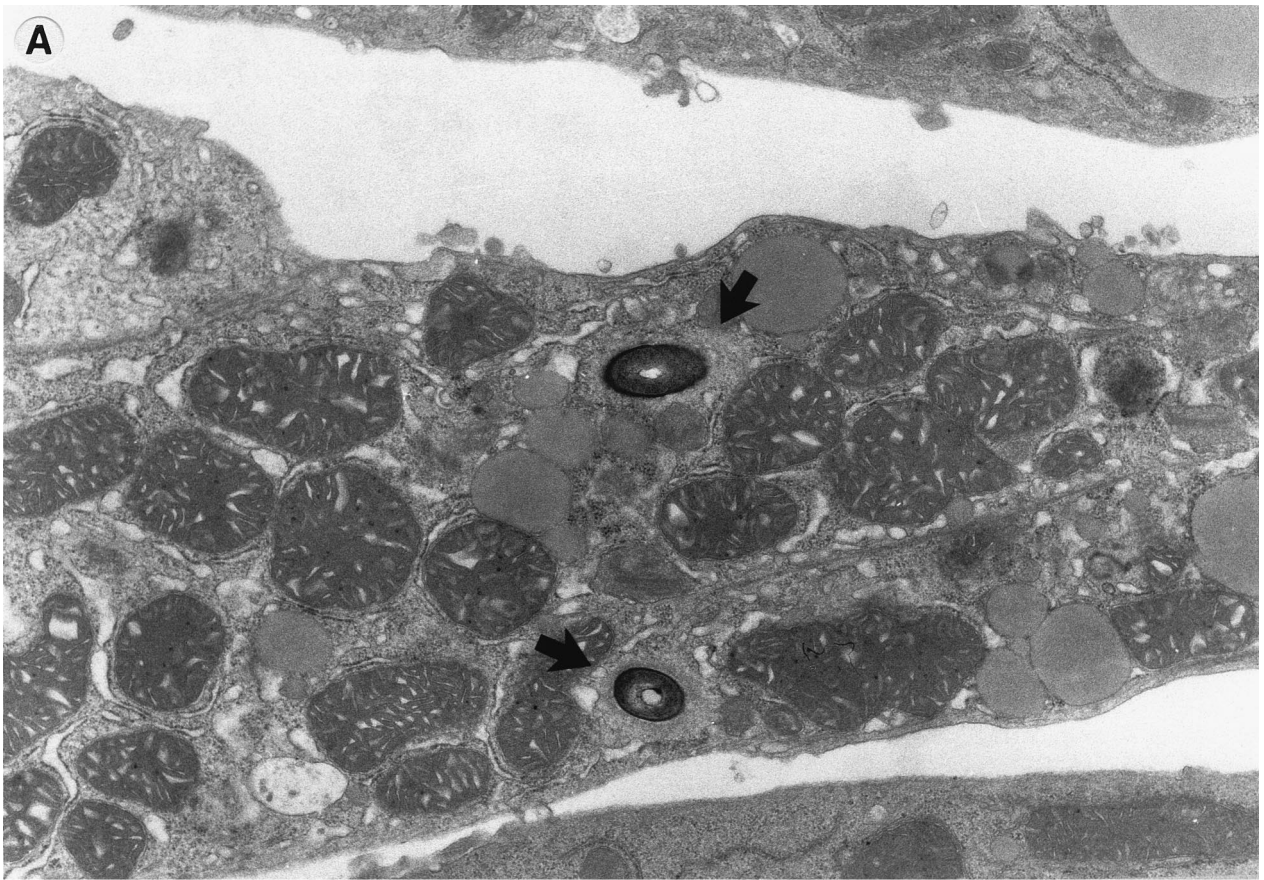




FIG. 6. Electron micrographs of *Listeria*-infected hepatocytes. Primary mouse hepatocyte cultures were inoculated with wild-type (BUG600 [A]) or *inIB* mutant (BUG947 [B]) *Listeria* (10:1 ratio of bacteria to cells). Following 4 h of incubation, the cell monolayer was washed, fixed, and processed for electron microscopy. Original magnification,  $\times 17,000$ ; arrows denote intracellular listeriae.

the literature as a mouse fibroblast cell line derived from embryonic liver [17]) (7). The uncertain relationship of the TIB73 cell line to authentic mouse hepatocytes could readily account for the disparity in our findings. Our results do agree, however, with a recent report demonstrating the role of internalin in promoting the proliferation of *Listeria* within a macrophage cell line (20). In the latter case, the number of wild-type listeriae in J774A.1 macrophages increased approximately 2.5  $\log_{10}$  during a 4-h incubation period; an *inLAB* mutant, on the other hand, failed to proliferate intracellularly.

A number of studies in addition to the one reported here have demonstrated the decreased capacity of internalin mutants to replicate *in vivo* (3, 4, 7, 16, 20). Gaillard et al., for example, reported 1 to 2  $\log_{10}$  fewer organisms recovered in the livers of mice on day 2 postinfection i.v. with *inLAB* mutant, relative to wild-type control, listeriae (7). This difference is most often attributed to a reduction in the number of mutants that enter permissive cells, e.g., the parenchymal cells of the liver (hepatocytes), where they can proliferate (3, 7). Alternatively, it has been suggested that the protein products of the internalin genes may promote the replication and/or dissemination of *Listeria* *in vivo* (2, 20). In this regard, it was reported recently that *irpA* mutants exhibited a consistent decrease in pathogenicity although the expression of *irpA* (*inIC*) had no effect on the uptake of *Listeria* by cells in culture (2). These results, taken together with our own demonstrating the impaired capacity of *inIB* mutants to escape from endocytic vacuoles, suggest that the protein products of the internalin operon may exert their greatest effect on the proliferation of intracellular organisms.

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