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E-Cadherin Is the Receptor for Internalin, a Surface Protein Required for Entry of L. monocytogenes into Epithelial Cells

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Summary

We report the first identification of a cellular receptor mediating entry of a gram-positive bacterium into nonphagocytotic cells. By an affinity chromatography approach, we identified E-cadherin as the ligand for internalin, an L. monocytogenes protein essential for entry into epithelial cells. Expression of the chicken homolog of E-cadherin (L-CAM) in transfected fibroblasts dramatically increases entry of L. monocytogenes and promotes that of a recombinant L. innocua strain expressing internalin but does not promote entry of the wild-type noninvasive L. innocua or that of an internalin-deficient mutant of L. monocytogenes. Furthermore, L-CAM-specific antibodies block internalin-mediated entry. In contrast to Salmonella, Listeria enters cells by a mechanism of induced phagocytosis occurring without membrane ruffling. This work reveals a novel type of heterophilic interactions for E-cadherin.

Introduction

Several microorganisms trigger their entry into nonphagocytic cells by a process, called induced phagocytosis, involving complex interactions between microbial factors and cell surface receptors. Entry has been extensively studied in the cases of Yersinia, Salmonella, and Shigella, three gram-negative bacteria (Falkow et al., 1992).

In the cases of Salmonella and Shigella, entry is a multifactorial process. It involves several bacterial factors secreted in the extracellular environment and probably directly into the host cell cytoplasm through a type III secretion pathway induced upon contact of bacteria with eukaryotic cells (Finlay, 1994; Parsot, 1994). Receptors for these proteins have not been identified. These factors probably interfere with intracellular signalization pathways, leading to important actin cytoskeleton rearrangements, such as membrane ruffling, that internalize bacteria. The term "trigger mechanism" has recently been proposed for this type of bacterial-induced phagocytosis (Swanson and Baer, 1995).

Yersinia is the only case where a bacterial ligand and its cellular receptor have been identified. Y. pseudotuberculosis and Y. enterocolitica produce three proteins, invasin, Ail and YadA that, when expressed into E. coli, are sufficient to promote an adhesive and invasive phenotype (Miller and Pepe, 1994). Among these three proteins the best characterized and most efficient one is invasin, a 103 kDa outer membrane protein that binds to β1 integrin receptors leading to bacterial entry by a "zipper mechanism" (Isberg and Tran Van Nhieu, 1994; Swanson and Baer, 1995). Invasin now represents the archetype of a bacterial ligand promoting entry.

We are studying the entry of Listeria monocytogenes into mammalian cells. L. monocytogenes is an opportunistic bacterium responsible for human food-borne infections leading to meningitis and abortion with a high mortality rate (Schlech III et al., 1983). The primary site of entry of L. monocytogenes has not been clearly identified. L. monocytogenes is able to multiply in Peyer's patches (MacDonald and Carter, 1980; Marco et al., 1992) and enterocytes (Racz et al., 1972), but it is not clear whether enterocytes are infected from their apical pole and represent a site of translocation across the intestinal barrier or if they are infected from their basolateral surface after translocation of the bacteria through M cells or between cells. Alternatively, infection of enterocytes could result from direct cell-to-cell spread of bacteria from infected M cells or macrophages. Following translocation, bacteria spread via the lymph and the blood to the spleen and the liver where most bacteria are killed (Lepay et al., 1985). In the immunocompromised host, active bacterial multiplication takes place in hepatocytes with further release of the bacteria into the blood and dissemination to the brain and the placenta. In vitro L. monocytogenes, in contrast to the closely related noninvasive nonpathogenic species L. innocua, enters a variety of mammalian cell lines.

We have previously identified an 80 kDa bacterial surface protein, internalin, required for entry of L. monocytogenes into epithelial cells and sufficient to confer invasiveness to L. innocua (Gaillard et al., 1991). Internalin is encoded by the *inlA* gene, which is part of a multigene family. Internalin is characterized at the sequence level by two different regions of repeats. The first region is made of fifteen 22 amino acid leucine-rich repeats (LRRs). The second region is formed of three consecutive repeats, two of 70 amino acids and one of 49 amino acids. In the carboxylic part of internalin, sequence homologies with other surface proteins of gram-positive cocci suggest that internalin is anchored to the cell wall peptidoglycan by covalent linkage via the threonine residue of the conserved motif LPXTG (Fischetti et al., 1990; Schneewind et al., 1995). In agreement with these structural features, internalin is detected on the bacterial surface, but an important fraction of the produced internalin is released in the culture supernatant (Dramsi et al., 1993b). Recent data indicate that only internalin present on the bacterial surface plays a role in entry (unpublished data). Apart from the LPXTG motif and an FATDK motif present in three copies in internalin and one copy at position 831–835 in invasin of Y. pseudotuberculosis (Isberg et al., 1987), internalin shares no homology with proteins of the databases.

In this work, we demonstrate that the cellular receptor for internalin is E-cadherin. E-cadherin is a Ca^{2+}-dependent cell–cell adhesion molecule that plays a critical role in cell sorting during development, formation of intercellular junctions and polarization of epithelial cell layers, and maintenance of adult tissue architecture. Cadherins are glycoproteins with a highly conserved cytoplasmic domain, a membrane-spanning region, and a large extracellular domain responsible for homophilic Ca^{2+}-dependent adhesion (Takeichi, 1990; Geiger and Ayalon, 1992; Kemler, 1993). This extracellular domain contains five protein modules whose tertiary structure has recently been solved (Overduin et al., 1995; Shapiro et al., 1995).

The cadherin family contains several subclasses with the main group consisting of classical cadherins, N-, R-, M-, P-, and E-cadherins (Geiger and Ayalon, 1992). N-cadherin is expressed in the nervous system, skeletal muscle, and cardiac muscle; R-cadherin, in the retina and the nervous sytem; M-cadherin, in skeletal muscles; and P-cadherin was originally identified in the placenta. E-cadherin is expressed in epithelial tissues, primarily in the skin, the liver, and the digestive track. In the digestive track, E-cadherin plays a crucial role in maintenance of the intestinal epithelium structure and is expressed at the basolateral face of enterocytes (Hermiston and Gordon, 1995).

In this paper, we identify internalin as a novel ligand for E-cadherin and thus demonstrate that, in addition to its role in cell–cell adhesion, E-cadherin can play a role in bacterial invasion of nonphagocytic cells.

Results

Mammalian Cells Bind to Internalin

A prerequisite in the identification of a cellular ligand for internalin was the demonstration that epithelial cells could bind to internalin. Internalin was purified from culture supernatant of an L. innocua strain expressing *inlA*. During purification, presence of internalin was monitored using a serum directed against a MalE–internalin fusion protein (Dramsi et al., 1993b).

Purified internalin presents a multiple band pattern when analyzed with SDS–polyacrylamide gel electrophoresis (SDS–PAGE; Figure 1A). Such a pattern has been described for cell wall–extracted protein A and protein M, two surface proteins from gram-positive bacteria, Staphylococcus aureus and Streptococcus pyogenes, respectively (Fischetti et al., 1985) and could be due to the presence of cell wall peptidoglycan covalently linked to the C-terminal portion of the protein or to a partial degradation of the protein. All bands detected in Coomassie blue staining reacted with the anti-MalE-internalin serum (data not shown). The amino terminal sequence of the five major bands, pooled together, was determined. It is identical to the amino-terminal sequence of mature internalin predicted from the *inlA* gene sequence (Dramsi et al., 1993a). The five bands thus correspond to internalin.

To determine if cells could bind to purified internalin, 96-well microtiter plates were coated with increasing amounts of internalin and incubated with Caco-2 cells.

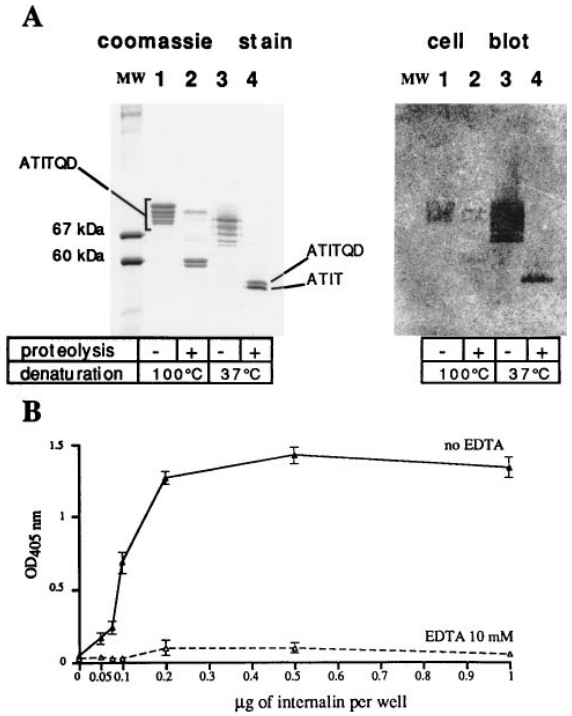

Figure 1. Caco-2 Cells Bind to Internalin

(A) SDS–PAGE profile of purified internalin stained with Coomassie blue (left) and cell blot: attachment of Caco-2 cells to proteins transferred to nitrocellulose membranes (right). Lanes 1–4: 1.4 µg of internalin subjected to a mild proteolytic treatment and to heat denaturation (see Experimental Procedures). MW: molecular mass standards. NH_2-terminal sequences determined are indicated.

(B) Caco-2 cells bind to surfaces coated with internalin and binding is inhibited by EDTA. Internalin was used to coat 96-well microtiter dishes. After incubation with a Caco-2 cells suspension with (dotted line) or without EDTA (plain line), wells were washed and the number of bound cells was determined by assaying for lysosomal hexosaminidase. OD_{405} is directly proportional to the number of cells per well. The experiment was done in quadruplicate.

Presence of bound cells was determined using an hexosaminidase colorimetric assay. Caco-2 cells were able to bind to wells coated with internalin and not to uncoated wells. Binding was concentration dependent and saturable. It was totally inhibited with 10 mM EDTA (Figure 1B).

To definitively establish that Caco-2 cells had bound to internalin and not to a minor contaminant present in the internalin preparation, fixation of Caco-2 cells to internalin was analyzed in "cell-blot" experiments. Internalin was subjected to SDS–PAGE and transferred to nitrocellulose filters. Filters were incubated with a suspension of Caco-2 cells, and cells bound to filters were stained with amido black. As shown in Figure 1A, Caco-2 cells bound to the five major bands of purified internalin. This binding was more efficient on internalin preincubated at 37°C than on internalin denatured by incubation at 100°C. When we used conditions allowing migration of purified internalin at different positions in the gel, the pattern of bound cells was always superimposed with the pattern of internalin bands revealed by Coomassie blue staining, demonstrating that Caco-2 cells bound

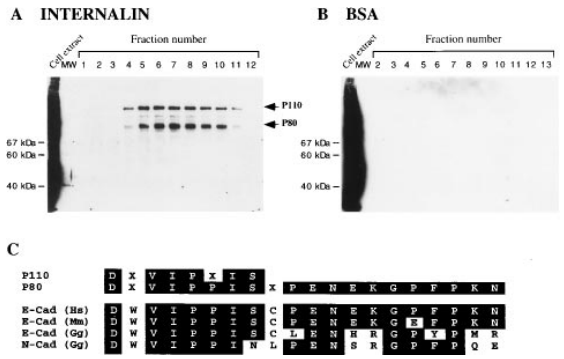

Figure 2. E-Cadherin Binds to Internalin

Surface biotin–labeled Caco-2 cell extracts were loaded onto affinity columns of internalin (A) and BSA (B) covalently linked to affigel-15. After washing bound proteins were eluted with EDTA. Fraction samples (5 μl) were analyzed by SDS–PAGE, transferred to nitrocellulose, and probed with streptavidin conjugated to horse radish peroxidase to detect biotin-labeled species with chemiluminescence. From left to right: total cell extracts, molecular mass standards (MW), and EDTA elution fractions. The two major biotinylated proteins eluted from the internalin column, P110 and P80, are indicated with arrows. (C) Amino acid sequence comparison of NH_2-terminal sequences from P110 and P80 and the most closely related cadherins. X, undetermined amino acid residue; E-Cad, E-cadherin; N-Cad, N-cadherin; Hs, Homo sapiens; Mm, Mus musculus; Gg, Gallus gallus.

specifically to internalin (Figure 1A). Binding was, as in the microtiter assay, inhibited by 10 mM EDTA (data not shown). Together, these results show that internalin is by itself able to bind to mammalian cells.

E-Cadherin Binds to Internalin

To identify the cellular receptor interacting with internalin, we subjected Caco-2 cell extracts to affinity chromatography on an internalin column. Nondifferentiated (4-day-old) Caco-2 cells, surface labeled with biotin, were extracted with n-octyl glucoside in the presence of divalent cations. The clarified extract was loaded on an internalin affinity column. After washing, two major proteins P110 and P80 of apparent molecular masses of 110 ± 10 kDa and 80 ± 10 kDa, respectively, could be eluted with EDTA (Figure 2A). As a control, the same extract was loaded on a column of bovine serum albumin (BSA). In this case, no biotinylated protein was recovered after EDTA elution (Figure 2B). When proteins that did not bind to BSA were loaded onto the internalin column, the same two proteins, P110 and P80, were eluted with EDTA, demonstrating that binding of these two proteins was internalin specific.

To identify P110 and P80, we determined their NH_2-terminal sequence. The two proteins displayed the same NH_2-terminal sequence, suggesting that P80 is a processed form or a degradation product of P110. Searches of sequence databases with the 20 amino acid NH_2-terminal sequence indicated that the two proteins were E-cadherin and its 84 kDa proteolytic fragment classically detected in cell extracts (Figure 2C). In Western blot experiments, P110 reacted with DECMA-1 (Vestweber and Kemler, 1985), a monoclonal antibody directed against the mouse E-cadherin and cross-reacting with human E-cadherin (data not shown). We concluded from these results that E-cadherin is the cellular ligand for internalin.

E-Cadherin Promotes Internalin-Dependent Binding and Entry into Cells

To test if binding of Caco-2 cells to internalin was strictly E-cadherin dependent, we examined whether anti-E-cadherin antibodies had inhibitory effects using the microtiter plate binding assay. Pretreatment of a Caco-2 cells suspension for 50 min at 37°C with 10 μg/ml or 100 μg/ml of DECMA-1 resulted in a 50% and 96% reduction, respectively, of Caco-2 cells binding. Thus, the ability of Caco-2 cells to bind to internalin is mostly E-cadherin mediated.

The role of E-cadherin in the interaction between cells and internalin was confirmed by testing the effect of E-cadherin expression on the ability to bind to internalin of four other cell lines: a mouse fibroblast cell line, S180, which expresses no known cadherin; two independent clones of stably transfected S180 cells expressing the chicken homolog of E-cadherin also called L-CAM (S180L-CAM1 and S180L-CAM2); and one clone of stably transfected S180 cells expressing chicken N-cadherin [S180N-cad (Gg)]. Only cells expressing L-CAM were able to bind to internalin although this binding was about 10 times less efficient than that of Caco-2 cells (data not shown). Cells expressing N-cadherin did not bind to internalin, indicating that internalin binds specifically to L-CAM and not to N-cadherin.

Internalin is required not only for binding to epithelial cells but also for bacterial entry (Gaillard et al., 1991). To test if E-cadherin (or L-CAM) can trigger bacterial internalization, we performed invasion assays with the four fibroblastic cell lines and four Listeria strains differing in internalin expression. Entry of wild-type L. monocytogenes was 8- to 14-fold higher in fibroblasts expressing L-CAM than in those expressing no cadherin or expressing N-cadherin (Figure 3A). This promoting effect of L-CAM expression on bacterial entry was internalin specific, since it was not observed with an *inlA*− mutant. This result was confirmed by testing entry of a recombinant L. innocua strain expressing internalin (Figure 3B). This strain only entered fibroblasts expressing L-CAM (2.5% to 4% of entry) and did not enter fibroblasts devoid of cadherin expression or expressing N-cadherin (0.01% to 0.02% of entry). The control L. innocua strain transformed with the vector alone was unable to enter any of the four cell lines tested. These results demonstrate that E-cadherin (or L-CAM), in addition to binding, promotes internalin-dependent entry of bacteria into cells.

Antibodies against L-CAM Block Internalin-Dependent Entry into Cells

Anti-L-CAM antibodies blocked entry of wild-type L. monocytogenes into S180L-CAM1 and S180L-CAM2 (77% and 75% of inhibition, respectively), as well as that of a recombinant L. innocua strain expressing internalin (92% and 96% of inhibition, respectively). Inhibition of entry was specific, since it was observed neither with S180 cells nor with S180N-cad (Gg) cells. This inhibition

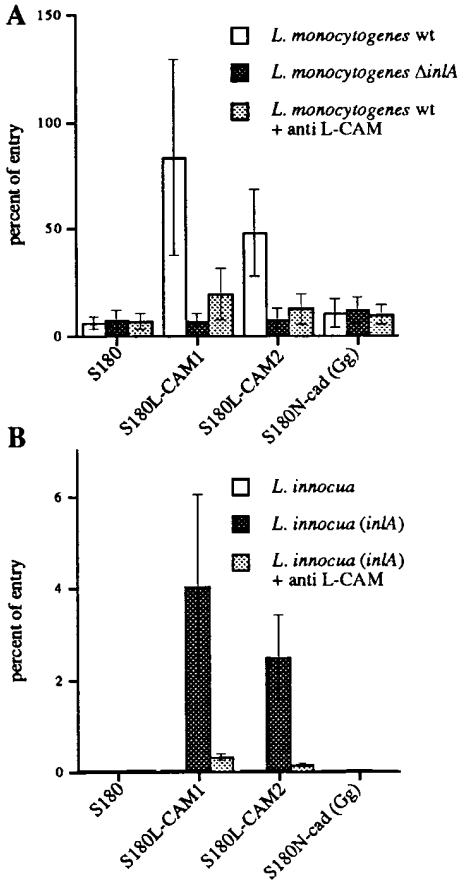

Figure 3. E-Cadherin Promotes Internalin-Dependent Entry into Mammalian Cells

Entry of bacterial strains was tested in four fibroblastic cell lines expressing either no cadherin (S180), L-CAM (the homolog of E-cadherin in chicken; S180L–CAM1 and S180L–CAM2), or chicken N-cadherin [S180N-cad (Gg)].

(A) Effect of cadherin expression on entry of L. monocytogenes. Two bacterial strains were tested in each cell line: a wild type L. monocytogenes strain (left and right bars) and an isogenic Δ*inlA* mutant (central bar).

(B) Effect of cadherin expression on entry of L. innocua. Two bacterial strains were tested in each cell line: L. innocua tranformed with *inlA* [L. innocua (pP1*inlA*), central and right bars] and L. innocua transformed with the cloning vector alone [L. innocua (pP1), left bar].

The inhibitory effect of anti-L-CAM antibodies was tested by incubating the cells with 50 μg/ml of anti-L-CAM serum 45 min before adding bacteria (right bar). Displayed is the data from three independent invasion assays.

was specific for the internalin-dependent pathway of entry, since no inhibition of the residual level of entry in any of the four cell lines was observed with bacterial strains devoid of internalin expression (Figure 3).

Entry of Listeria into Cells Is Strikingly Different from That of Salmonella

To observe entry of L. monocytogenes into nondifferentiated Caco-2 cells, we used scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Consistent with previous observations (Gaillard et al., 1994; Temm-Grove et al., 1994), bacteria were mostly present at the periphery of the Caco-2 cell islets, i.e., on nondifferentiated cells devoid of brush border microvilli. Bacteria in the process of entering cells were observed in diplike structures. They were covered at different extents by thin folds of plasma membrane (Figures 4A and 4B). At the site of bacterial contact, the intimately apposed membrane was undulated, suggesting that the bacterium had some direct but extremely local effect on the membrane structure (Figures 4C and 4D). The cell surface was similar in regions devoid of bacteria and in regions with entering bacteria, suggesting that apart from the local invagination no dramatic morphological changes of the cell surface were induced by invading bacteria.

Since entry of L. monocytogenes into Caco-2 cells is not solely dependent on internalin, to focus our analysis on internalin-mediated entry, we observed by SEM and TEM, entry of L. innocua (*inlA*) into S180L-CAM2 cells. The morphology of the entry appeared identical to that of L. monocytogenes into Caco-2 cells (Figures 5C and 5F). This entry process was a bona fide induced phagocytosis since no bacterial entry could be detected with S180L-CAM2 cells infected with L. innocua (Figure 3B). It was thus distinct from low efficiency classical phagocytosis observed with some cell lines when cultured in vitro.

To compare entry of Listeria with that of other invasive bacteria, we infected S180L-CAM2 cells with L. innocua (*inlA*), S. typhimurium strain SL1344, and a Y. pseudotuberculosis strain, YPIIIc, cured of its virulence plasmid. Salmonella and Yersinia were both able to invade S180L-CAM2 cells with high efficiency (44% and 40% of entry, respectively). When analyzed by SEM and TEM, cells infected with Salmonella presented the previously described local dramatic morphological changes known as membrane ruffles (Figures 5A and 5D) (Francis et al., 1992). No such structures were observed with cells infected with L. innocua (*inlA*) or Yersinia (Figures 5B, 5C, 5E, and 5F). In these two latter cases, the entry process was not associated with morphological changes.

Entry of each of the three bacteria requires actin polymerization since it is inhibited by cytochalasin D (Falkow et al., 1992). We compared the extent of accumulation of polymerized actin (F-actin) at the site of entry. This quantitative study was performed using laser scanning confocal microscope analysis of S180L-CAM2 cells fixed 10 min after infection with Salmonella, Listeria, and Yersinia. F-actin was revealed by rhodamine-phalloidin staining and bacteria by indirect immunofluorescence. In each case, we calculated the percentage of cell-associated bacteria that colocalized with areas of "F-actin accumulation." Such areas were defined as areas where the intensity of the F-actin signal was higher than an arbitrary threshold of 63 estimated in noninfected cells. In the case of Salmonella, 71% ± 15% of cell-associated bacteria (n = 154) colocalized with a cellular area (larger than 10 μm^2) of "F-actin accumulation" versus 30% ± 17% in the case of Listeria (n = 103) and 16% ± 19% in the case of Yersinia (n = 224). A Student unpaired t test revealed no significant difference (p = 0.1109) between the percentage values of Yersinia and Listeria, whereas the percentage value for Salmonella appeared

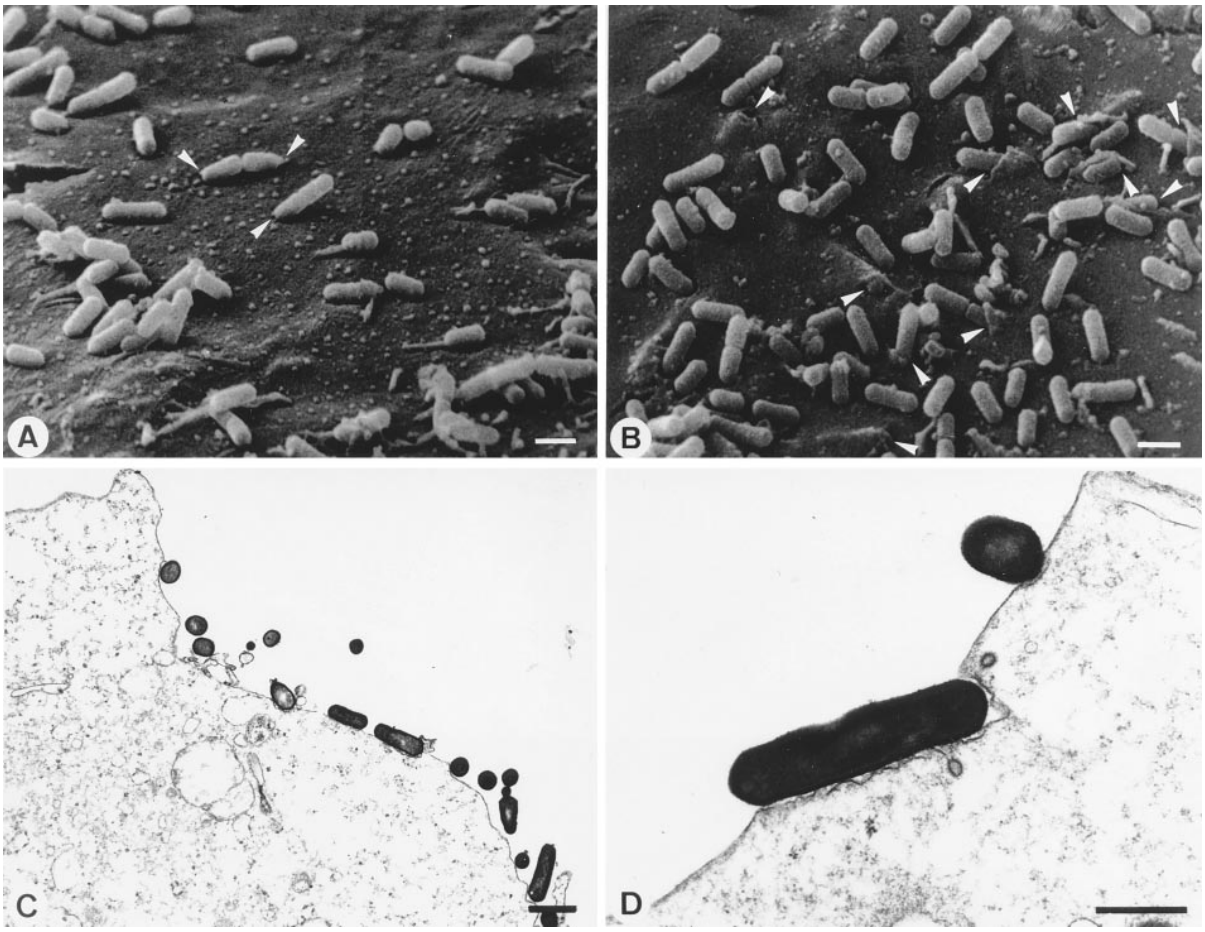

Figure 4. Electron Micrographs of Early Events of Caco-2 Cells Infection by L. monocytogenes

Cells were fixed 3 min (A) and 15 min (B–D) after infection. (A) and (B), SEM. Bacteria at different stages of invasion are indicated with arrowheads. (C) and (D), TEM at low and high magnification, respectively. The bacteria enter the cell without dramatic morphological changes of the cell surface. Scale bar, 0.5 μm.

significantly different from that of Yersinia and Listeria ($p < 0.0001$). In conclusion, in contrast to Salmonella, which induces the accumulation of high levels of F-actin during entry, Listeria and Yersinia do not.

Discussion

We have identified E-cadherin as the first cellular receptor involved in entry of a gram-positive bacterium into epithelial cells. E-cadherin binds to L. monocytogenes internalin, which represents a new ligand for cadherin heterophilic interactions. This finding has implications in the understanding of both entry processes used by pathogens, and signal transduction cascades involving external signals and cadherins.

Our study on the internalin-mediated entry into cells expressing different types of cadherins shows that internalin can interact with human E-cadherin and chicken L-CAM but not with chicken N-cadherin. This result demonstrates a strong specificity of internalin for the E-cadherin group, in agreement with the interspecies functional conservation of E-cadherins. Indeed, although chicken E-cadherin shares only 65% of homology with human E-cadherin and may not be the exact homolog of human E-cadherin (Pouliot, 1992), it is the chicken cadherin most closely related to E-cadherin. The two molecules present similar spatial and temporal pattern of expression and have the same specificity of adhesion (Edelman et al., 1983). The stronger adhesion of internalin to Caco-2 cells, which express human E-cadherin, compared with cells expressing chicken L-CAM probably reflects different affinities of internalin for those two cadherins.

The region of E-cadherin and that of internalin involved in the E-cadherin–internalin interaction is unknown. The NH_2-terminal extracellular cadherin domain containing the highly conserved tripeptide HAV is involved in selective homophilic binding specificity of E-cadherin (Blaschuk et al., 1990; Nose et al., 1990). Although no sequence homology between internalin and the HAV-containing sequence of E-cadherin has been found, internalin might bind to the NH_2-terminal domain of E-cadherin since antibodies blocking homophilic cell–cell binding block the promoting effect of E-cadherin expression on bacterial entry. The NH_2-terminal domain of internalin contains 15 LRRs and could be the region interacting with E-cadherin since LRR-proteins are usually involved in strong protein–protein interactions (Kobe

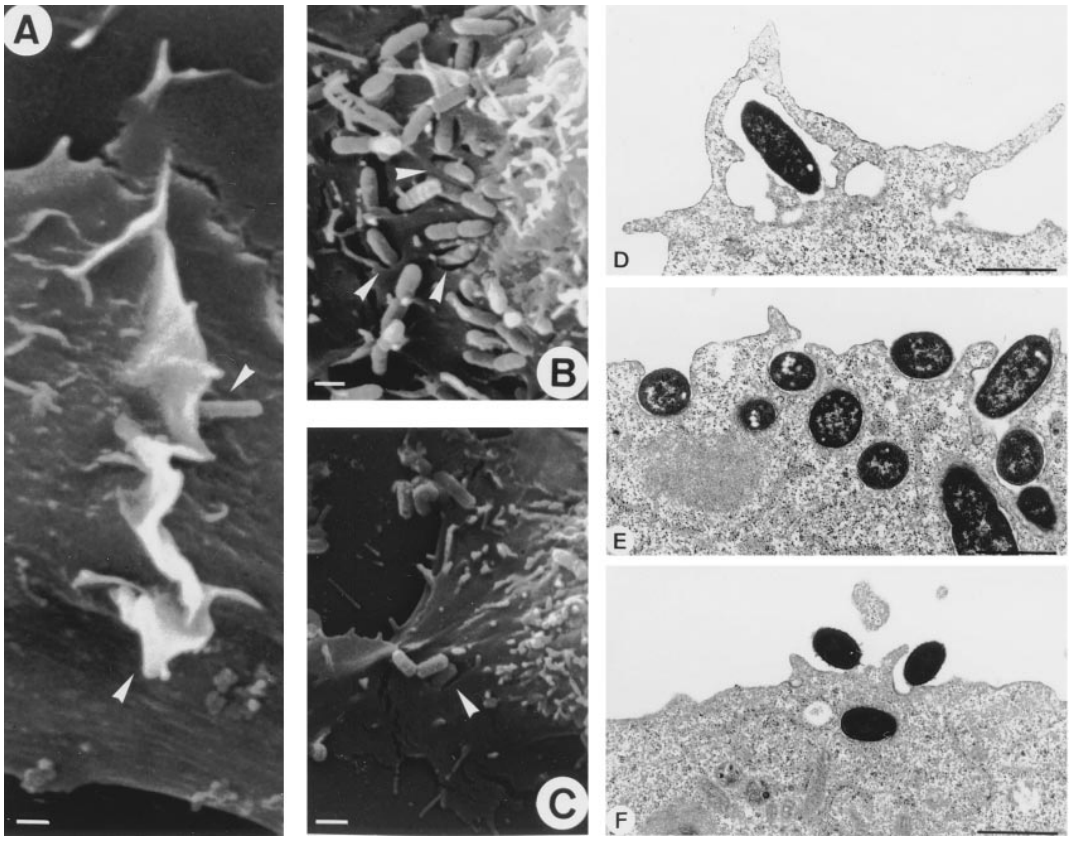

Figure 5. Comparative Analysis of the Entry of Salmonella, Yersinia, and Listeria into S180L-CAM2 Cells

SEM (A–C) and TEM (D–F) of early events of S180L-CAM2 cells infection by S. typhimurium (A and D), Y. pseudotuberculosis (B and E) and L. innocua (*inlA*) (C and F). Cells were fixed 10 min after infection. In cells infected with S. typhimurium membrane ruffles are observed (A and D). Such spectacular structures were neither observed on uninfected cells (data not shown) nor on cells infected with Y. pseudotuberculosis or L. innocua (*inlA*). In these two latter cases, bacterial entry appeared as zipper phagocytosis with bacteria "sinking" in dip-like structures (B, C, E, and F). Scale bar, 1 μm.

and Deisenhofer, 1994). As for E-cadherin homophilic interactions, calcium is probably required for internalin–E-cadherin interaction since EDTA inhibited cell binding on immobilized internalin and allowed elution of E-cadherin from internalin columns. Whether internalin can compete with E-cadherin for E-cadherin binding, thus disrupting adherens junctions between cells, and whether this disruption could destabilize the tight junctions and allow bacteria to migrate between enterocytes or other cells expressing E-cadherin are unknown.

Entry of L. monocytogenes into cells requires actin polymerization, since it is blocked by cytochalasin D (Gaillard et al., 1987). E-cadherin is connected to the actin cytoskeleton via its cytoplasmic carboxy-terminal domain and at least two cytoplasmic proteins, α- and β-catenins (Kemler, 1993; Hinck et al., 1994; Knudsen et al., 1995). The requirement of actin polymerization for the entry process of L. monocytogenes suggests that integrity of the cytoplasmic domain of E-cadherin is necessary for the productive internalin–E-cadherin interaction and subsequent internalization of the bacterium, as it is essential for the homophilic adhesive function of the extracellular domain (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990).

Our comparative morphological analysis and the quantitative analysis of the accumulation of F-actin at the site of entry of Listeria, Salmonella, and Yersinia showed that entry of Listeria, in contrast to that of Salmonella, is not associated with intense F-actin accumulation and is similar to that of a Yersinia strain cured of its virulence plasmid and entering via the invasin/integrin interaction. Our results on Yersinia are in agreement with a previous study using a recombinant E. coli strain expressing the invasin (*inv*) gene (Finlay and Ruschkowski, 1991). Using another recombinant E. coli strain expressing invasin, Young et al. (1992) reported F-actin accumulation at the site of entry. In that study, detection of areas of F-actin accumulation was probably due to overexpression of invasin, since the *inv* gene was under the control of a strong inducible promoter. The mechanism of entry of Listeria into cells is thus closely related to the zipper mechanism previously proposed for Yersinia (Isberg, 1991) or described in the case of classical C3 receptor–mediated phagocytosis by professional phagocytes (Kaplan, 1977). It is different from the "trigger mechanism" of entry of Salmonella (Swanson and Baer, 1995). Although induced phagocytosis of the three bacterial pathogens requires active actin polymerization, the extent of this process clearly differs in the "trigger mechanism" and in the "zipper mechanism" of

phagocytosis. Interestingly, a difference in cytochalasin D sensitivity was reported for phagocytosis by Fc and C3 receptor (Kaplan, 1977) suggesting that, in both phagocytosis and induced phagocytosis, actin polymerization plays a critical role.

Listeria and Yersinia have developed a similar "molecular strategy" to invade cells involving interaction of a unique bacterial surface protein (invasin and internalin) with one type of cell surface adhesion molecule (β1-integrin and E-cadherin). Despite these similarities, Listeria and Yersinia probably enter cells by distinct pathways, since they use different receptors that could trigger different cascades of intracellular signals leading to bacterial engulfment. For example, entry of Yersinia can be inhibited by staurosporin, a broad-range protein kinase inhibitor, whereas this inhibitor was reported to have no effect on Listeria entry. In contrast, entry of both organisms is inhibited by tyrosine kinase inhibitors such as genistein (Rosenshine et al., 1992; Tang et al., 1994; Velge et al., 1994). In the case of L. monocytogenes, the signaling events are probably triggered by binding of internalin to E-cadherin. Interestingly, recent data indicated direct links between cadherins and some signaling pathways (Mason, 1994; Kirkpatrick and Peifer, 1995). The EGF receptor is able to bind to β-catenin leading to β-catenin phosphorylation and disassembly of the cadherin–catenin complex from the actin filament network (Hoschuetzky et al., 1994). N-cadherin and β-catenin are substrates for the tyrosine kinase $p60^{v\text{-}src}$ and phosphorylation leads to inactivation of the N-cadherin-dependent cell adhesion (Hamaguchi et al., 1993). The cascade of signaling events and the intracellular factors that are associated with Listeria entry into nonphagocytic cells remain to be identified. Note that internalin represents a new type of heterophilic ligand for E-cadherin and that in contrast to N-cadherin for which two heterophilic ligands, the FGF receptor (Mason, 1994; Williams et al., 1994) and the rat soluble superoxide dismutase (Willems et al., 1995), have been suspected, only one (the surface $\alpha^E\beta_7$ integrin of intraepithelial lymphocytes) has been identified so far in the case of E-cadherin (Cepek et al., 1994; Karecla et al., 1995).

E-cadherin is a characteristic marker of epithelial cells. In vivo, in differentiated enterocytes, E-cadherin is only present on the basolateral surface of the cell (Boller et al., 1985; Cepek et al., 1994; Hermiston and Gordon, 1995), suggesting that L. monocytogenes does not penetrate the intestinal barrier by the apical pole of enterocytes. Our results favor a mechanism of translocation through M cells as a primary step in infection. Entry into enterocytes would represent a secondary step taking place at the basolateral pole. This hypothesis is in agreement with previous observations that, in vitro, Listeria preferentially invades Caco-2 cell islets at their periphery, i.e., where cellular basolateral surfaces are accessible (Gaillard et al., 1994; Temm-Grove et al., 1994).

Existence of an internalin-independent pathway of entry of L. monocytogenes into cells has been previously suggested (Gaillard et al., 1991; Dramsi et al., 1995). Results of our invasion assays indicate that this pathway is efficient for entry into fibroblasts, since the percentage of entry of the internalin negative mutant in any of the four fibroblasts tested and that of the wild type L. monocytogenes into fibroblasts expressing no E-cadherin were high (6%–12% of entry) compared with the reported percentage of entry of L. monocytogenes into Caco-2 cells (1%–3 % of entry) (Gaillard et al., 1991; Dramsi et al., 1993b). L. innocua does not express the bacterial factors required for this internalin/E-cadherin–independent pathway, since it does not enter fibroblast cells and, when transformed with the L. monocytogenes internalin gene, still enters less efficently than wild-type L. monocytogenes (2.5%–4% versus 48%–83%).

E-cadherin and internalin are both members of a family of surface proteins (Gaillard et al., 1991; unpublished data). In many tissues, more than one cadherin can be found and cadherins either are present in subpopulations of cells or are coexpressed in the same cell (Geiger and Ayalon, 1992). It is tempting to speculate that different members of the internalin family could interact with more or less affinity, with different members of the cadherin family. The nature and the relative amount of cadherin expressed in one particular cell type could be the basis for the species and cell tropism of L. monocytogenes.

Experimental Procedures

Bacterial Strains, Cell Lines, and Media

Listeria strains were grown in brain heart infusion (Difco) broth or plates at 37°C. For invasion assays we used a wild-type L. monocytogenes strain EGD (BUG 600) and its Δ*inlA* isogenic derivative (BUG 947) and a recombinant L. innocua strain expressing internalin (BUG 991) and its isogenic counterpart harboring the plasmid vector alone (BUG 994; Dramsi et al., 1995). For BUG 991 and BUG 994, erythromycin was added at 5 μg/ml in brain heart infusion. BUG 531 was grown in TGY broth (30 g/l Bio Trypcase [Bio Mérieux], 20 g/l yeast extract [Difco], 0.7 g/l H_2KPO_4, 4.12 g/l Na_2HPO_4 ($2H_2O$)). S. typhimurium strain SL1344 and Y. pseudotuberculosis strain YPIIIc were grown in Luria–Bertani broth.

Caco-2 cells were used between passages 70 and 90 and grown as described (Dramsi et al., 1995). S180 (Dunham and Stewart, 1953), S180L-CAM1, S180L-CAM2, and S180N-cad (Gg) (Mège et al., 1988; Matsuzaki et al., 1990) were grown in Dulbecco's modified Eagle's medium (DMEM; Techgen) with 15% fetal calf serum (GIBCO) in 10% CO_2.

Internalin Purification

Internalin was purified from culture supernatant of BUG 531 (Gaillard et al., 1991) grown 48 hr at 37°C with aeration (220 rpm) in TGY broth containing 1% glucose and 100 μg/ml spectinomycin. Proteins of the culture supernatant precipitating between 45% and 60% ammonium sulfate were harvested by centrifugation for 30 min at 4000 g at 4°C, resuspended in 70 ml TA buffer (50 mM Tris–HCl; 0.01% sodium azide; pH 7.5) and desalted and equilibrated in TA buffer by dialysis and ultrafiltration (cutoff value of 30,000). The sample was loaded on a 5 ml Hitrap Q column (Pharmacia) at 5 ml/min and internalin was eluted with a 100 ml salt gradient from 0 to 0.2M NaCl in TA buffer. Fractions of 1.5 ml were collected and those containing internalin were pooled, concentrated by ultrafiltration, and loaded on a Sephacryl S300 HR (or high resolution) column (26 mm in diameter and 55 cm in length) at 4 ml/min. Fractions of 2 ml were collected and those containing internalin were pooled and concentrated by ultrafiltration (cutoff value of 50,000). Protein concentration was determined with the bicinchoninic acid system (Pierce). Typically, 5 mg of purified internalin was obtained from 2 l of bacterial culture.

Purification of the Internalin Receptor

Internalin or BSA were covalently coupled to Affi-gel 15 (BioRad) in 0.1 M MOPS (pH 7.5) following manufacturer's instructions. Caco-2

cells were grown to 80% confluence in twelve 150 cm^2 flasks. Biotinylation and extraction of surface proteins, as well as affinity chromatography of the internalin receptor on the 0.9 ml internalin column, were performed as described in (Isberg and Leong, 1990). Proteins eluted with EDTA were collected in 200 μl fractions and 10 μl of each fraction were analyzed by SDS–PAGE on 8% polyacrylamide gels. Following electroblotting to nitrocellulose filters, biotinylated proteins were detected by probing filters with streptavidin covalently linked to horse radish peroxidase (Pierce) and chemiluminescent detection (ECL, Amersham).

NH_2-Terminal Sequence Determination

Proteins separated by SDS–PAGE were transferred overnight at 33 V in transfer buffer (Tris–HCl 50 mM, boric acid 50 mM) at room temperature to Immobilon P filters (Millipore) in a trans-Blot cell apparatus (BioRad). NH_2-terminal sequences were determined using an Applied Biosystems 473A sequencer.

Cell Binding Assays with Internalin-Coated Surfaces

To detect cell binding to internalin in microtiter plates, 96-well Maxisorp microtiter plates (Nunc) were coated 1 hr at 37°C with 100 μl per well of internalin solution at various concentrations in phosphate-buffered saline (PBS)/Ca^{2+}/Mg^{2+}. Wells were blocked 2 hr at 37°C with 200 μl per well of a 1% BSA solution in PBS/Ca^{2+}/Mg^{2+} and washed three times with PBS/Ca^{2+}/Mg^{2+}. To assay for cell attachment to internalin, aliquots (50 μl) of Caco-2 cells (10^6 cells per ml in DMEM containing 0.4% BSA) were incubated 1 hr at 37°C in each well. Wells were washed with 250 μl of DMEM and the number of attached cells was quantitated by the hexosaminidase assay (Landegren, 1984). The inhibitory effect of EDTA was tested by adding 10 mM EDTA to the Caco-2 cells suspension as well as to the DMEM used to wash the wells.

To determine the effect of anti E-cadherin antibodies (DECMA-1), 10, 50 and 100 μg/ml of the monoclonal antibody or 100 μg/ml of rabbit IgG used as a control were added to the 10^6 cells per milliliter suspension 50 min at 37°C before and during the cell binding assay.

"Cell-blot" experiments were used to visualize attachment of Caco-2 cells to proteins immobilized on filter replicas of polyacrylamide gels. Internalin untreated or subjected to a mild proteolytic treatment was incubated in Laemli sample buffer at 37°C or 100°C before SDS–PAGE analysis and transfer to nitrocellulose filters. Filters were processed for cell fixation as described (Isberg and Leong, 1988). Mild proteolytic treatment of internalin was obtained by incubating 200 μg/ml of purified protein with 343 U/ml of mutanolysin (Sigma) 30 min at 37°C in TA buffer (pH 6.5). The proteolytic degradation observed was not due to action of mutanolysin but to a contaminating proteolytic activity in the mutanolysin solution since this degradation activity was inhibited by phenylmethylsulfonyl fluoride (Schneewind et al., 1995; unpublished data).

Invasivity Assays

Invasivity assays were performed in 24-well tissue culture plates. Listeria strains were grown at 37°C to an OD_{600} of 0.8–1. The Yersinia strain YPIIIc was grown overnight at 22°C without agitation in 10 ml Luria–Bertani broth in a loosely capped 15 ml Falcon tube. Entry of this strain into cells is invasin dependent, since this strain had been cured of its virulence plasmid and thus had lost the plasmid-encoded invasion genes *ail* and *yadA*. The Salmonella strain SL1344 was grown overnight at 37°C with slow agitation in 25 ml Luria–Bertani, diluted to an OD_{600} of 0.17 and incubated for an additional 30 min under the same conditions. Before infection, bacteria were washed twice in DMEM and diluted in DMEM to a concentration of 10^7 bacteria per milliliter. Cells ($\sim 10^5$ cells per well) were washed with DMEM and incubated 45 min at 37°C in DMEM with or without rabbit polyclonal antibodies directed against L-CAM (Gallin et al., 1983) used at 50 μg/ml. Bacterial suspension (0.5 ml per well) was added to mammalian cells. After 1 hr incubation at 37°C, cells were washed twice with DMEM, overlaid with DMEM containing gentamicin (5 mg/l), and incubated 2 hr at 37°C. Cells were washed twice with DMEM and trypsinized to determine the total amount of cells per well at the end of the invasion assay. Trypsinized cells were lyzed by addition of Triton X-100 (0.2% final) and the number of viable bacteria was assessed on agar plates.

TEM and SEM

For TEM, 2×10^5 Caco-2 cells or 2×10^4 S180L-CAM2 cells were seeded in 35 mm diameter Petri dishes. Two days later, cells infected with 10^9 L. monocytogenes, 7×10^8 L. innocua (*inlA*), 10^8 Salmonella, or 7×10^8 Yersinia were washed twice with PBS, fixed in situ in (1.6% glutaraldehyde; 1% tannic acid in 0.1 M phosphate buffer [pH 7.4]), washed three times with phosphate buffer, postfixed 1 hr at room temperature with 1% osmium in the same buffer, washed twice with distilled water, and incubated overnight in 1% uranyl acetate in distilled water. After dehydration in a graded ethanol series, cells were flat embedded in epoxy resin. Specimens were prepared for horizontal sectioning along the plane of growth, cut with a diamond knife using a Reichert Ultracut-S microtome. Sections (70 nm) were picked up on regular 200 mesh, thin-bar copper grids, contrasted with uranyl acteate and lead citrate, and observed on a Philips CM12 electron microscope operating under standard conditions at 80 kV.

For SEM, cells were grown on 12 mm diameter glass coverslips and infected as for TEM. Infected cells were washed twice with PBS, fixed in situ in (1.6% glutaraldehyde; 1% tannic acid in 0.1M phosphate buffer [pH 7.4]), rinsed twice 5 min in the same buffer complemented with 3.7% sucrose, and postfixed in 1% osmium in 0.1 M cacodylate buffer. After one rinse in the same buffer, one rinse in distilled water, and one 5 min wash in 50% ethanol, cells were stained for 30 min in 50% ethanol and 0.5% (v/v) uranyl acetate, dehydrated in a graded series of ethanol, and treated 10 min with hexamethyldisilane (Bray et al., 1993). After drying, coverslips were mounted on pins, coated with gold using a Polaron E5100 SEM coating unit and examined on a JEOL JSM-35CF scanning electron microscope.

Scanning Confocal Microscopy

S180L-CAM2 cells (4×10^4) were seeded on glass coverslips in 6-well tissue culture plates. Two days later, cells infected with 6×10^6 L. innocua (*inlA*), 4×10^6 Salmonella, or 3×10^6 Yersinia were washed twice with PBS and fixed in situ with 3% paraformaldehyde in PBS. Infected cells were stained with DAPI and rhodamine-conjugated phalloidin. Bacteria were immunolabeled using a rabbit polyclonal serum specific for each bacterial species, an antilipopolysaccharide serum for Salmonella, serum PSTIII for Yersinia, serum R11-ISA for Listeria, and an fluorescein isothiocyanate–conjugated goat anti-rabbit secondary antibody (Nordic). Samples were mounted with mowiol and observed in a laser-scanning confocal microscope (Wild Leitz) at low magnification (40×).

Database Searches and Statistical Analysis

The Blast algorithm (Altschul et al., 1990) was used to search the nonredundant protein compilation of the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD). Statistical analysis of the data was performed with the InStat software (GraphPad).

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