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# Ultrastructural Study of *Listeria monocytogenes* Entry into Cultured Human Colonic Epithelial Cells

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**Evidence that *Listeria monocytogenes* enters Caco-2 cells through the apical surface is presented. Attachment of bacteria to host cells seems to induce modifications of microvilli which are either in direct contact with the bacterial surface or in close vicinity, resulting in the formation of lamellipodia involved in the cellular uptake of the bacteria. Such modifications are not induced by *L. monocytogenes* SLCC 53, which carries a deletion in the *prfA* gene, although attachment of this mutant to Caco-2 cells occurs. *Listeria innocua* does not attach well to Caco-2 cells and also fails to cause structural alterations of the microvilli. Treatment of confluent monolayers of Caco-2 cells with ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), which disrupts intercellular junctions, greatly reduced the uptake of *Listeria* cells. Attachment and invasion of *L. monocytogenes* was not accompanied by accumulation of filamentous actin around the entering bacterial cell.**

*Listeria monocytogenes* is a gram-positive bacterium which can cause serious and sometimes fatal infections in pregnant women, newborns, and immunocompromised patients. Outbreaks of listeriosis have been shown to be associated with ingestion of contaminated food, and there is evidence that the intestine is the usual site of entry of this organism (5, 17). Therefore, a critical step in the pathogenesis of listeriosis is the invasion of intestinal epithelial cells by this organism, and it is still unclear how listeriae enter these cells. *L. monocytogenes* has been shown to be capable of invading and multiplying intracellularly within the human enterocyte-like cell line Caco-2 (8). Caco-2 cells express typical enterocytic differentiation and polarization under standard culture conditions (14). The use of polarized cells, such as MDCK and Caco-2 cells, has been very useful in understanding the invasion mechanism of intestinal pathogens such as *Salmonella* (6, 7) and *Shigella* (13) spp. In this communication, we present ultrastructural evidence that *L. monocytogenes* enters polarized Caco-2 cells through the apical surface.

The human colon carcinoma cell line Caco-2 (ECACC 86010202) was cultured at 37°C in the presence of 5% $CO_2$ in minimal essential medium (GIBCO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% nonessential amino acids, penicillin (100 μg/ml), and streptomycin (100 μg/ml). *L. monocytogenes* EGD (provided by S. H. E. Kaufmann, Ulm, Germany), *L. monocytogenes* NCTC 7973, *L. monocytogenes* SLCC 53 (a mutant carrying a deletion in the *prfA* gene) (10), and *Listeria innocua* 6a were the strains used to infect Caco-2 cells. At 48 h before infection, $4 \times 10^4$ Caco-2 cells were seeded onto coverslips in a 24-well tissue culture plate; they became nearly confluent at the time of infection. *L. monocytogenes* was 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 minimal essential medium without supplements. Host cells were washed with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. A 1-ml bacterial suspension containing a 100-fold excess of bacteria over host cells was added to each well in tissue culture plates. These plates were then incubated at 37°C in the presence of 5% $CO_2$. At 30 min postinfection, cells were washed with PBS–1 mM $CaCl_2$–0.5 mM $MgCl_2$, fixed in 2.5% buffered glutaraldehyde (50 mM cacodylate [pH 7.2], 50 mM KCl, 2.5 mM $MgCl_2$) for 30 min, 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 distilled water. Alternatively, cells were fixed with 6.75% buffered glutaraldehyde (0.1 M phosphate, pH 7.4) at 4°C for 18 h and finally washed with distilled water. Cells attached to coverslips were then stepwise dehydrated in acetone and critical-point dried with $CO_2$. Specimens were sputtered with 30-nm gold. Photographs were taken with a Zeiss scanning electron microscope (DSM 962; Zeiss, Oberkochen, Germany).

In nearly confluent cell cultures, *L. monocytogenes* was found to attach to and enter polarized Caco-2 cells at the apical surface. A representative overview of an epithelial cell with several bacteria is shown in Fig. 1. On our specimens, the basolateral and the basal sides of epithelial cells were not accessible to analysis by scanning electron microscopy. Therefore, we cannot exclude the possibility that listeriae may also enter Caco-2 cells along these surfaces. However, there was no particular concentration of bacteria at intercellular junctions, as reported for *Shigella flexneri* (13). In some cells, in which differentiation and microvillar development were not uniform (Fig. 1), listeriae were found attached to areas with microvilli. In the initial stage, *L. monocytogenes* was observed to interact with microvilli (Fig. 2A). This interaction appeared to stimulate the host cell plasma membrane in the vicinity of the attached bacteria to form lamellipodium-like projections (Fig. 2B and C). The morphology of some of these plasma membrane protrusions suggests that they were generated by lateral fusion of neighboring microvilli (Fig. 2B and C arrowheads). At a more advanced stage of entry, lamellipodia entangled predominantly one of the bacterial tips (Fig. 2D), whereas in the final stage, bacterial cells were almost completely covered by the plasma membrane of the host cell (Fig. 2E). The process of uptake with *L. monocytogenes* EGD was similar to that

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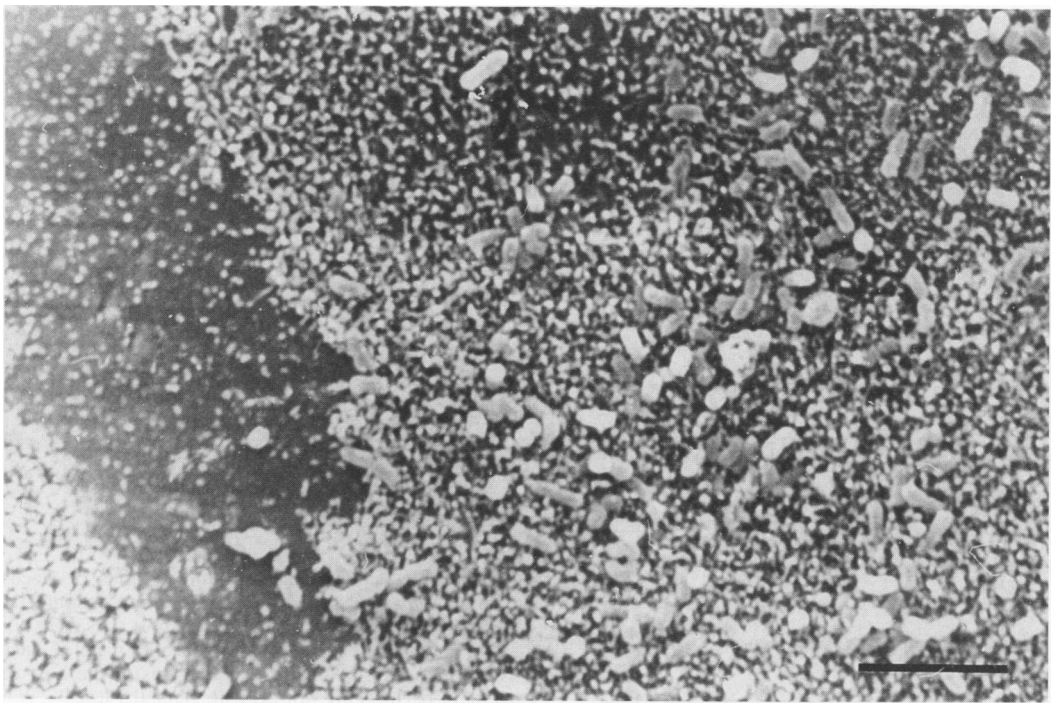

FIG. 1. Scanning electron micrograph of *L. monocytogenes* NCTC 7973 interacting with the apical surface of Caco-2 cells at 30 min postinfection.

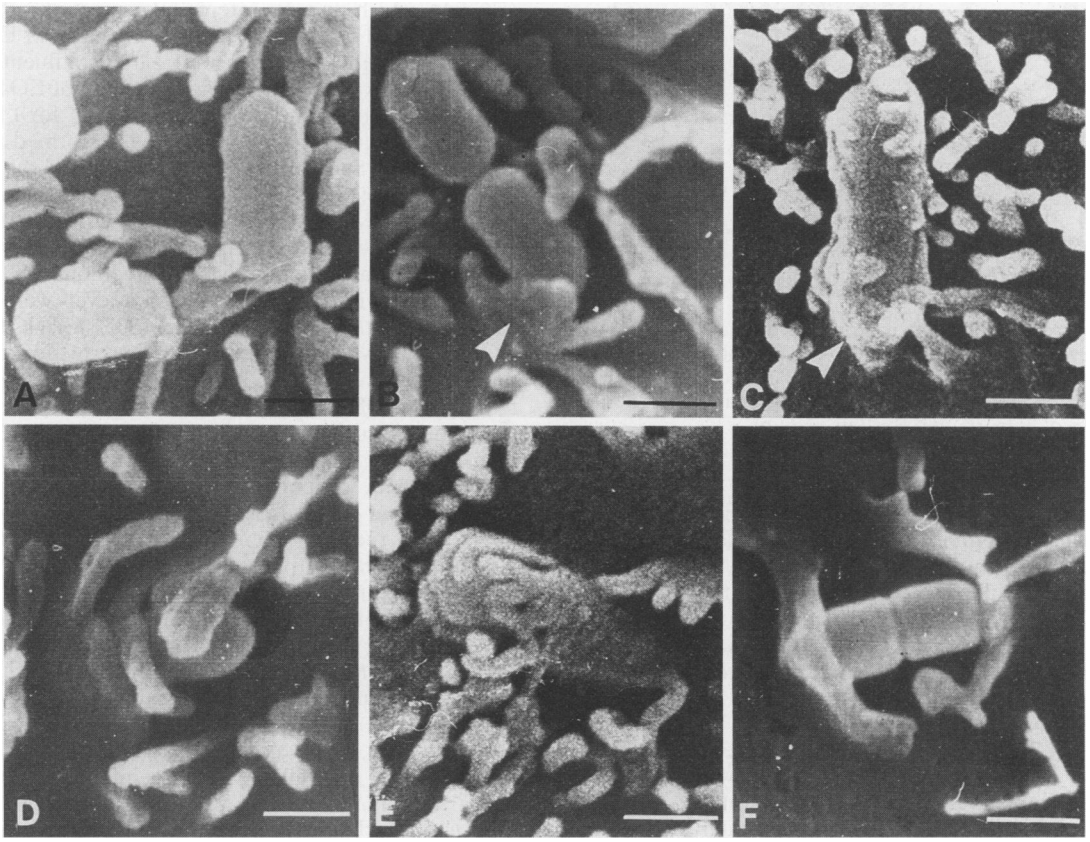


FIG. 2. Scanning electron micrographs of *L. monocytogenes* NCTC 7973 at different stages of invasion of Caco-2 cells (30 min postinfection). (A) Bacteria interacting with microvilli. (B to D) Progressive generation of lamellipodia which entangle one of the bacterial tips; arrowheads denote lamellipodia which may have been generated by lateral fusion of microvilli. (E) Late stage of entry. (F) Dividing bacterium engulfed on both tips by lamellipodia. Bars, 0.5 μm.

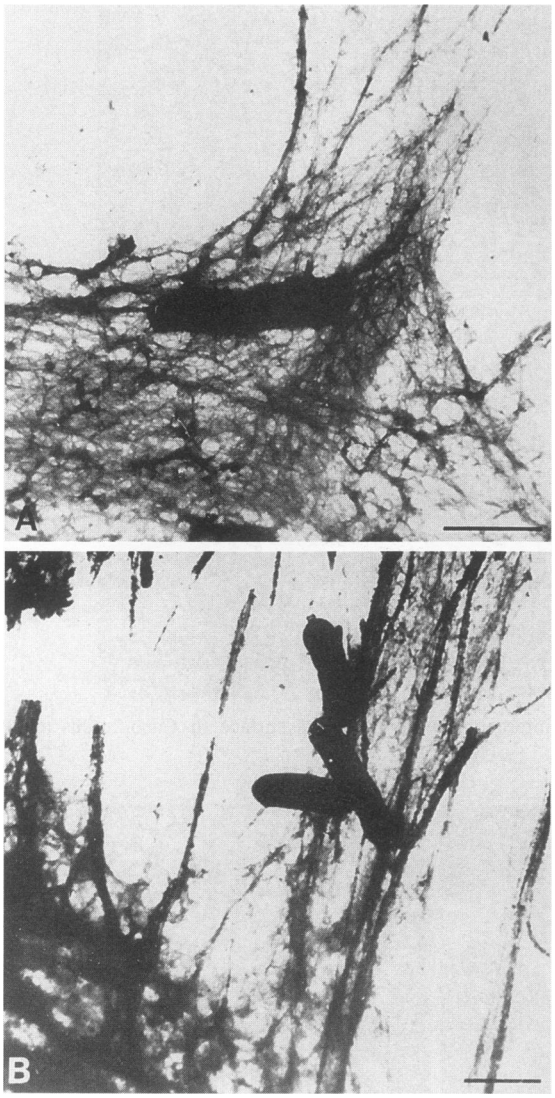


FIG. 3. Transmission electron micrographs of Caco-2 cells infected with *L. monocytogenes* NCTC 7973. At 30 min postinfection, cells were fixed, detergent extracted, and stained with uranyl acetate to visualize actin filaments. No electron-dense material is seen around bacteria at different stages of entry (A and B). Bars, 0.5 μm.

observed with strain NCTC 7973 (data not shown), although this strain, which is commonly used for laboratory studies, was less invasive (18). On most occasions when bacteria in the process of entry could be seen, it appeared that one of the bacterial tips entered first and that the entry took place in the longitudinal direction. For one dividing bacterium it was observed that both ends were partially engulfed by membrane protrusions (Fig. 2F).

These observations clearly indicate that *L. monocytogenes* can enter Caco-2 cells from the apical surface, in contrast to the case with *S. flexneri*, which has been shown to enter the same host cells exclusively from the basolateral side (13). This is interesting, since these organisms show similar intracellular behaviors, which include lysis of the phagosomal membrane, interaction with the host cell cytoskeleton, and cell-to-cell spread. The uptake of *S. flexneri* has been reported to be greatly enhanced by the treatment of Caco-2 cells with ethyl-

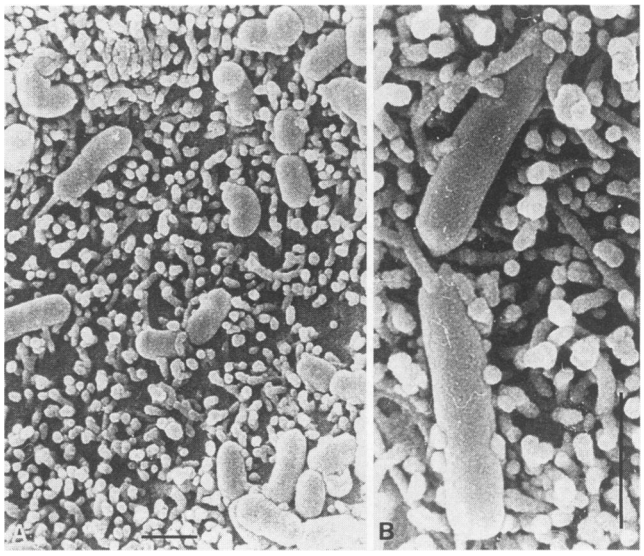


FIG. 4. Scanning electron micrographs of Caco-2 cells infected with *L. monocytogenes* SLCC 53 (30 min postinfection). Bacteria are passively lying on the surface of the host cells, with no modifications of the microvilli or the host cell surface. In panel B, bacteria are perhaps in the process of entry. Modifications of the host cell membrane to engulf bacteria are not evident. Bars, 0.5 μm.

ene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), which disrupts intercellular junctions, thus exposing the basolateral surface (13). To test the effect of EGTA on the uptake of *L. monocytogenes*, we treated confluent monolayers of Caco-2 cells (4 days old) with 100 μmol of EGTA in Hanks balanced salt solution (HBSS) without $Ca^{2+}$ for 1 h before and during the invasion assay, which was performed as described previously (9). Such treatment greatly reduced the uptake of *L. monocytogenes*: strains NCTC 7973 and EGD showed 7.2 and 9.6%, respectively, of the invasion observed with untreated controls. However, when the assay was done in Hanks balanced salt solution containing $Ca^{2+}$ (without EGTA) after 1 h of treatment with EGTA, the invasion levels were comparable to those with the controls (97 and 100% for NCTC 7973 and EGD, respectively), indicating that the reduction in uptake was due to depletion of $Ca^{2+}$ in the extracellular medium and not to any other effect of EGTA on Caco-2 cells. The observation that the exposure of the basolateral surface by EGTA treatment does not affect the uptake when $Ca^{2+}$ is present in the assay medium supports the conclusion that uptake of *L. monocytogenes* occurs from the apical surface. However, since uptake was reduced when $Ca^{2+}$ was absent from the assay medium, basolateral penetration in addition to apical uptake cannot be rigorously excluded by these experiments.

The exact role of $Ca^{2+}$ in the uptake of *L. monocytogenes* is not known. $Ca^{2+}$ is an important regulator of cytoskeletal structure and a number of other functions, such as motility, phagocytosis, and intracellular signalling in eucaryotic cells (2, 12). Polarized epithelial cells have a rigid cytoskeleton and microvilli with a structural backbone consisting of vertically oriented actin microfilaments cross-linked by "bundling proteins" such as fimbrin and villin (11). Figure 2 suggests that following attachment of bacteria, the microvilli become flexible, expand, and fuse laterally to cover the bacterial body. Such changes would involve actin depolymerization as well as actin polymerization. It has been reported that depending on the $Ca^{2+}$ concentration, villin could induce severing of actin

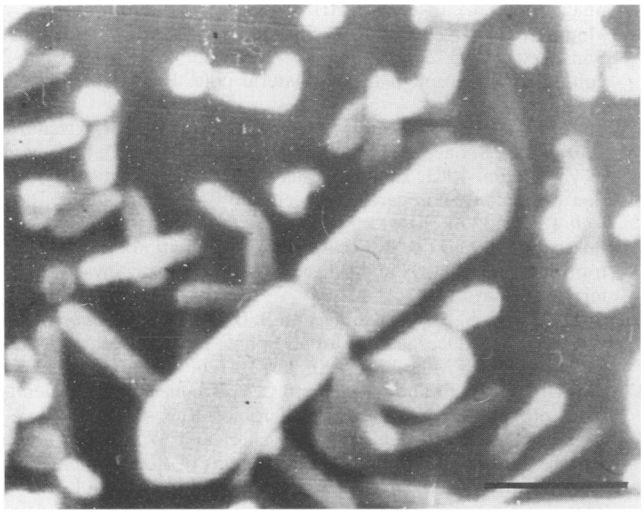

FIG. 5. Scanning electron micrograph of Caco-2 cells infected with *L. innocua* (30 min postinfection). Bacteria are lying passively on the cellular surface. Bar, 0.5 μm.

microfilaments or nucleation, capping, and bundling (11). Thus, calcium may be important for bringing about cytoskeletal rearrangements required for *L. monocytogenes* uptake. *Salmonella typhimurium*, which also invades from the apical surface, causes a major disruption of the brush border, called membrane ruffles, involving cytoskeletal rearrangements and intracellular free $Ca^{2+}$ fluxes (1, 7). However, no such disruption of the brush border was observed with *L. monocytogenes*. Further, in *S. typhimurium*, a chelator of intracellular calcium inhibited the uptake by HeLa cells, but extracellular calcium chelators such as EGTA had no effect on invasion (16). Internalization of *S. flexneri* into HeLa cells, on the other hand, occurred without an increase in intracellular $Ca^{2+}$ concentration (3) and was enhanced in Caco-2 cells in the presence of extracellular $Ca^{2+}$ chelators because of disruption of intercellular junctions and exposure of the basolateral surface (13). The different mechanisms used by these enteroinvasive pathogens raise interesting issues. Binding of *L. monocytogenes* to the surface of epithelial cells may trigger a signal in these cells that causes rearrangements in the various cytoskeletal components. Membrane protrusions which engulf the listeriae appear to be the end result. Although the intracellular interaction with actin appears to be similar in *Shigella* and *Listeria* cells, they might bind to different surface receptors, thereby leading to different mechanisms of entry into the host cell.

To study the cytoskeletal rearrangements induced by *L. monocytogenes*, we infected Caco-2 cells grown on coverslips, and at 15, 20, and 30 min, the coverslips were washed in PBS, permeabilized with 0.5% Triton X-100, and fixed in acetone at −20°C. These coverslips were stained with antibodies against listeriolysin O and p60 and with tetramethyl rhodamine isothiocyanate-conjugated anti-rabbit immunoglobulin G to visualize bacteria and with fluorescein isothiocyanate-conjugated Phalloidin (Sigma Chemical Co.) to look for filamentous actin as described previously (9). No accumulation of filamentous actin around bacteria could be seen at this stage of invasion. This was further confirmed by detergent extraction of cells grown on Formvar-coated electron microscopic grids as described by Young et al. (19). The cells were infected with *L. monocytogenes* and fixed and permeabilized at 30 min by a modification of the method described by Young et al. (19). After fixation in 1% glutaraldehyde, the grids were postfixed with 2% osmium tetroxide, dehydrated in graded ethanol, and stained with 1% uranyl acetate in ethanol for 5 min before treatment with hexamethyldisilazane (Sigma Chemical Co.). The grids were observed with a transmission electron microscope (EM 900; Zeiss). As shown in Fig. 3, bacteria in contact with the host cell surface could be seen, and there was no accumulation of actin filaments around these bacteria. In this respect also, the process of invasion of *L. monocytogenes* into epithelial cells differed from that of *Salmonella*, *Shigella*, and *Yersinia* spp. and enteropathogenic *Escherichia coli*, since actin and actin-binding proteins have been shown to accumulate around these bacteria during the process of invasion (15). This suggests that these bacteria use different mechanisms of invasion, perhaps because of interaction with different surface receptors.

Analysis of an *L. monocytogenes prfA* mutant (strain SLCC 53) (10) showed many bacterial cells adhering to the apical surface; bacteria entering the host cells were only rarely observed (Fig. 4). Alterations of the microvilli, which were clearly visible during entry of the wild-type bacteria into the host cells, were not seen with the *prfA* mutant. This is consistent with the previous observation that this *prfA*-defective strain adheres to Caco-2 cells, albeit at a reduced rate (about 30% of the efficiency of the wild-type bacteria), but is hardly invasive (18). These observations suggest that a *prfA*-dependent gene product is required in order to induce the observed structural changes in the host cell surface and the subsequent phagocytosis by non-professional phagocytes, like the Caco-2 cells. A possible bacterial factor which may be responsible for these events is internalin (InlA), which has been shown to be necessary for invasion of *L. monocytogenes* into Caco-2 cells. The expression of internalin seems to depend on PrfA (4). The avirulent and noninvasive *L. innocua* bacteria adhere to the Caco-2 cells very poorly. Although the number of bacteria used was the same as for *L. monocytogenes*, less than 1% of the number of *L. monocytogenes* bacteria could be seen on the cell surface, suggesting that *L. innocua* does not adhere to these host cells. This is in line with previous observations (18).

The few *L. innocua* cells which were observed by electron microscopy are lying passively on the host cell surface. No modifications of the host cell surface could be seen (Fig. 5). These results suggest that structural alterations on the host cell surface of non-professional phagocyte cells are caused only by the interaction of virulent *L. monocytogenes* strains with the epithelial host cells.

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## REFERENCES

1. **Biliska, J. B., J. E. Galan, and S. Falkow.** 1993. Signal transduction in the mammalian cell during bacterial attachment and entry. Cell **73:**903–920.
2. **Brundage, R. A., K. E. Fogarty, R. A. Tuft, and F. S. Fay.** 1991. Calcium gradients underlying polarization and chemotaxis of eosinophils. Science **254:**703–706.
3. **Clerc, P. L., B. Berthon, M. Claret, and P. J. Sansonetti.** 1989. Internalization of *Shigella flexneri* into HeLa cells occurs without an increase in cytosolic $Ca^{2+}$ concentration. Infect. Immun. **57:** 2919–2922.
4. **Dramsi, S., C. Kocks, C. Forestier, and P. Cossart.** 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes*

is regulated by the bacterial growth state, temperature and the pleiotropic activator *prfA*. Mol. Microbiol. **9:**931–941.
5. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
6. **Finlay, B. B., and S. Falkow.** 1990. *Salmonella* interaction with polarized human intestinal Caco-2 epithelial cells. J. Infect. Dis. **162:**1096–1106.
7. **Finlay, B. B., B. Gumbiner, and S. Falkow.** 1988. Penetration of *Salmonella* through a Madin-Darby kidney epithelial cell monolayer. J. Cell Biol. **107:**221–230.
8. **Gaillard, J., P. Berche, J. Mounier, S. Richard, and P. J. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55:**2822–2829.
9. **Karunasagar, I., G. Krohne, and W. Goebel.** 1993. *Listeria ivanovii* is capable of cell-to-cell spread involving actin polymerization. Infect. Immun. **61:**162–169.
10. **Leimeister-Wächter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty.** 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. Proc. Natl. Acad. Sci. USA **87:**8336–8340.
11. **Leiser, J., and B. A. Molitoris.** 1993. Disease processes in epithelia: the role of actin cytoskeleton and altered surface polarity. Biochim. Biophys. Acta **1225:**1–13.
12. **Marks, P. W., and F. R. Maxfield.** 1990. Local and global changes in cytosolic free calcium in neutrophils during chemotaxis and phagocytosis. Cell Calcium **11:**181–190.
13. **Mounier, J., T. Vasselon, R. Hellio, M. Lesourd, and P. J. Sansonetti.** 1992. *Shigella flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole. Infect. Immun. **60:** 237–248.
14. **Pinto, M., S. Robine-Leon, M. Appay, M. Kedinger, N. Triadore, E. Dussaux, B. Lacroix, P. Simon-Assman, K. Haffen, J. Fogh, and A. Zweibaum.** 1983. Enterocyte-like differentation and polarization of human colon carcinoma cell line Caco-2 in culture. Biol. Cell **47:**323–330.
15. **Rosenshine, I., and B. B. Finlay.** 1993. Exploitation of host signal transduction pathways and cytoskeletal functions by invasive bacteria. Bioessays **15:**17–24.
16. **Ruschkowski, S., I. Rosenshine, and B. B. Finlay.** 1992. *Salmonella typhimurium* induces an inositol phosphate flux in infected epithelial cells. FEMS Microbiol. Lett. **95:**121–126.
17. **Schuchat, A., B. Swaminathan, and C. V. Broome.** 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. **4:**169–183.
18. **Sokolovic, Z., J. Riedel, M. Wuenscher, and W. Goebel.** 1993. Surface-associated, PrfA-regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. Mol. Microbiol. **8:**219–227.
19. **Young, V. B., S. Falkow, and G. K. Schoolnik.** 1992. The invasin protein of *Yersinia enterocolitica*: internalization of invasin-bearing bacteria by eucaryotic cells is associated with reorganization of the cytoskeleton. J. Cell Biol. **116:**197–207.