

Listeria ivanovii Is Capable of Cell-to-Cell Spread Involving Actin Polymerization

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Listeria ivanovii has been considered to be pathogenic to animals but has rarely been found associated with human infections. It has been claimed that *L. ivanovii* lacks the *actA* gene, which in *L. monocytogenes* encodes a protein required for interaction with host cell actin. Using fluorescence microscopy and electron microscopy, we demonstrate that *L. ivanovii* can invade mammalian cells, lyse the phagosomal membrane, polymerize host cell actin, reorganize actin to form tails, and spread from cell to cell. However, no DNA homologous to the *actA* gene could be detected by polymerase chain reaction. Further, *L. ivanovii* lacks the 90-kDa surface protein which in *L. monocytogenes* is encoded by *actA*. Despite the ability to spread from cell to cell, *L. ivanovii* differed significantly from *L. monocytogenes* in being unable to form plaques on monolayers of 3T3 fibroblast cells.

Apart from *Listeria monocytogenes*, *L. ivanovii* is the only other *Listeria* sp. which is considered to be pathogenic. However, although *L. ivanovii* has been reported to infect animals, causing abortions, neonatal sepsis, and enteritis (5, 22, 23), human infections are very rare. This organism has also been isolated from healthy animals, human carriers, and the environment (22, 23).

Recently, development of tissue culture models of infection and molecular genetic approaches have led to significant advances in the knowledge of the mechanism of virulence in *L. monocytogenes*. This organism is capable of invading a number of cell systems, and intracellular events have been studied by using the macrophage-like cell line J774, bone marrow derived macrophages, peritoneal macrophages and the enterocyte-like cell line Caco-2 (1, 16, 19, 21). Following internalization, *L. monocytogenes* escapes from the host vacuole and enters the cytoplasm, where it multiplies rapidly. The ability of *L. monocytogenes* to lyse the phagolysosomal membrane depends on the secretion of a hemolytic factor termed listeriolysin O (LLO) (3, 6). Following entry into the cytoplasm, *L. monocytogenes* becomes surrounded by cytoplasmic actin filaments (19, 25, 26), which rearrange to form a tail; this is associated with bacterial movement to the peripheral membrane and subsequent spread to neighboring cells (7, 26). A 90-kDa surface protein encoded by *actA* has been reported to be required for actin assembly in *L. monocytogenes*, and this gene has been reported to be absent in *L. ivanovii* (8, 13, 28). Although *L. ivanovii* has also been reported to be able to invade Caco-2 cells (10), further intracellular events have not been reported so far. *L. ivanovii* produces strong hemolysis on sheep blood agar, in contrast to the weak hemolysis produced by *L. monocytogenes*. The hemolysins of *L. ivanovii* have been purified (15, 27). An SH-activated hemolysin genetically (12), biochemically, and serologically related to LLO is produced by this organism. In *L. monocytogenes*, this hemolysin is an important virulence factor which enables the organism to escape from the phagosomal membrane following internalization by mammalian cells (6, 11). In addition, a 27-kDa hemolytic sphingomyelinase C produced by *L. ivanovii*, which appears

(11, 14, 27) to be responsible for the halo of incomplete hemolysis synergistically enhanced by *Rhodococcus equi* exosubstances (CAMP factor), has been identified. To understand the differences in the virulence of *L. monocytogenes* and *L. ivanovii*, we undertook a study of intracellular behavior of the latter microorganism.

MATERIALS AND METHODS

Bacterial strains. *L. ivanovii* ATCC 19119 (SLCC 2379), *L. monocytogenes* NCTC 7973, and *L. monocytogenes* EGD (provided by S. H. E. Kaufmann) were the wild-type strains used. Two transposon (Tn1545)-generated mutants of *L. ivanovii*, designated 44/2 and 8/6 and described by Kreft et al. (14), were used. Mutant 44/2 was negative for sphingomyelinase and a 27-kDa protein. Mutant 8/6 was negative for LLO, the 27-kDa protein, and lecithinase but showed sphingomyelinase activity and the CAMP reaction with *R. equi*. *L. monocytogenes* SLCC53 was a spontaneous mutant of strain NCTC 7973 harboring a deletion in its *prfA* gene, which regulates the production of listeriolysin (18). An ActA mutant described by Domann et al. (8) was also used.

Mammalian cell cultures. The human colon carcinoma cell line Caco-2 (ECACC 86010202) was cultured in minimal essential medium (GIBCO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a 5% CO₂ incubator. Mouse fibroblast cell line 3T3 (ECACC 88031146) and mouse macrophage-like cell line J774 (ATCC TIB 67) were cultured in RPMI medium (GIBCO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a 5% CO₂ incubator.

Infection of cells. Essentially the method described by Gaillard et al. (10) was used for cell infection. At 24 h before infection, Caco-2 or 3T3 cells were trypsinized and the cell concentration was adjusted to 2×10^5 /ml. A 1-ml portion of cell suspension in medium without penicillin and streptomycin was seeded out in each well of a 24-well tissue culture plate. Bacteria were grown in brain heart infusion broth for 18 h at 37°C under aeration. Bacterial cultures were centrifuged, washed in phosphate-buffered saline (PBS), and resuspended in the respective tissue culture media without

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supplements to obtain a concentration of 2×10^5 bacteria per ml. Host cells were washed with PBS containing calcium and magnesium [PBS(Ca,Mg)]. The bacterial suspensions (1 ml) were added to each well in tissue culture plates, and the plates were centrifuged at $500 \times g$ for 10 min at room temperature and incubated at 37°C . At 1 h (this was considered the invasion period), the cells were washed three times with PBS(Ca,Mg), and 1 ml of complete medium without penicillin and streptomycin but containing gentamicin (10 $\mu\text{g/ml}$) was added to each well. At this concentration of gentamicin, extracellular bacteria would be killed but intracellular bacteria would not be affected. To study invasion, 1 h after the addition of gentamicin the cells were washed three times with PBS(Ca,Mg) and lysed by addition of 1 ml of distilled water; 10-fold dilutions were plated on brain heart infusion agar plates. To study intracellular multiplication, the same procedure was followed and platings were done at different time intervals after the addition of gentamicin. Each experiment was done in duplicate and performed three times.

Fluorescence staining of F-actin, actin-binding proteins, and bacteria. Caco-2 cells were seeded out on coverslips in tissue culture plates and infected as described above. At different time intervals after the addition of gentamicin, cells were washed with PBS(Ca,Mg) and permeabilized as described by Mounier et al. (19). Cells were fixed in acetone at -20°C , dried, and labeled for indirect immunofluorescence. To observe *Listeria* cells, 1/100 dilutions of antisera raised in rabbits against both LLO and p60 were used and revealed by incubation in a 1/40 dilution of goat anti-rabbit rhodamine-conjugated immunoglobulin G (Sigma). For staining of F-actin, coverslips were incubated in a 10-U/ml solution of fluorescein isothiocyanate-labeled phalloidin (Sigma). For locating actin-binding proteins, monoclonal antibodies against the following were used: α -actinin (BM-75.2; Sigma) filamin (FIL-2; Sigma), vinculin (VIN 11-5; Sigma), and myosin (2F12.A9; Immunotech); they were revealed by TRITC-conjugated goat antibody against mouse immunoglobulin G (Sigma).

Electron microscopy. Caco-2 cells were seeded out on coverslips and infected as described above. At different time intervals, coverslips were washed with PBS(Ca,Mg), fixed in 2.5% buffered glutaraldehyde (50 mM cacodylate [pH 7.2], 50 mM KCl, 2.5 mM MgCl_2) for 30 min at 4°C , washed with cacodylate buffer, postfixed for 1 h at 4°C with 2% buffered osmium tetroxide (50 mM cacodylate [pH 7.2]), and washed with water. Cells were then dehydrated and embedded in Epon 812 (Serva, Heidelberg, Germany). Serial ultrathin sections were stained with uranyl acetate and lead citrate. Photographs were taken with a Zeiss EM 900 electron microscope at 80 kV.

Plaque assay. The ability of *Listeria* spp. to form plaques on 3T3 cells was tested by using the technique described by Kuhn et al. (17). A 2-ml volume of 3T3 cells ($2 \times 10^5/\text{ml}$) was seeded out into 12-well tissue culture plates and infected as described above. After a 2-h infection, an agarose overlay containing 0.5% agarose in RPMI medium, 20% fetal calf serum, 2 mM L-glutamine, and 5 μg of gentamicin per ml was added to each well and the plates were incubated at 37°C for 24 h. A second overlay containing 0.5% agarose in RPMI, 0.01% neutral red, and 40 μg of gentamicin per ml was added, and the plates were observed for plaques after an additional incubation at 37°C for 24 h.

Extraction of surface proteins. *Listeria* strains grown in brain heart infusion broth for 18 h at 37°C with shaking were diluted 1:10 with fresh brain heart infusion broth and incu-

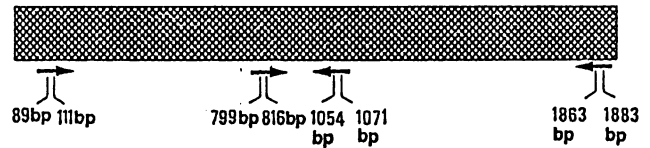


FIG. 1. Schematic diagram of the *actA* gene, showing the binding sites of PCR primers.

bated under the same conditions for 3 h to obtain cells in logarithmic phase. These cells were washed in PBS and resuspended and incubated for 1.5 h in a minimal medium which allows preferential synthesis of *prfA*-controlled gene products (23a). The bacterial cells were centrifuged and washed in PBS, and surface proteins were labeled with Sulfo NHS Biotin (Pierce) and extracted by using sodium dodecyl sulfate (SDS) as described by Kocks et al. (13). Proteins were separated on SDS-7.5% polyacrylamide slab gels and transferred to nitrocellulose filters. After overnight saturation with PBS containing 0.5% gelatin, the filters were incubated for 1.5 h with peroxidase-conjugated streptavidin (Pierce) in PBS containing 0.5% gelatin and 0.1% Tween 20. After being washed in the same buffer, the filters were developed with 0.5 mg of 4-chloro-1-naphthol per ml and 0.03% H_2O_2 in Tris HCl buffer (pH 7.6).

PCR amplification of *actA* gene fragments. The following polymerase chain reaction (PCR) primers (Fig. 1) were used to amplify *actA* gene fragments in *L. monocytogenes* and to look for similar signals in *L. ivanovii*: 5'-CCACCA CCTACGGATGA-3' (positions 799 to 816), 5'-ACTAGA ATCTAGCGAGG-3' (positions 1054 to 1071), 5'-GACAGA TAGCGAAGATTCTAGTC-3' (positions 89 to 111), and 5'-CGCCCCTAAAGAGAACACGC-3' (positions 1863 to 1883) derived from the published sequence of *actA* (8, 27). A loopful of test culture was suspended in 100 μl of distilled water and lysed by heating at 110°C for 5 min. Thermal cycling consisted of denaturation for 1 min at 95°C , annealing of primers for 1 min at 52 or 45°C , and primer extension for 1 min at 72°C (30 cycles).

RESULTS

Invasion and intracellular growth of wild-type and mutant *L. ivanovii*. *L. ivanovii* was able to invade both Caco-2 and 3T3 cells (Table 1). Interestingly, the efficiency of invasion was greater than that of *L. monocytogenes* EGD, and this was evident particularly in 3T3 cells. It should, however, be pointed out that both *Listeria* species were able to invade Caco-2 cells more efficiently than they invaded 3T3 cells.

Both the listeriolysin-negative mutant (mutant 8/6) and the sphingomyelinase-negative mutant (mutant 44/2) were able to invade Caco-2 cells (Fig. 2). However, the former appeared to be slightly less efficient and was unable to multiply in the host cells. The latter mutant, on the other hand, multiplied in the host cells, and its growth curve was comparable to that of the wild type. Wild-type *L. ivanovii* was also capable of multiplication in the phagocytic macrophage-like cell line J774, and the rate of multiplication was similar to that of *L. monocytogenes* EGD (Fig. 3).

Intracellular behavior of *L. ivanovii*. After 1 h of infection of monolayers with *Listeria* spp., a bactericidal concentration of gentamicin was added to kill extracellular bacteria. This period was considered the invasion phase. At different time intervals after this period of invasion, observations

TABLE 1. Invasion of mammalian cell lines by *L. monocytogenes* and *L. ivanovii*

Bacterial strain	Host cell	No. of bacteria inoculated ^a	No. of intracellular bacteria at 1 h ^a	Percent invasion
<i>L. monocytogenes</i> EGD	Caco-2	$(3.1 \pm 0.3) \times 10^5$	$(6.0 \pm 0.8) \times 10^4$	19.35
<i>L. ivanovii</i>	Caco-2	$(4.4 \pm 0.3) \times 10^5$	$(2.1 \pm 0.1) \times 10^5$	47.73
<i>L. monocytogenes</i> EGD	3T3	$(1.5 \pm 0.3) \times 10^5$	$(5.3 \pm 0.2) \times 10^2$	0.35
<i>L. ivanovii</i>	3T3	$(9.8 \pm 0.2) \times 10^4$	$(9.7 \pm 0.5) \times 10^3$	9.90

^a Data are means and standard deviations for three experiments.

were made by both light and electron microscopy. *L. ivanovii* left the phagosomal membrane more slowly than *L. monocytogenes* did. At 30 min postinvasion, only 36% of *L. ivanovii* cells had lysed the phagosomal membrane while 89% of *L. monocytogenes* cells had already entered the cytoplasm. At 1 h postinvasion, few *L. ivanovii* cells were still found within the phagosomal membrane (Fig. 4a) but most of them were free in the cytoplasm (Fig. 4b) and a few of them already had polymerized actin around them (Fig. 4c). To estimate the percentage of bacteria with actin coats, bacteria labeled with anti-p60 and anti-listeriolysin antibodies were counted by indirect immunofluorescence and the presence of actin around these bacteria was checked by phalloidin staining. As shown in Table 2, at 1 h only about 14% of *L. ivanovii* cells had polymerized actin around them while 79% of *L. monocytogenes* EGD cells were surrounded by polymerized actin. These results were also confirmed by electron microscopy. At 2 h, 32% of *L. ivanovii* cells had actin coats (Table 2), and already some of the actin was rearranged into tails (Fig. 4d and 5a). Electron-microscopic observations indicate that at 2 h intracellular movement and cell-to-cell spread occurred. This is illustrated in Fig. 4e, which shows a bacterial cell projecting into the neighboring cell through a pseudopodium, and in Fig. 4f, in which a cross section of one such bacterium enclosed in a double membrane can be seen. The conclusion that cell-to-cell spread occurred is further supported by results of experiments shown in Fig. 5. Monolayers infected at a low multiplicity of infection (one bacterium per host cell) were stained with Giemsa at different time points. At 1 h (Fig. 5a), only a few bacteria could be seen per field and many cells were uninfected. At 4 h (Fig. 5b), multiplication had occurred and neighboring cells were infected. At 6 h (Fig. 5c), bacteria could be seen in almost all cells.

The sphingomyelinase-negative mutant 44/2 of *L. ivanovii* was also observed to multiply intracellularly, polymerize actin, and spread from cell to cell (data not shown). For the listeriolysin-negative mutant 8/6, intracellular multiplication was not apparent (Fig. 3) and most of the bacteria were trapped inside the phagosomal membrane (Fig. 4g). However, a few bacteria escaped from the phagosomal membrane and polymerized actin, which was rearranged into tails (Fig. 6d).

Involvement of actin-binding proteins. Indirect immunofluorescence with monoclonal antibodies against the various actin-binding proteins revealed that α -actinin was localized around bacteria that had polymerized actin (Fig. 6b) and that this protein was also associated with actin tails. These results were confirmed by immunogold labeling and electron microscopy (Fig. 6c). Other proteins such as tropomyosin, filamin, myosin, and vinculin were not found associated with bacteria.

Search for the *actA* gene and its product in *L. ivanovii*. Two sets of PCR primers were used to amplify *actA* gene fragments in *L. monocytogenes* and to look for similar signals in *L. ivanovii* (Fig. 1). Neither set of primers yielded amplification products with *L. ivanovii* chromosomal DNA under high-stringency or low-stringency conditions (data not shown). Since the *actA* gene product has been shown to be a 90-kDa surface protein, we tried SDS extraction of surface proteins of *L. monocytogenes* and *L. ivanovii*. We observed that the 90-kDa protein was missing in the *actA*-negative mutant of *L. monocytogenes* EGD and in *L. ivanovii* (data not shown). To further confirm the absence of protein encoded by *actA* in *L. monocytogenes*, surface proteins separated by SDS-gel electrophoresis were transferred onto nitrocellulose filters and Western immunoblot assays were performed by using antibodies raised against two synthetic peptides corresponding to amino acid residues 41 to 54 and

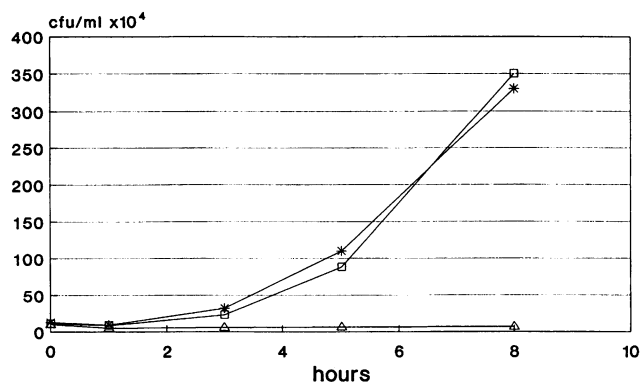


FIG. 2. Multiplication of *L. ivanovii* wild type (*) and mutants 44/2 (□) and 8/6 (Δ) in Caco-2 cells (values represent means of three experiments).

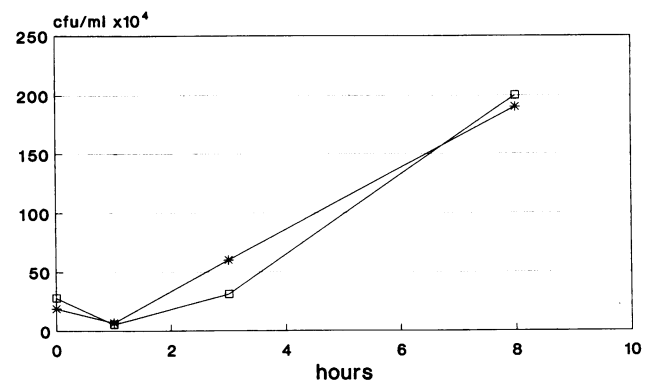


FIG. 3. Multiplication of *L. monocytogenes* (*) and *L. ivanovii* (□) in J774 cells (values represent means of three experiments).

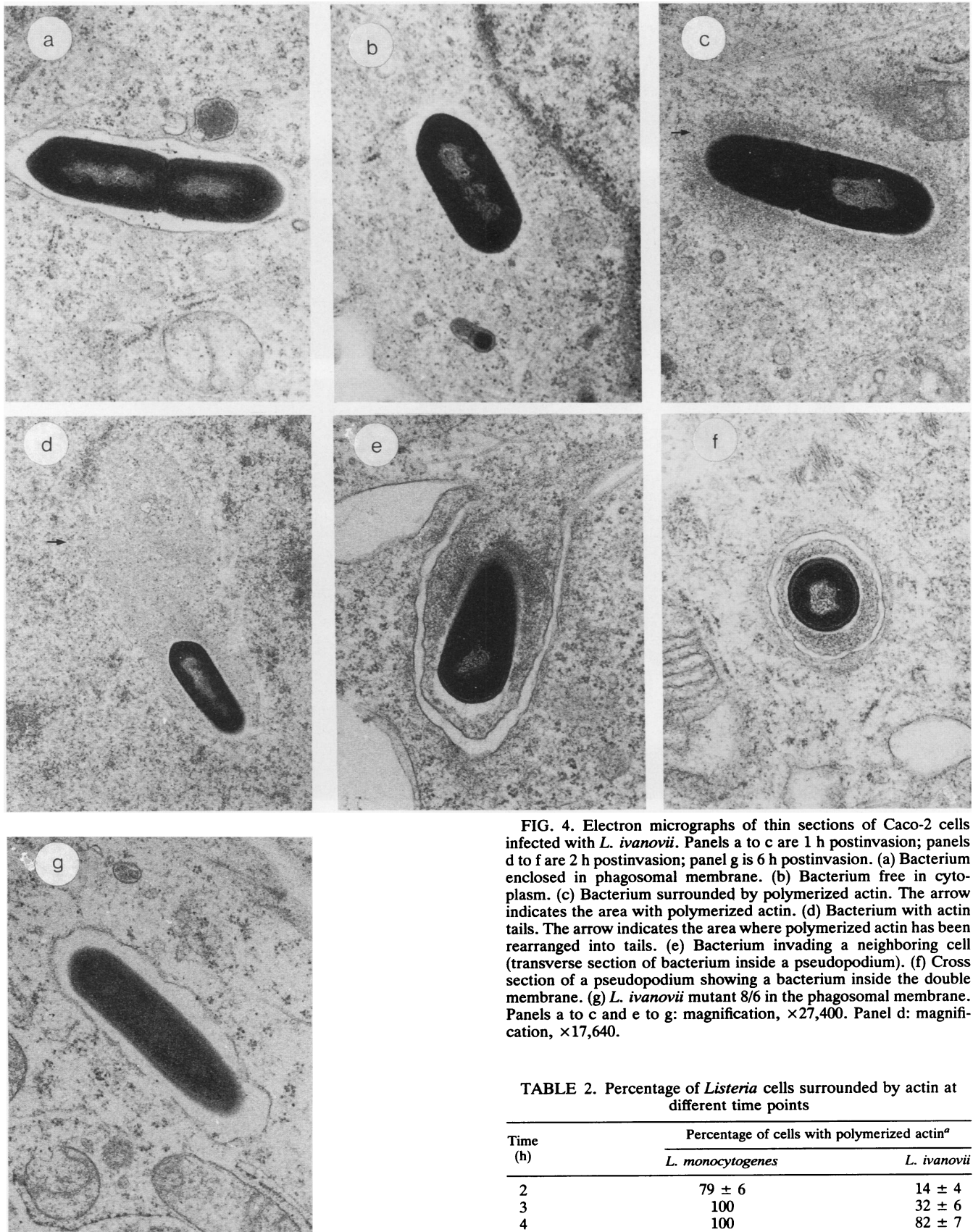


FIG. 4. Electron micrographs of thin sections of Caco-2 cells infected with *L. ivanovii*. Panels a to c are 1 h postinvasion; panels d to f are 2 h postinvasion; panel g is 6 h postinvasion. (a) Bacterium enclosed in phagosomal membrane. (b) Bacterium free in cytoplasm. (c) Bacterium surrounded by polymerized actin. The arrow indicates the area with polymerized actin. (d) Bacterium with actin tails. The arrow indicates the area where polymerized actin has been rearranged into tails. (e) Bacterium invading a neighboring cell (transverse section of bacterium inside a pseudopodium). (f) Cross section of a pseudopodium showing a bacterium inside the double membrane. (g) *L. ivanovii* mutant 8/6 in the phagosomal membrane. Panels a to c and e to g: magnification, $\times 27,400$. Panel d: magnification, $\times 17,640$.

TABLE 2. Percentage of *Listeria* cells surrounded by actin at different time points

Time (h)	Percentage of cells with polymerized actin ^a	
	<i>L. monocytogenes</i>	<i>L. ivanovii</i>
2	79 ± 6	14 ± 4
3	100	32 ± 6
4	100	82 ± 7

^a Data are means and standard errors for three experiments.

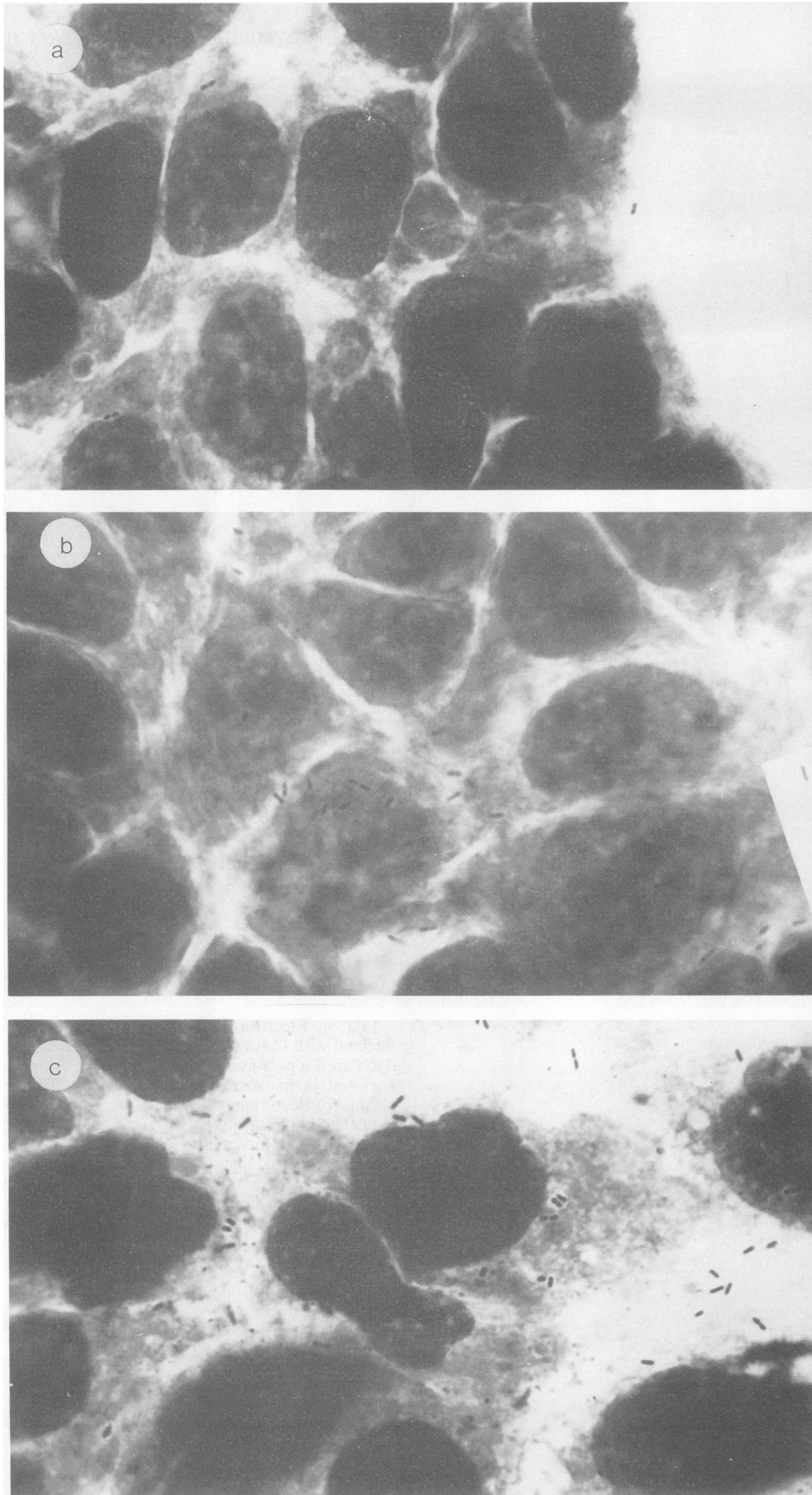


FIG. 5. *L. ivanovii*-infected Giemsa stained Caco-2 cells 1 h (a), 4 h (b), and 6 h (c) postinvasion. Magnification, $\times 1,500$.

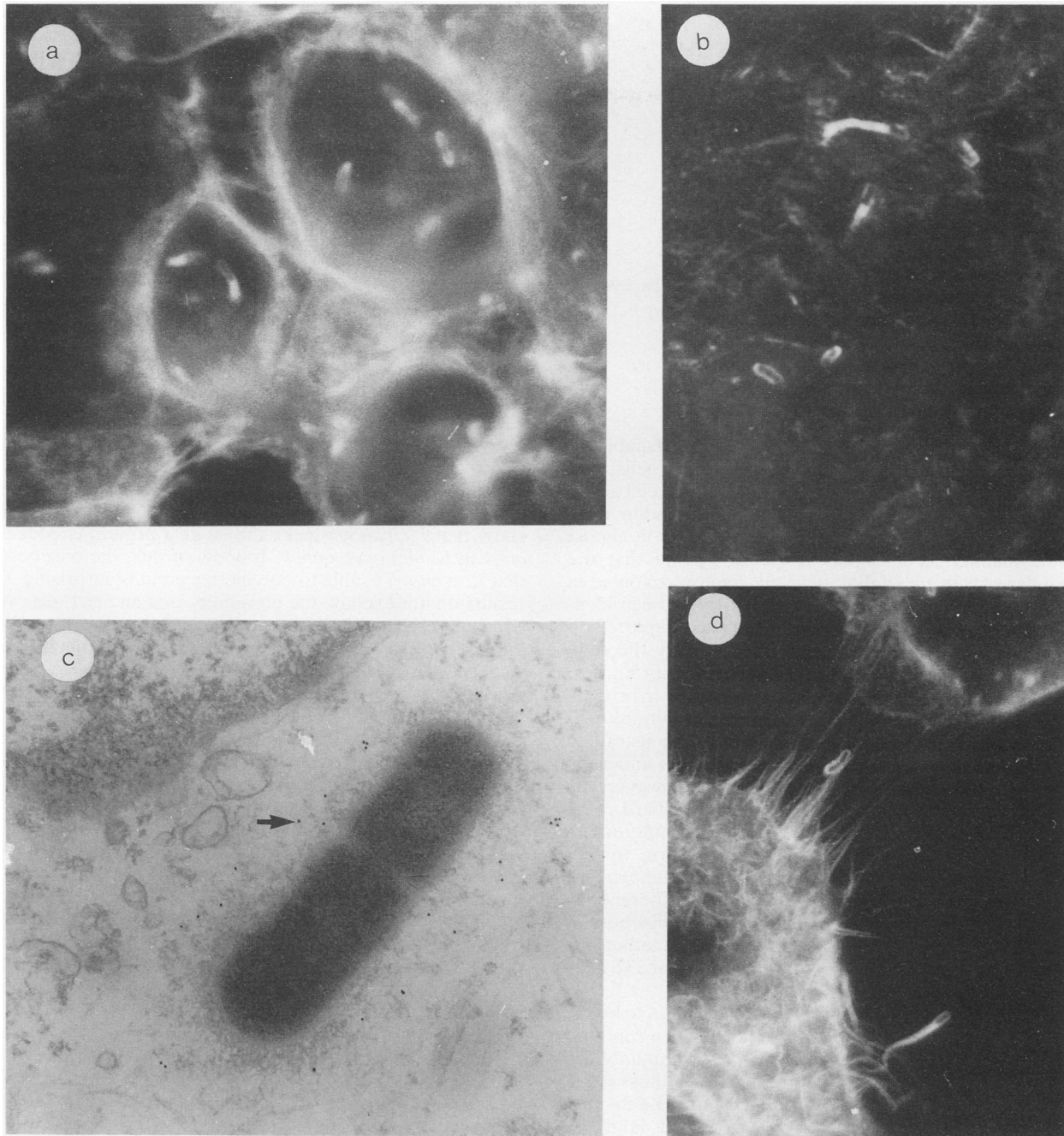


FIG. 6. *L. ivanovii*-infected Caco-2 cells 4 h postinvasion (panels a to c). (a) Stained with fluorescein isothiocyanate-labeled phalloidin showing bacteria with polymerized actin and tails. (b) Stained with monoclonal antibody to α -actinin and TRITC-labeled anti-mouse immunoglobulin G showing colocalization of α -actinin around bacteria and in actin tails. (c) Electron-microscopic picture of thin section stained with monoclonal antibody to α -actinin and immunogold labeled anti-mouse immunoglobulin G. The arrow indicates the area of gold localization. (d) *L. ivanovii* 8/6 mutant 6 h postinvasion stained with phalloidin. There are few cells with polymerized actin. Panels a, b, and d: magnification, $\times 2,000$. Panel c: magnification, $\times 44,800$.

85 to 97 of the ActA polypeptide. However, no reaction could be detected in *L. ivanovii* (data not shown).

Plaque formation by *Listeria* spp. *L. monocytogenes* strains are capable of forming plaques on monolayers of 3T3 fibroblast cells overlaid with agarose containing a concentration of gentamicin sufficient to kill extracellular bacteria. Plaques represent areas of host cells destroyed by bacterial infection and therefore reflect the ability of the bacteria to

multiply and spread from cell to cell. As shown in Fig. 7, *L. ivanovii* did not form plaques on monolayers of 3T3 cells. Microscopic observations indicated that at 48 h, when plaques were visible in *L. monocytogenes*-infected wells, numerous *L. ivanovii* cells were found within the fibroblasts but the host cells did not lyse (data not shown). Plaques with *L. ivanovii* were not observed even after extended incubation.

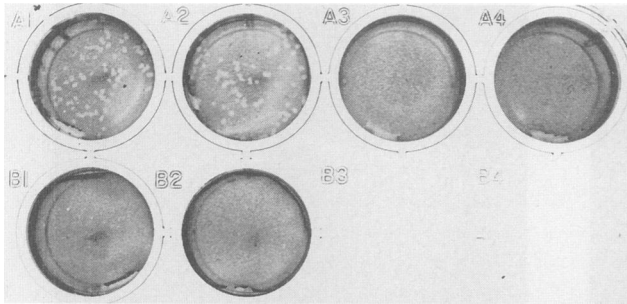


FIG. 7. Plaque assay with *Listeria* spp. A1, A2, *L. monocytogenes* EGD. A3, A4, *L. ivanovii*. B1, B2, uninfected cells.

DISCUSSION

The above results show that *L. ivanovii* is capable of invading nonprofessional phagocytic mammalian cells, lysing the phagosomal membrane, polymerizing host cell actin, rearranging the actin in the form of tails, and spreading from cell to cell. Up to this point, this species behaves in almost the same manner as *L. monocytogenes*, except that the intracellular events are slightly delayed. Factors involved in most of these events in *L. monocytogenes* have been identified. Invasion of mammalian cells has been reported to require internalin, an 80-kDa protein encoded by the gene *inlA* (9). Whether *L. ivanovii* possesses *inlA* is not known. Gaillard et al. (9) did not find any signal in DNA hybridization tests with *L. ivanovii* under stringent conditions. Although they detected several bands under conditions of low stringency, the appearance of signals in *L. innocua* under the same conditions makes interpretation difficult. However, the results presented here confirm the report of Gaillard et al. (10) that *L. ivanovii* is capable of invading the enterocyte-like cell line Caco-2 and extend the observation to 3T3 fibroblasts. Kuhn et al. (16) earlier noted that *L. ivanovii* was unable to invade 3T6 cells. The reasons for the difference in the behavior of *L. ivanovii* toward 3T3 and 3T6 cells, both of which are mouse fibroblasts, is not known. Whether the collagen- and hyaluronic acid-secreting property of 3T6 cells accounts for this difference should be studied.

The first step after entry into the host cell appears to be the lysis of the phagosomal membrane. This has been reported to be mediated by LLO since LLO-negative mutants are unable to grow intracellularly and they reside in host vacuoles (6, 11, 26). Since *L. ivanovii* produces an SH-activated hemolysin closely related to LLO (12, 15), it is to be expected that *L. ivanovii* would be able to lyse the phagosomal membrane and enter the host cell cytoplasm. The results obtained with *L. ivanovii* mutants provide convincing evidence for the role of listeriolysin of this organism in lysing the phagosomal membrane. The nonhemolytic mutant 8/6 was able to invade Caco-2 cells but was trapped inside the phagosomal membrane and was therefore unable to multiply. However, a very small proportion of these mutants were able to enter the cytoplasm and polymerize actin. Similar results have been reported for LLO-negative mutants of *L. monocytogenes* (19). It has been suggested that in the absence of LLO, phospholipase C might account for the lysis of the vacuole (19); this explanation might hold good for even *L. ivanovii*, which also possesses phospholipase C activity (20). Further, sphingomyelinase produced by *L. ivanovii* also may contribute to the lysis of the membrane in

this small number of mutant cells. In addition, few of these bacteria may represent revertants of the mutant strain.

Intracellular movement in *L. monocytogenes* is associated with the ability to polymerize actin and reorganize the actin filaments in the form of tails. Mutants defective in actin polymerization are unable to spread and grow as microcolonies (8, 13). Actin polymerization has been shown to require a 90-kDa surface protein encoded by *actA* (8, 13). More than one gene product may be involved in this process since a mutant which is able to polymerize actin but is still incapable of reorganizing it into tails and spreading from cell to cell has been described (17). The results presented here clearly show for the first time that *L. ivanovii* is capable of polymerizing host cell actin, reorganizing it into tails, and spreading from cell to cell. Using PCR, we have not been able to detect DNA sequences homologous to *actA* in *L. ivanovii*. The region amplified between bp 799 and 1071 contained an internal repeat region which is a likely candidate for a conserved region of *actA*. Further, part of this region has been reported to be homologous to the proline-rich region of vinculin (8, 13). This confirms the results of Vazquez-Boland et al. (28), who were unable to detect *actA* in *L. ivanovii* by Southern hybridization. Our results further show that *L. ivanovii* lacks the 90-kDa protein encoded by *actA* in *L. monocytogenes*. In view of this, the observation that *L. ivanovii* is able to polymerize actin is surprising. Our results do not exclude the possibility that an *actA* gene with a high divergence in DNA sequence exists in *L. ivanovii* and that the 90-kDa ActA protein is more labile in this microorganism than in *L. monocytogenes*. It is also possible that another gene product is involved in actin polymerization in *L. ivanovii*. A 120-kDa surface protein which is entirely unrelated to ActA has been reported to be required for the polymerization step in *Shigella flexneri*, which shows almost the same type of intracellular behavior as *L. monocytogenes* (2).

Of the various actin-binding proteins tested, only α -actinin was found to be associated with polymerized actin in *L. ivanovii*. α -Actinin has been known to cross-link actin filaments and to bind to diacylglycerol and palmitic acid, leading to increased actin bundle formation (24). Further, α -actinin has been shown to dissociate profilin-actin complexes in vitro and promote polymerization to give cross-linked filaments (29). The role of α -actinin in *Listeria*-associated actin assembly is not known.

Electron-microscopic and light-microscopic observations clearly indicate that *L. ivanovii* is capable of cell-to-cell spread. Lecithinase encoded by *plcB* has been suggested to be involved in this process in *L. monocytogenes*, and DNA sequences hybridizing to *plcB* have been detected in *L. ivanovii* (28). Despite the ability to multiply intracellularly and spread from cell to cell, the inability of *L. ivanovii* to form plaques on monolayers of 3T3 cells is surprising. This suggests that a cytotoxic factor is probably involved in the destruction of host cells by *L. monocytogenes* but is lacking in *L. ivanovii*. The mechanisms by which intracellular bacteria may kill host cells are unclear. In *S. flexneri*, a contact hemolysin has been suggested to account for cell killing. The membrane-damaging function has been suspected to have deleterious effects on the endoplasmic reticulum, Golgi apparatus, or mitochondria (4). The mechanism of cytotoxicity in *Listeria* spp. is not known. Invasive *S. flexneri* strains induce programmed cell death (apoptosis), whereas *L. monocytogenes* does not (30). Since *L. ivanovii* is capable of lysing the phagosome, spreading to the neighboring cell, and lysing the double membrane and yet lacks cytotoxicity,

membrane-damaging function alone cannot explain the cytotoxicity of *L. monocytogenes*. It is tempting to speculate that lack of cytotoxicity might account for the lower virulence of *L. ivanovii* than of *L. monocytogenes*.

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