INFECTION AND IMMUNITY, Dec. 1997, p. 5309–5319 Vol. 65, No. 12
0019-9567/97/$04.00+0

Internalin of *Listeria monocytogenes* with an Intact Leucine-Rich Repeat Region Is Sufficient To Promote Internalization

MARC LECUIT,[1] HÉLÈNE OHAYON,[2] LAURENCE BRAUN,[1]
JÉRÔME MENGAUD,[1] AND PASCALE COSSART[1]*

Unité des Interactions Bactéries-Cellules[1] and Station Centrale de Microscopie Electronique,[2] Institut Pasteur, 75724 Paris Cedex 15, France

Received 8 August 1997/Accepted 24 September 1997

Listeria monocytogenes **can use two different surface proteins, internalin (InlA) and InlB, to invade mammalian cells. The exact role of these invasiveness factors in vivo remains to be determined. In cultured cells, InlA is necessary to promote *Listeria* entry into human epithelial cells, such as Caco-2 cells, whereas InlB is necessary to promote *Listeria* internalization in several other cell types, including hepatocytes, fibroblasts, and epithelioid cells, such as Vero, HeLa, CHO, or Hep-2 cells. We have recently reported that the InlA receptor on Caco-2 cells is the cell adhesion molecule E-cadherin and demonstrated that nonpermissive fibroblasts become permissive for internalin-mediated entry when transfected with the gene coding for LCAM, the chicken homolog of the human E-cadherin gene. In this study, we demonstrate for the first time that the internalin protein alone is sufficient to promote internalization into cells expressing its receptor. Indeed, internalin confers invasiveness to both *Enterococcus faecalis* and internalin-coated latex beads. As shown by transmission electron microscopy, these beads were phagocytosed via a "zipper" mechanism similar to that observed during the internalin–E-cadherin-mediated entry of *Listeria*. Moreover, a functional analysis of internalin demonstrates that its amino-terminal region, encompassing the leucine-rich repeat (LRR) region and the inter-repeat (IR) region, is necessary and sufficient to promote bacterial entry into cells expressing its receptor. Several lines of evidence suggest that the LRR region would interact directly with E-cadherin, whereas the IR region would be required for a proper folding of the LRR region.**

Listeria monocytogenes is a ubiquitous gram-positive bacillus responsible for listeriosis, a severe animal and human foodborne disease characterized by central nervous system infections and fetal or neonatal infections associated with a high mortality rate (26). Similar to the gram-negative enteropathogenic bacteria *Salmonella*, *Shigella*, and *Yersinia* spp., *L. monocytogenes* has the capacity to induce its own uptake into nonphagocytic mammalian cells (6, 12, 14, 16). The ability to enter and reside in mammalian cells is critical for pathogenicity of *L. monocytogenes*. While *Salmonella* and *Shigella* enter cells via a "trigger mechanism," characterized by the formation of large membrane projections involving the interaction of type III secreted bacterial proteins with cellular targets and leading to a dramatic rearrangement of the actin cytoskeleton (33), *Listeria* enters cells like *Yersinia* does, by a so-called "zipper" mechanism (19, 29, 33) characterized by an intimate interaction between the bacterial body and the host cell membrane leading to the progressive engulfment of the bacterium. This process requires direct interaction between a bacterial surface protein, the internalin of *Listeria* or the invasin of *Yersinia*, and a mammalian cell surface receptor, E-cadherin or β1 integrins, respectively (13, 18, 20, 29).

To date, two surface proteins of *L. monocytogenes*, internalin (or InlA) and InlB, encoded by two genes (*inlA* and *inlB*) organized in an operon, are considered to play a major role in *Listeria* internalization into cultured cells (9, 13). InlA and InlB have common structural features also shared by other proteins constituting the internalin multigene family, i.e., two repeat regions, the leucine-rich repeat (LRR) region and the B-repeat region, separated by a highly conserved inter-repeat (IR) region (10, 13). The carboxy-terminal region of InlA contains an LPXTG motif, a signature sequence necessary for sorting and cell wall anchoring of many surface proteins of gram-positive bacteria, such as protein A of *Staphylococcus aureus* (SPA) (13, 32). Our laboratory recently reported that this region is necessary for anchoring internalin on the bacterial surface and that internalin exposed on the surface is capable of promoting entry (23).

Domain-swapping experiments have shown that the InlA cell wall anchoring region is also able to anchor SPA to the staphylococcal cell wall (31). InlB has no LPXTG motif in its carboxy-terminal region, but it contains repeated sequences beginning with the amino acids GW (13). We have recently shown that these GW repeats are necessary and sufficient to anchor InlB to the bacterial surface, even when the protein is added externally, a property reminiscent of the cell surface association of other proteins in gram-positive bacteria, such as the amidases of staphylococci (2).

Whereas InlB appears to allow *L. monocytogenes* entry into a wide variety of cell types such as hepatocytes, fibroblasts, and epithelioid cells, such as Vero, Hela, CHO, and Hep2 cells (17), InlA exhibits a more restricted tropism, limited to date to cells of epithelial origin such as the human enterocytic cell line Caco-2 or to the LCAM-transfected S180-fibroblastic cell line 2B2 (13, 29). This cell specificity of InlA-mediated entry is probably due to the nonubiquitous expression of the InlA receptor, E-cadherin in Caco-2 cells and LCAM (the proposed chicken homolog for human E-cadherin) in 2B2 cells.

Internalin confers an invasive phenotype to *L. innocua*, a noninvasive *Listeria* species closely related to *L. monocytogenes* (13, 29), but the mechanism by which internalin interaction with E-cadherin leads to entry is still unknown, and the existence of a cofactor common to both *L. innocua* and *L. mono-*

* Corresponding author. Mailing address: Unité des Interactions Bactéries-Cellules, Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris Cedex 15, France. Phone: (33-1)-45 68 88 41. Fax: (33-1) 45 68 87 06. E-mail: pcossart@pasteur.fr.

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s)	Selection (μg/ml)[a]	Abbreviated name[b]	Source
E. coli				
MC1061				Casadaban et al. (4)
DH5α				Gibco BRL
BL21(DE3)pLysS				Novagen
BUG1450	BL21(DE3)pLysS (ppE23)	Kan (30)		This work
L. innocua				
BUG499				J. Rocourt
BUG1390	BUG499(pGM10)	Ery (5)	*L. innocua* (*inlA*)2	This work
BUG1489	BUG499(pGM29)	Cm (7)	*L. innocua* (*inlA*)3	This work
BUG1395	BUG499(pGM13)	Ery (5)	*L. innocua* (*inlAΔ15LRRs*)	This work
BUG1469	BUG499(pGM20)	Ery (5)	*L. innocua* (*inlAΔ9LRRs*)	This work
BUG1471	BUG499(pGM21)	Ery (5)	*L. innocua* (*inlAΔ6LRRs*)	This work
BUG1441	BUG499(pGM18)	Ery (5)	*L. innocua* (*inlAΔIR*)	This work
BUG1396	BUG499(pGM14)	Ery (5)	*L. innocua* (*inlAΔBR*)	This work
BUG1444	BUG499(pGM19)	Ery (5)	*L. innocua* (*inlAΔPA*)	This work
BUG1435	BUG499(pGM16)	Ery (5)	*L. innocua* (*LRRs-SPA*)	This work
BUG1438	BUG499(pGM17)	Ery (5)	*L. innocua* (*LRRs-BSPA*)	This work
BUG1462	BUG499(pGM19)	Ery (5)	*L. innocua* (*BSPA*)	This work
BUG1460	BUG499(pGM25)	Ery (5)	*L. innocua* (*LRRs-IR-SPA*)	This work
BUG1496	BUG499(pGM30)	Cm (7)	*L. innocua* (*LRRs-IR-SPA*)3	This work
E. faecalis				
BUG1491			*E. faecalis* OG1X	P. Courvalin
BUG1493	BUG1491(pGM29)	Cm (7)	*E. faecalis* (*inlA*)3	This work
BUG1497	BUG1491(pGM30)	Cm (7)	*E. faecalis* (*LRRs-IR-SPA*)3	This work
S. epidermidis				
BUG1477			*S. epidermidis* BM3302	O. Chesneau
BUG1478	BUG1477(pGM29)	Cm (7)	*S. epidermidis* (*inlA*)3	This work
BUG1498	BUG1477(pGM30)	Cm (7)	*S. epidermidis* (*LRRs-IR-SPA*)3	This work

[a] Kan, kanamycin; Ery, erythromycin; Cm, chloramphenicol.
[b] (*inlA*)2 indicates that *inlA* is under the control of its promoter, and (*inlA*)3 and (*LRRs-IR-SPA*)3 indicate that *inlA* and *LRRs-IR-SPA*, respectively, are under the control of the *spa* promoter.

cytogenes and necessary for internalin-mediated entry has not been excluded.

In this work, we demonstrate that internalin is sufficient to promote internalization. We establish that noninvasive bacteria, unrelated to the genus *Listeria* and expressing internalin, or inert particles, such as internalin-coated latex beads, enter cells in an internalin–E-cadherin-dependent manner. We also demonstrate that the first two parts of the internalin protein, the LRR and IR regions, are necessary and sufficient for internalin-mediated entry. These data, together with our previous finding that among anti-internalin antibodies, only anti-LRR antibodies had an inhibitory effect on entry (28), demonstrate the crucial role of internalin LRR region in the entry process. We propose that the LRR region is the region interacting with the internalin receptor, whereas the IR region is required for the correct folding of the LRRs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Luria-Bertani medium was used for growth of *Escherichia coli* strains, and brain heart infusion (Difco Laboratories, Detroit, Mich.) was used for growth of *Listeria innocua*, *Enterococcus faecalis*, and *Staphylococcus epidermidis* strains. *E. coli* strains containing pUC19 derivatives were cultivated in the presence of ampicillin; *E. coli* and *L. innocua* strains containing pAT19 derivatives were cultivated in the presence of erythromycin; and *E. coli*, *L. innocua*, *E. faecalis*, and *S. epidermidis* strains containing pRB474 derivatives were cultivated in the presence of chloramphenicol. The antibiotic concentrations used for selection are indicated in Table 1.

Transfer of plasmids. Plasmids were introduced into *E. coli* by transformation, into *L. innocua* by conjugation or electroporation (22, 34), and into *E. faecalis* by electroporation (7). For electroporation of *S. epidermidis*, we used the following protocol kindly provided by O. Schneewind. Overnight *S. epidermidis* cultures at 37°C in tryptic soy broth (Difco) were diluted into fresh medium and grown until the optical density at 600 nm reached 0.4. The culture was centrifuged at 4°C at 4,500 × *g*. The cell pellet was resuspended in an equal volume of 0.5 M filtered sterilized sucrose, centrifuged at 4°C at 4,500 × *g*, and resuspended in 0.5 volume of 0.5 M sucrose and incubated on ice for 30 min. After a final centrifugation at 4°C at 4,500 × *g*, the cell pellet was resuspended in 0.1 volume of 0.5 M sucrose. Forty microliters of the cell suspension was mixed with 2 μl of plasmid DNA in an Eppendorf tube and incubated on ice for 1 min. The mixture was transferred to a cold 0.2-cm Bio-Rad Gene Pulser cuvette, and electroporation was performed with the following parameters: 25 μF, 2.5 kV, and 200 Ω. Medium (300 μl) was quickly added, the components were mixed, and the cells were plated directly onto agar plates with the appropriate antibiotic. Colonies appeared after overnight growth at 37°C.

Plasmid constructions. All of the plasmid constructions are derived from ppE10, a pUC19 derivative harboring the *inlA* promoter region and the *inlA* gene on a 3.3-kb *Sph*I-*Sac*I fragment (23). The *Sph*I-*Sac*I restriction fragments of ppE10 derivatives harboring each of the different constructs described below were subcloned in the shuttle vectors pAT19 (35) and/or pRB474 (modified by R. Brückner from pRB374 [3] by replacing the kanamycin resistance cassette with a chloramphenicol resistance cassette), between these two restriction sites, giving rise to pGM plasmids (see Table 2 for nomenclature). PCR fragments were sequenced with the T7 sequencing kit (Pharmacia Biotech).

Construction of plasmids harboring the *inlA* gene under the control of the *spa* (*SPA* gene) promoter. For subcloning of the *spa* promoter upstream of and in frame with the start codon of *inlA*, a 1,005-nucleotide *Sph*I-*Cla*I restriction fragment of ppE10 was replaced with a 273-nucleotide fragment, giving rise to ppE27. This 273-nucleotide fragment was obtained after digestion with *Sph*I and *Cla*I of a 1,200-nucleotide PCR-amplified product obtained from ppE10 with the primers OML22 (5′-ACATGCATGCGAAAAAAACGATATGTATG-3′), harboring an *Sph*I site, and OML6 (5′-GCTCGAGGTTTACTGGTGCATTTGTC C-3′), situated at the end of the LRR coding region. A 262-nucleotide PCR-amplified product of the *spa* promoting region (from plasmid pRIT5:*spa* harboring the *spa* promoter and the full-length *spa* gene, a gift of O. Schneewind [32]) was obtained with primers OML23 (5′-ACTTGCATGCCGGAGAGGGT AGC-3′), containing an *Sph*I site, and OML24 (5′-CGGCATCATGATTAATA CCCCCTGTATGTATTTG-3′), containing an *Nla*III site. This PCR-amplified

TABLE 2. Plasmids used in this study

Plasmid[a]	Host(s)	Marker(s)[b]	Relevant characteristic(s)	Source
pUC19	*E. coli*	Amp	Cloning vector	Yanisch Perron (38)
pAT19	*E. coli*, *L. innocua*	Amp Ery	Shuttle vector	Trieu-Cuot (35)
pRB474	*E. coli*, *L. innocua*, *E. faecalis*, *S. epidermidis*	Amp Cm	Shuttle vector	Brückner (3)
pRIT5:*spa*	*E. coli*	Amp Cm	pRIT5 derivative carrying *spa* promoter and *spa*	Schneewind (32)
ppE10/pGM10	*E. coli*, *L. innocua*	Amp Ery	pUC19/pAT19 derivatives carrying *inlA* promoter and *inlA*	Lebrun (23) and this work
ppE27	*E. coli*	Amp	pPE10 derivative deleted for *inlA* promoter	This work
ppE28/pGM29	*E. coli*, *L. innocua*, *E. faecalis*, *S. epidermidis*	Amp Cm	pUC19/pRB474 derivatives carrying *spa* promoter and *inlA*	This work
ppE13/pGM13	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *inlAΔ15LRRs*	Mengaud (28) and this work
ppE20/pGM20	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *inlAΔ9LRRs*	This work
ppE21/pGM21	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *inlAΔ6LRRs*	This work
ppE18/pGM18	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *inlAΔIR*	This work
ppE14/pGM14	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *inlAΔBR*	Mengaud (28) and this work
ppE19/pGM19	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *inlAΔPA*	This work
ppE16/pGM16	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *LRRs-SPA*	This work
ppE17/pGM17	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *LRRs-BSPA*	This work
ppE19/pGM19	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *LRRs-BSPA*	This work
ppE25/pGM25	*E. coli*, *L. innocua*	Amp Ery	pPE10/pGM10 derivatives carrying *LRRs-IR-SPA*	This work
pGM30	*E. coli*, *L. innocua*, *E. faecalis*, *S. epidermidis*	Amp Cm	pGM29 derivative carrying *LRRs-IR-SPA*	This work
ppE23	*E. coli* BL21(DE3)pLysS	Kan Cm	pET28b derivative carrying *LRRs* of *inlA*	This work

[a] ppE refers to *E. coli* plasmids; pGM refers to shuttle plasmids.
[b] Amp, ampicillin; Ery, erythromycin; Cm, chloramphenicol; Kan, kanamycin.

product was digested by *Nla*III, generating compatible ends with the *Sph*I site, and cloned at the *Sph*I site of ppE27, reconstituting only one *Sph*I site situated upstream of the *spa* promoter. The orientation of the subcloned fragment was checked by PCR with the primers OML23 and OML6. The plasmid harboring the *spa* promoter fused to the start codon of *inlA* was named ppE28.

ppE28 was digested with *Sph*I and *Sac*I, and the restriction fragment harboring the *spa* promoter fused to *inlA* was subcloned at the same restriction sites in pRB474, a shuttle vector for gram-positive bacteria, giving rise to pGM29. pGM29 was transferred by electroporation in *L. innocua*, *E. faecalis*, and *S. epidermidis.*

Construction of internalin variants. Construction of the two internalin variants InlAΔ15LRRs and InlAΔBR has been described previously (28). The internalin variants InlAΔIR, InlAΔ9LRRs, and InlAΔ6LRRs were constructed as follows (see Fig. 4).

The internalin variant InlAΔIR corresponds to an internalin lacking its IR region (amino acids [aa] 414 to 517). It was constructed by replacing the 1,390-nucleotide *Bcl*I-*Kpn*I restriction fragment of ppE10 with a 930-nucleotide PCR-amplified product from ppE10, which had been digested by *Bgl*II and *Kpn*I, and was obtained with primer OML20 (5′-GAAGATCTTTTATTGACTGAACCA GCT-3′), which contains a *Bgl*II restriction site, and primer OML18 (5′-GGG GTACCTCTCCTTGATTCTAG-3′), which contains a *Kpn*I site.

The internalin variant InlAΔPA corresponds to an internalin lacking its pre-anchor (PA) region (aa 714 to 765). It was constructed by replacing the 492-nucleotide *Pfl*MI-*Kpn*I restriction fragment of ppE10 with a 184-nucleotide PCR-amplified product from ppE10, which had been digested by *Pfl*MI and *Kpn*I, and was obtained with primer OML19 (5′-CGAGCCAGCAACTGGAC TCCCTACAACTGGC-3′), which contains a *Pfl*MI site, and primer OML18.

The internalin variant InlAΔ9LRRs corresponds to an internalin lacking its first nine LRRs (aa 74 to 260, with addition of an arginine residue at position 75). It was constructed by deletion of a 558-nucleotide fragment after digestion of ppE10 by *Bgl*II and *Bst*EII; the resulting deleted *inlA* gene was treated with T4 polymerase to generate blunt ends, which were in-frame ligated.

The same strategy was used to construct the internalin variant InlAΔ6LRRs, which corresponds to an internalin lacking its last six LRRs (aa 261 to 413). It was constructed by deletion of a 461-nucleotide fragment after digestion of ppE10 by *Bst*EII and *Bcl*I; the resulting deleted *inlA* gene was treated with T4 polymerase to generate blunt ends, which were in-frame ligated.

Construction of internalin-protein A chimeric proteins (see Fig. 6). The 633-aa chimeric protein LRRs-SPA is a fusion of the first 414 aa of InlA to the carboxy-terminal region of SPA (aa 305 to 524). It was constructed by replacing the *Bcl*I-*Kpn*I digestion fragment of ppE10 with a 750-nucleotide PCR-amplified product from plasmid pRIT5:*spa* that had been digested with the same restriction enzymes. This PCR product was obtained with the primers OML3bis (5′-GAAGATCTTGACGATCCTTCGGTGAGC-3′), which contains a *Bgl*II site, and OML4 (5′-GCGGTACCATTTCAAATAAGAATGTGTT-3′), which contains a *Kpn*I site.

The 801-aa chimeric protein LRRs-BSPA is a fusion of the first 414 aa of InlA to a longer carboxy-terminal region of SPA (aa 137 to 524). It was constructed by replacing the *Bcl*I-*Kpn*I digestion fragment of ppE10 with a 1,260-nucleotide PCR-amplified product from plasmid pRIT5:*spa* obtained with the primers OML11 (5′-GAAGATCTTACTAACGTTTTAGGTGAAGC-3′) and OML4, which contain a *Bgl*II site and a *Kpn*I site, respectively, and that had been digested with these two enzymes.

The 461-aa chimeric protein BSPA is a fusion of the first 73 aa of InlA (upstream of the LRRs) to the last 388 aa of SPA (aa 137 to 524). It was constructed by replacing the *Bgl*II-*Kpn*I fragment of *inlA* with an OML11-OML4 PCR product from plasmid pRIT5:*spa* that had been digested by *Bgl*II and *Kpn*I.

The 737-aa chimeric protein LRRs-IR-SPA is a fusion of the first 517 aa of InlA to the carboxy-terminal region of SPA (aa 305 to 524). It was constructed by replacing the *Bst*XI-*Kpn*I digestion fragment of ppE10 with a 750-nucleotide PCR-amplified product from plasmid pRIT5:*spa* digested with the same restriction enzymes. This PCR product was obtained with primers OML21 (5′-CTGC AGAACCAAAGAAGTGGACGATCCTTCGGTGAGC-3′), which contains a *Bst*XI site, and OML4.

Covalent coupling of internalin to latex beads. Internalin was purified as previously described (29). A total amount of 400 μg of internalin in 80 μl of 15 mM sodium acetate buffer (pH 5.0) was mixed in an Eppendorf tube with 200 μl of a 2% aqueous suspension of carboxylate-modified latex Texas red fluorescent beads (1 μm in diameter; Molecular Probes). After 15 min of incubation on a rocker shaker at room temperature, 1.6 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carboiimide (EDAC) was added, the solution was vortexed, and the pH was adjusted to 6.5 with diluted NaOH. The reaction mixture was incubated on a rocker shaker for 2 h at room temperature. Glycine was then added to a final concentration of 100 mM, and the mixture was incubated for 30 min at room temperature. The beads were then centrifuged for 20 min at 2,000 × *g*, and the supernatant was removed. Beads were washed three times in 50 mM phosphate-buffered saline (PBS) and then were finally resuspended in 50 mM PBS plus 1% bovine serum albumin (BSA). For control beads, the covalent coupling of BSA to beads was done by the same procedure.

Cell cultures, invasion assays, and immunofluorescence labeling. Caco-2 cells and 2B2 cells (S180 fibroblasts stably transfected with the LCAM gene) were grown as described previously (29). L2071 cells and LE6 cells (L2071 parental fibroblasts and L2071 fibroblasts stably transfected with the LCAM gene, respectively; a gift of R. Brackenbury [5]) were cultivated in Dulbecco's modified Eagle's medium (Gibco) plus 10% fetal calf serum and in the presence of 400 μg of G418 per ml for LE6 cells.

Invasion assays were performed in 24-well plates with a gentamicin killing assay as described previously (10). Bacteria were added to cells at a multiplicity

of infection (MOI) of 100. After 1 h of infection and 2 h of incubation in the presence of gentamicin, the cells were lysed and the lysates were plated for bacterial counting. The percentage of invasion was determined by dividing the number of surviving bacteria at the end of the test by the number of bacteria present in the inoculum. The gentamicin concentration used (10 μg/ml for *L. innocua* and *S. epidermidis* and 50 μg/ml for *E. faecalis*) was shown to kill all extracellular bacteria in less than 2 h. Invasion assays were done in three independent experiments, each time in duplicate.

To test the invasive properties of InlA- or BSA-coated latex beads, L2071 and LE6 cells were grown on glass coverslips. Latex beads were added to cells at a rate of 100 beads/cell. After 1 h of incubation and five washes with PBS, the cells were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature, rinsed three times in PBS, and incubated for 1 h with anti-BSA rabbit polyclonal antibodies (dilution, 500; Sigma). This anti-BSA antibody reacts with InlA- and BSA-coated beads, since both types of beads were incubated in PBS plus 1% BSA at the final blocking step of the coupling. The coverslips were washed three times in PBS and incubated for 1 h with goat anti-rabbit polyclonal antibodies conjugated with fluorescein isothiocyanate (FITC); the cells were then permeabilized with PBS plus 0.4% Triton X-100 (Sigma) for 5 min, rinsed three times in PBS, and incubated with FITC-phalloidin (Sigma; 0.1 μg/ml). After three final rinses in PBS, coverslips were examined with an Optiphot-2 Nikon microscope and observed by phase-contrast microscopy and epifluorescence or examined with a laser-scanning confocal microscope (Wild Leitz). With this labeling technique, F-actin was labeled in green and the extracellular beads were labeled in green and red, whereas intracellular beads, which were not accessible to the antibodies during the labeling steps, appeared only in red (see Fig. 2B).

Immunogold labeling of bacteria and transmission electron microscopy. For immunogold labeling, mid-log-cultured bacteria were harvested by centrifugation at 4,000 × *g*, washed with PBS, and resuspended at room temperature for 30 min in 500 μl of PBS and 500 μl of filtered 8% paraformaldehyde in phosphate buffer (0.1 M [pH 7.2]). Drops of 15 to 35 μl were deposited on parafilm and adsorbed for 5 min onto freshly hydrophilized (by glow discharge) Formvar carbon-coated nickel grids. The grids were subsequently floated, Formvar facing down, on the following reagents: PBS–50 mM NH_4Cl (2 min), PBS–1% normal goat serum (5 min), G6.1 or K18.4 anti-internalin monoclonal antibodies diluted in PBS–1% BSA–1% normal goat serum (1 h), PBS (four washes, 1 min each), immunogold electron microscopy goat anti-mouse conjugates (10-nm-diameter particles; British BioCell International) diluted 1/20 in PBS–0.01% fish skin gelatin (Sigma G7765) (1 h), PBS (one wash, 1 min), distilled water (three washes, 1 min each), 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) (2 min), and distilled water (3 washes, 2 min each). Grids were observed under a Philips CM12 electron microscope operating under standard conditions at 80 kV.

For transmission electron microscopy assays of cells incubated with beads, cells were seeded 2 days before the experiments into 35-mm-diameter dishes (Corning) at a density of 8×10^4 cells per ml in cell culture medium. Cells incubated with InlA-coated beads (100/cell) were washed twice in PBS, fixed in situ at 4°C in 1.6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), washed three times with phosphate buffer, postfixed for 30 min at room temperature with 2% osmic acid in the same buffer, washed three times in distilled water, and dehydrated in a graded ethanol series. The ethanol was removed, and 1 ml of *N*-butylglycidyl ether was added to each petri dish. The dishes were allowed to stand until the cell monolayer appeared rippled (1 to 2 min). After gentle agitation with a rocking motion, a thin sheet of cells separated and floated freely to the surface of the liquid. This floating sheet was transferred to an Eppendorf tube and centrifuged. The supernatant was removed and replaced with epoxy-1,2-propane. The sheet was then embedded in an epoxy resin (Epikote 812). After sectioning and counterstaining with uranyl acetate and lead citrate, microscopic observations were performed with a Philips CM12 and/or a Jeol 1010 electron microscope operating at 80 kV. Because of treatment with epoxy-1,2-propane, beads were dissolved or partially dissolved and were thus visualized as "bead ghosts" (see Fig. 3).

Purification of internalin LRRs6×His. The LRR-encoding sequence of *inlA* was amplified by PCR from plasmid ppE10 with the primers OML5 (5′-GCCA TGGCTACAATTACACAAGATACT-3′), which contains an *Nco*I site, and OML6 (5′-GCTCGAGGTTTACTGGTGCATTTGTCC-3′), which contains an *Xho*I site, and cloned in frame upstream from the His tag coding sequence in the pET28b expression vector (Novagen), giving rise to pPE23. The nucleotide sequence of the PCR-amplified product was verified by sequencing. For expression of the recombinant protein, the construct was transferred into *E. coli* BL21(DE3)pLysS (Novagen). Strain BL21(DE3)pLysS containing ppE23 was grown in 2YT culture medium (Bacto tryptone, 16 g/liter; yeast extract, 10g/liter; NaCl, 5 g/liter) with 30 μg of kanamycin per ml at 37°C with shaking until the early log phase, and expression was induced by addition of 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 3 h of induction, bacteria were harvested by centrifugation, resuspended in I40 buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole [pH 7.5]), frozen at −20°C, thawed, and sonicated on ice. Cell debris were removed by centrifugation, and protein was purified from the supernatant by metal affinity chromatography according to the manufacturer's instructions (Novagen). LRRs6×His was eluted with I300 buffer (20 mM sodium phosphate, 0.5 M NaCl, 300 mM imidazole [pH 7.5]), concentrated to the desired volume in TA buffer (50 mM Tris HCl, 0.01% sodium azide [pH 7.5]) with Centriprep 30 and Centricon 30 devices (Amicon), and gel filtrated on a Sephacryl S300HR column. Elution fractions containing purified LRRs6×His were pooled and then concentrated with Centriprep 30 and Centricon 30 devices, and the protein was finally frozen at −80°C in TA buffer until use. The protein concentration was determined with the bicinchoninic acid (BCA) system (Pierce).

RESULTS

Internalin is sufficient to promote internalization. Caco-2 cells or fibroblastic S180 cells transfected with the LCAM gene encoding the chicken homolog of human E-cadherin (2B2 cells) are permissive to *L. monocytogenes* or *L. innocua* cells expressing internalin (13, 29). In the present study, we have also used a couple of new cell lines, L2071 and LE6, to test internalin-mediated entry. L2071 cells are fibroblastic cells of murine origin expressing no detectable level of endogenous cadherin, and LE6 cells are transfected L2071 cells expressing LCAM (5). As expected, no internalin-dependent entry of *L. monocytogenes* or *L. innocua* expressing internalin was detected in L2071, whereas the LE6 cells were permissive for internalin-mediated entry (data not shown). These results confirmed our previous results obtained with the cell line couple, S180 and 2B2, that LCAM behaves as a receptor for internalin (29).

In order to better understand the molecular aspects of internalin-dependent entry, we first investigated whether internalin is sufficient to promote internalization of gram-positive bacteria unrelated to the genus *Listeria* into cells expressing its receptor.

(i) Expression of internalin in noninvasive gram-positive bacteria confers invasiveness. To allow heterologous expression of internalin, two strong promoters, the *vegII* promoter of *B. subtilis* and the *spa* promoter of the gene encoding SPA were placed in tandem upstream of *inlA* in the shuttle vector pRB474, giving rise to pGM29 (Table 2). pGM29 was introduced by electroporation into several noninvasive gram-positive bacteria. We first used *S. aureus*. Preliminary gentamicin survival assays with *S. aureus* showed that the background level of entry of this bacterial species in Caco-2 or 2B2 cells was as high as that of *L. innocua* expressing internalin, rendering the interpretation of invasion tests with *S. aureus* expressing internalin difficult. We thus introduced pGM29 into the following bacterial strains: *L. innocua*, *E. faecalis*, and *S. epidermidis*, which are noninvasive for Caco-2 cells (Fig. 1B) and 2B2 cells (data not shown). Expression of internalin in these bacteria was evaluated by Western blotting (data not shown) and by immunogold labeling (Fig. 1A). These experiments showed that internalin is expressed, at a similar level, on the surface of these three types of recombinant bacteria.

The ability of these bacteria expressing internalin to enter the Caco-2 cell line was evaluated with the gentamicin survival assay. Arbitrarily, the level of entry of *L. innocua* cells expressing InlA was standardized to 100, and the levels of entry of the other bacteria were calculated as relative values. As shown in Fig. 1B, these assays demonstrated that (i) heterologous expression of internalin in *L. innocua* conferred invasiveness, as previously described (13); (ii) internalin heterologous expression in *E. faecalis* also conferred invasiveness; and (iii) internalin heterologous expression in *S. epidermidis* did not confer invasiveness (Fig. 1B) or adhesiveness (data not shown). Similar experiments were performed with 2B2 cells and gave the same results. We also used the double immunofluorescence labeling technique previously described in reference 36 and were unable to detect any intracellular internalin-expressing *S. epidermidis* cells, in agreement with the invasion assays. No adhesion to the plastic surface or formation of bacterial biofilm of *S. epidermidis* cells expressing or not expressing internalin

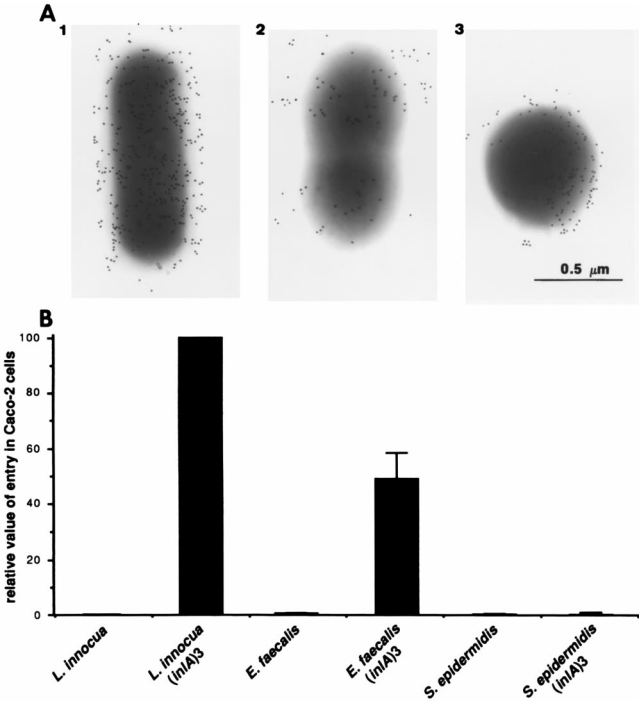

FIG. 1. Heterologous expression of internalin in *L. innocua*, *E. faecalis*, and *S. epidermidis*. (A) Internalin detection on the surface of *L. innocua* (*inlA*)3 (A1), *E. faecalis* (*inlA*)3 (A2), and *S. epidermidis* (*inlA*)3 (A3). (B) Invasiveness assays with Caco-2 cells of *L. innocua*, *L. innocua* (*inlA*)3, *E. faecalis*, *E. faecalis* (*inlA*)3, *S. epidermidis*, and *S. epidermidis* (*inlA*)3. The level of entry of *L. innocua*(*inlA*)3 has been reported to 100. The level of entry of the other bacteria is given as a relative value. Results are means ± standard deviations of three different experiments, each time done in duplicate.

was observed (data not shown). Taken together, these results demonstrate that internalin expression in *L. innocua* and in *E. faecalis* promotes entry. This is not the case for *S. epidermidis.*

(ii) Internalin-coated latex beads enter cells expressing the internalin receptor. In order to definitively determine if internalin is sufficient to promote entry or if a bacterial cofactor common to *L. monocytogenes*, *L. innocua*, and *E. faecalis* but absent in *S. epidermidis* is required for the internalin-mediated entry process, we coated 1-μm-diameter latex beads with purified internalin and determined their ability to enter cells expressing the internalin receptor. Preliminary experiments with Caco-2 cells showed that the background level of entry of various types of latex beads (from 1 to 6 μm in diameter) was high. This nonspecific entry was shown to preferentially occur in the borders of the cell islets, where internalin-mediated entry also preferentially occurs (15). The same technical problems were encountered with 2B2 cells. We therefore used the other two cell lines, L2071 and LE6. In these cells, the background level of entry of 1-μm-diameter latex beads covalently coated with internalin was extremely low. We thus tested the entry of internalin-coated beads in these cells and used BSA-coated beads as a negative control.

As shown in Fig. 2A, internalin-coated beads associated very efficiently with LE6 cells, whereas no cell association of beads was observed with L2071 cells. BSA-coated beads did not associate with LE6 or L2071 cells. Epifluorescence and confocal microscopy observation then allowed us to distinguish intracellular beads (appearing in red) from extracellular beads (appearing in red and green after FITC labeling [see Materials and Methods]). As shown in Fig. 2B, the efficiency of bead uptake was very high, roughly more than a third of associated beads being intracellular. In control experiments with nontransfected L2071 cells and/or BSA-coated beads, no intracellular beads were observed. The specificity of these experiments was further strengthened by the observation of the inhibitory effect on entry of anti-internalin or anti-LCAM antibodies, as previously described for *Listeria* internalin-mediated entry (data not shown) (28, 29). We then examined the morphological changes that occurred during entry of internalin-coated beads by using transmission electron microscopy. As shown in Fig. 3, internalin-coated beads were closely apposed to the cytoplasmic membrane during the whole entry process and entered cells through the so-called "zipper mechanism," as has been described in detail for internalin-mediated entry of *L. monocytogenes* and *L. innocua* cells expressing internalin (29).

Taken together, these results demonstrate that the presence of internalin on the surface of a particle is sufficient to induce its phagocytosis into cells expressing the internalin receptor. These data also suggest that the noninvasiveness of *S. epidermidis* cells expressing internalin was probably due to antiphagocytic factors present in this bacterial species, whose nature remains to be determined.

The LRR and IR regions are necessary for entry. Internalin being sufficient to induce entry, we generated deletions in the *inlA* gene to determine the regions critical for its function. These deletions were designed to obtain six internalin variants lacking either the first nine LRRs (InlAΔ9LRRs), the last six LRRs (InlAΔ6LRRs), all of the LRRs (InlAΔ15LRRs), the IR region (InlAΔIR), the B-repeat region (InlAΔBR), or the PA region, which is situated between the B-repeat region and the cell wall anchor region (InlAΔPA) (Fig. 4A). These genetic constructs were introduced into *L. innocua* by conjugation or electroporation. Expression of internalin variants into *L. innocua* was verified by Western blotting experiments with crude bacterial extracts by using monoclonal antibodies against internalin. We used the antibody K18.4, which recognizes the B-repeat region, to detect the first three constructs and the antibody G6.1, which recognizes the LRRs, to detect the other three (see reference 28 and our unpublished data). The variant proteins were all expressed (Fig. 4B). Immunogold labeling with K18.4 or G6.1 was performed with recombinant *L. innocua* cells to demonstrate and quantify the cell surface exposition of the internalin variants by using *L. innocua* cells expressing internalin as a control. (Note that all these constructs are expressed under the *inlA* promoter.) The average number of gold particles associated with bacteria was determined in each case and is expressed as a relative value of the average number of gold particles associated with *L. innocua* expressing wild-type internalin, arbitrarily standardized at 100. As shown in Fig. 5A, surface expression of all of the internalin variants was at least equivalent to that of internalin in *L. innocua*, suggesting that all of these constructs had kept the ability to be anchored to the cell wall and were exposed to the bacterial surface in a similar way.

We then tested the ability of *L. innocua* cells expressing each internalin variant to enter into cells permissive for internalin-mediated entry, such as Caco-2 cells or 2B2 cells. Invasion assays with these two cell lines gave similar results. Invasion assays with 2B2 cells, as shown in Fig. 5B, demonstrated that InlAΔBR and InlAΔPA conferred an invasiveness level comparable to that of full-length internalin, whereas the other variants, with deletion of a part (nine or six) or all of the LRRs or of the IR region did not confer invasiveness to *L. innocua* anymore. These results show that the two amino-terminal regions of internalin, namely LRRs and IR, are both necessary for internalin-mediated entry to occur.

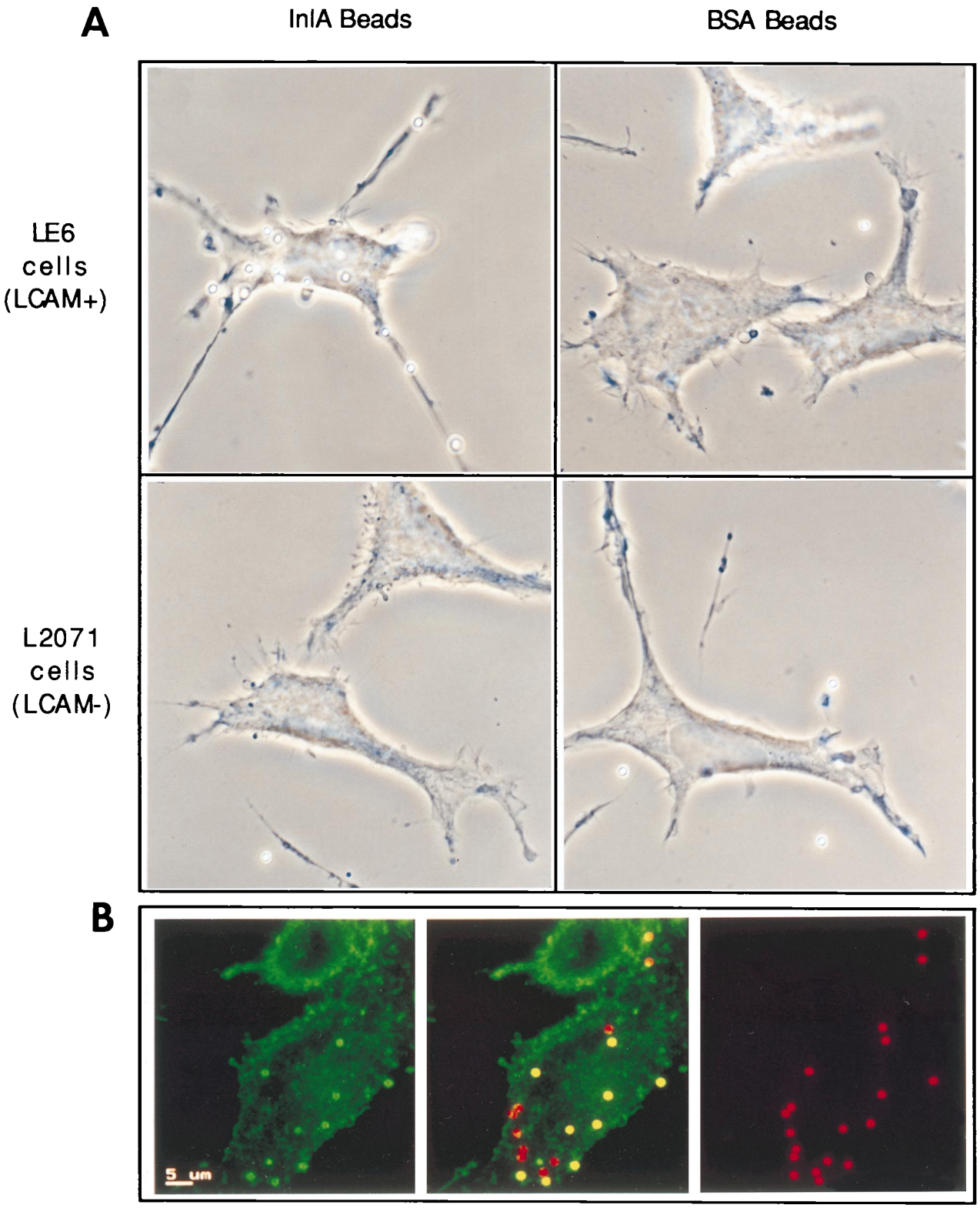

FIG. 2. Cell association and cell invasion of InlA-coated beads. (A) Phase-contrast observation of beads associating with LE6 cells expressing LCAM (top panel) and L2071 cells (bottom panel) with either InlA-coated beads (left panel) or BSA-coated beads (right panel). (B) Confocal laser-scanning micrographs of LE6 cells incubated with InlA-coated beads. External beads and actin cytoskeleton were FITC labeled, thus appearing in green (left). All of the beads (internal and external) are Texas red labeled by the manufacturer (right). The central image is a superposition of the lateral ones. Internal beads are only Texas red labeled, and external beads are FITC and Texas red labeled, and thus appear in yellow.

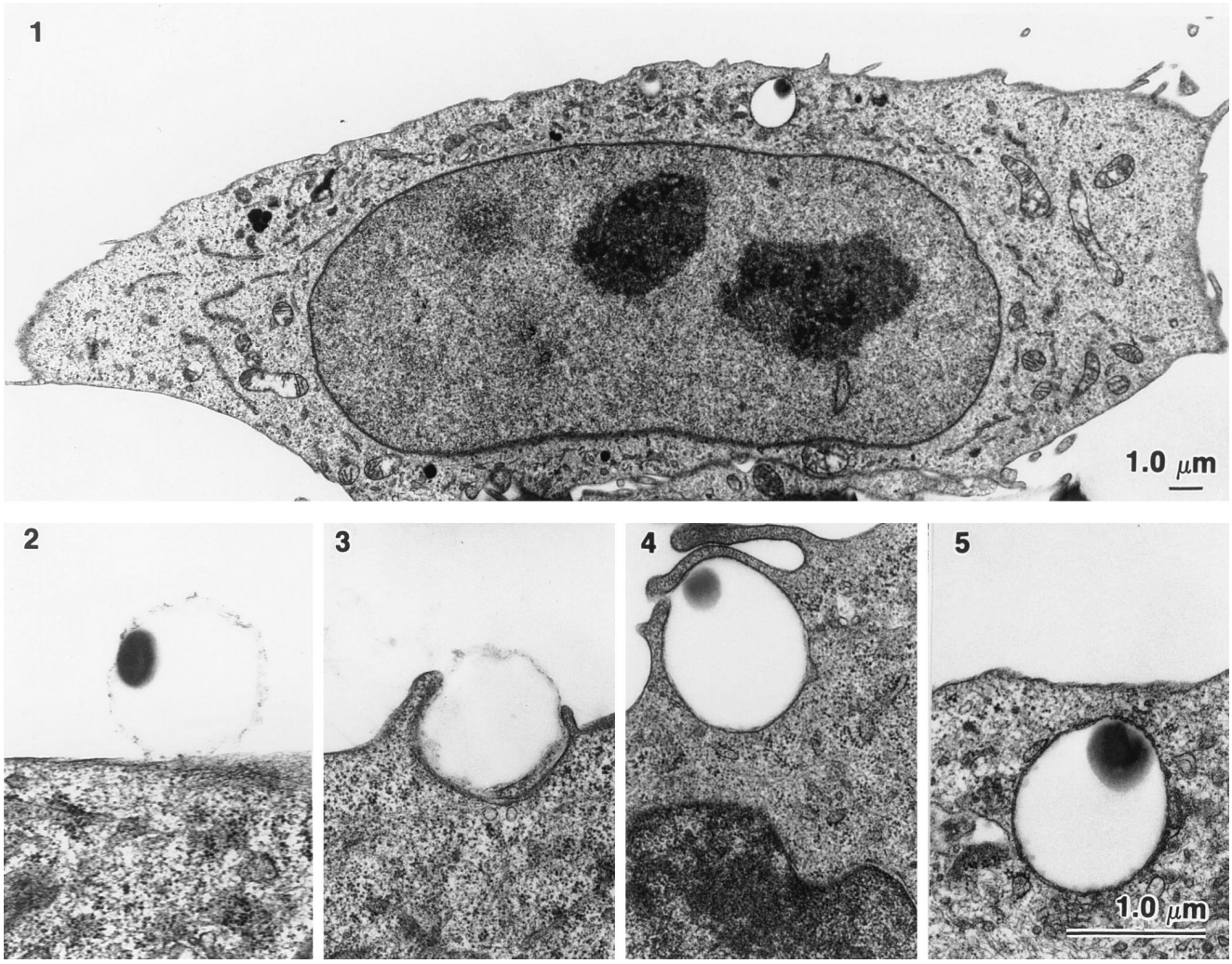

FIG. 3. Transmission electron micrographs of InlA-coated latex beads entering LE6 cells. (1) Global view of an LE6 cell having phagocytosed two InlA-coated latex beads. (2 to 5) InlA-coated latex beads at different stages of cell invasion, exemplifying the "zipper" phenotype of internalin-mediated entry of the InlA beads. Treatment with epoxy-1,2-propane partially dissolved the beads, which are thus visualized as "bead ghosts."

A chimeric protein fusing the LRR and IR regions of internalin to the carboxy-terminal region of SPA is sufficient for entry. To identify the minimal portion of internalin required for its function, we made use of chimeric proteins obtained by fusing the LRRs or the LRRs-IR regions to the carboxy-terminal region of SPA. Protein A, like internalin, contains at its carboxy-terminal end a cell wall anchoring signal (32). Four gene fusions were designed to obtain LRRs-SPA (633 aa), LRRs-BSPA (801 aa), LRRs-IR-SPA (737 aa), and BSPA (461 aa) (Fig. 6A). The gene fusions were introduced in *L. innocua*, and protein expression was verified by Western blotting with the G6.1 antibody, as shown in Fig. 6B. Surface expression of these proteins was also semiquantitatively evaluated by immunogold labeling, as previously done for the internalin variants. All of the chimeric proteins were surface exposed and were expressed at least as well as internalin, showing in addition that the cell wall anchoring signal of SPA was functional in *Listeria* (data not shown).

We tested the invasiveness conferred by these chimeric proteins. LRRs-SPA, LRRs-BSPA, and BSPA were not able to confer any invasiveness to *L. innocua*, in contrast to the chimeric protein LRRs-IR-SPA, which was as efficient as internalin in promoting bacterial entry (Fig. 7). The plasmid pGM30, encoding LRRs-IR-SPA under the control of the *spa* promoter, was then introduced into *E. faecalis* and *S. epidermidis*. LRRs-IR-SPA surface expression was similar to that of internalin in these bacteria, and LRRs-IR-SPA was as efficient as internalin at conferring invasiveness to *E. faecalis* and, in agreement with the results presented above failed to confer invasiveness to *S. epidermidis* (data not shown).

Taken together, these results demonstrate that surface expression of all LRRs and IR is not only necessary for internalin-mediated entry into cells, but it is also sufficient.

Unlike internalin, the purified LRR polypeptide does not bind cells permissive for internalin-mediated entry. We have previously shown that among nine anti-internalin antibodies, only anti-LRR monoclonal antibodies had an inhibitory effect on entry, suggesting that internalin LRRs play a crucial role in its function (28) and that the IR region is not responsible for its functional specificity. In addition, the IR region is the most conserved region among proteins of the internalin multigene family (10). In contrast, LRRs are different in number and nature among members of the family. Furthermore, the common hallmark of LRRs is to be classically involved in protein-

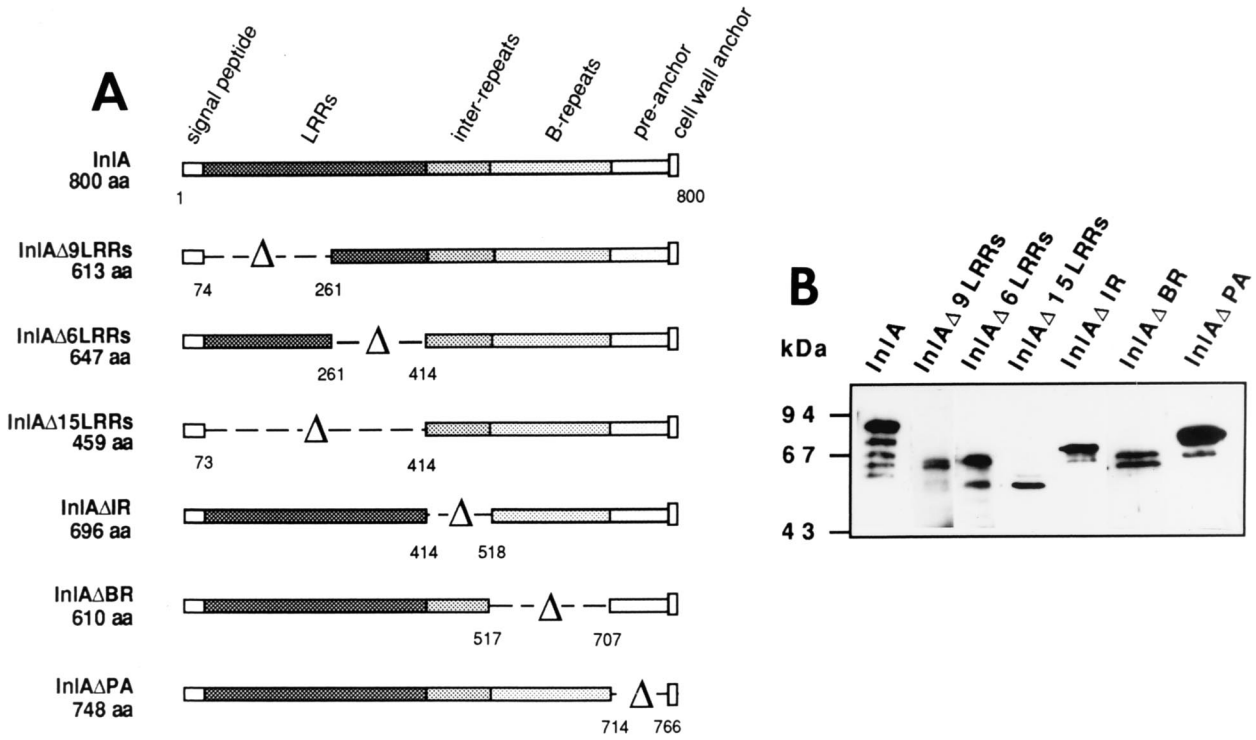

FIG. 4. Schematic representation of internalin variants and Western blotting analysis of these variants. (A) Wild-type InlA is represented on the top. From its amino-terminal part to its carboxy-terminal part (from left to right), InlA is made of a signal peptide, an LRR region, an IR region (IR), a B-repeat region (BR), a PA region, and a cell wall anchor region. Each internalin variant, InlAΔ9LRRs, InlAΔ6LRRs, InlAΔ15LRRs, InlAΔIR, InlAΔBR, and InlAΔPA, is represented. The lacking region is symbolized by a dotted line and Δ. The total length of the truncated protein is indicated, and the numbers at each end of internalin or of internal deletions indicate the amino acid positions in the wild-type protein. (B) Immunoblotting analysis of InlA variants expressed in *L. innocua*. Aliquots of total protein extracts of each strain expressing InlA variants were boiled in sodium dodecyl sulfate sample buffer and applied to a sodium dodecyl sulfate–10% polyacrylamide gel. Separated proteins were transferred onto nitrocellulose and were first incubated with the K18.4 monoclonal antibody for the four left lanes and the G6.1 monoclonal antibody for the three right lanes and then incubated with an antimouse horseradish peroxidase-conjugated antibody. Conjugated antibodies were detected by enhanced chemiluminescence (ECL system; Amersham) as described previously (9).

protein interactions (21). It was thus tempting to hypothesize that internalin LRRs interact directly with the internalin cellular receptor.

To test this hypothesis, we purified a recombinant LRR polypeptide with a carboxy-terminal tag of six histidine residues (LRRs6×His). Using the hexosaminidase colorimetric assays that we previously used to demonstrate that Caco-2 cells bind to purified internalin (29), we tested whether Caco-2 cells were able to bind to LRRs6×His. As shown in Fig. 8, we were unable to detect any cell association with LRRs6×His, suggesting that the purified protein was not able to bind cells expressing the internalin receptor. Although one cannot exclude that this purified protein was denatured, this result, together with the absence of any invasive phenotype in *L. innocua* cells expressing InlAΔIR, LRRs-SPA, and LRRs-BSPA, suggests that the internalin LRRs, in the absence of IR, are unable to interact with internalin receptor.

DISCUSSION

Internalin is necessary and sufficient to promote entry. The results presented in this paper definitely establish that the internalin protein alone is sufficient to promote internalization. Previous data had shown that heterologous expression of internalin in *L. innocua* confers invasiveness to this noninvasive *Listeria* species and suggested that internalin was sufficient to promote entry (13). However, *L. innocua* is the *Listeria* species that is most closely related to *L. monocytogenes*, and *inl*-like genes are present in this bacterium (10), suggesting that a cofactor common to these two species and required to promote entry could exist. The results presented here showing that *E. faecalis* cells expressing internalin became invasive reinforced the hypothesis that internalin was sufficient. However, the fact that *S. epidermidis* cells expressing internalin remained noninvasive reactivated the possibility that a cofactor present in *L. monocytogenes*, *L. innocua*, and *E. faecalis* but absent in *S. epidermidis*—which is more distantly related to the genus *Listeria* than is *E. faecalis* (8)—was required for internalin-mediated entry. Alternatively, *S. epidermidis* could possess an antiphagocytic factor blocking internalin-mediated entry. The bead experiments have clarified this point. Only internalin-coated beads entered cells expressing its receptor, definitely demonstrating that internalin is, on the bacterial side, the sole actor required for internalin-mediated entry into cells expressing its receptor. Internalin-coated beads entered cells by a zipper mechanism, showing that the internalin-only-mediated entry of these inert particles was similar to *L. monocytogenes* internalin-mediated entry and to *Yersinia* invasin-mediated entry into cells. Since the affinity chromatography procedure used to identify the internalin receptor led to the identification of a single protein (i.e., E-cadherin) (29), it is likely that the sole internalin interaction with E-cadherin at the bacterium-cell interface is the starting point for cell invasion. This points out an additional parallelism between internalin–E-cadherin-mediated entry of *Listeria* and invasin–β1 integrin-mediated entry of *Yersinia* (12, 27). The regions of E-cadherin required for

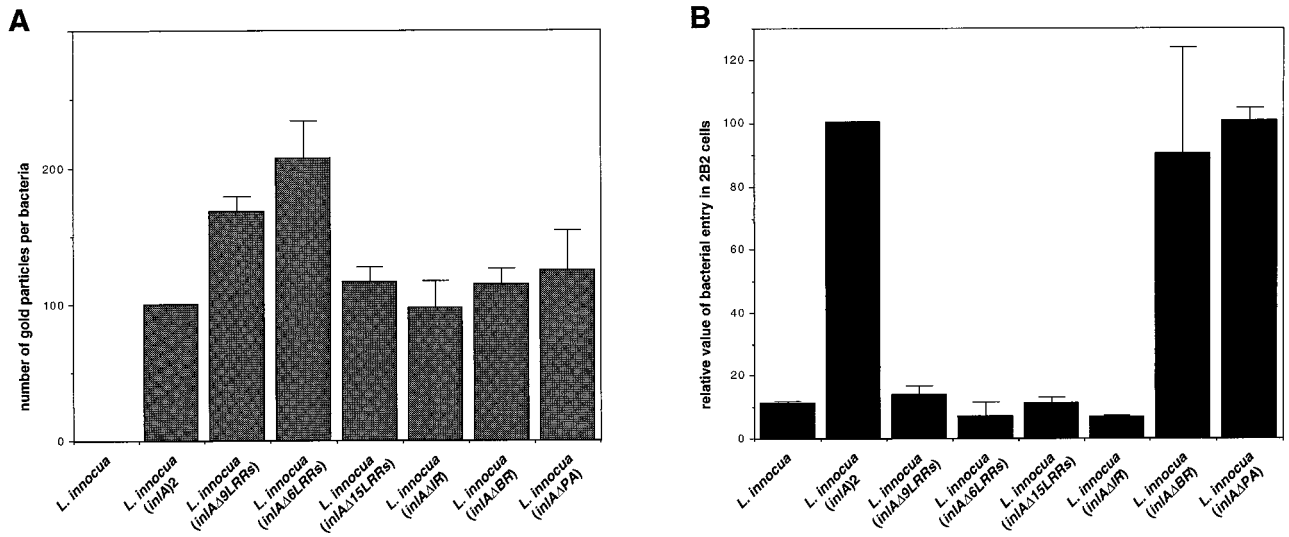

FIG. 5. Cell surface exposition of InlA and InlA variants and invasiveness conferred by these proteins. (A) Immunogold labeling of *L. innocua* strains expressing either internalin or each of the internalin variants. Each bar is the mean ± standard deviation of the number of gold particles present at the surface of about 20 bacteria and is given as a relative value of the number of gold particles present at the surface of *L. innocua* (*inlA*)2, which was reported as 100. (B) Invasiveness assays of *L. innocua*, *L. innocua* (*inlA*)2, and *L. innocua* expressing each internalin variant in 2B2 cells. The level of entry of *L. innocua* (*inlA*)2 has been artificially reported as 100. The level of entry of the other bacteria is given as a relative value. Results are means ± standard deviations of three different experiments done each time in duplicate.

internalin- and E-cadherin-mediated entry, as well as the putative intracytoplasmic factors and the signal transduction pathways, are not known and are currently under investigation. Interestingly, InlB also appears to be sufficient to promote InlB-dependent entry (1). Its cellular receptor still remains to be identified, but it will be interesting to compare the extent of homology between InlA- and InlB-mediated entries and important to evaluate their mutual contribution to *L. monocytogenes* host invasion in vivo during the infectious process.

The B-repeat and PA regions are not required for internalin-dependent entry. The results presented here showing that the B-repeat and PA regions of internalin play no role in internalization are in agreement with those of our previous experiments showing that monoclonal antibodies directed against the B-repeat or PA regions had no inhibitory effect on entry (28). The demonstration that these two regions are not required for internalin-induced phagocytosis establish that the total sequence of internalin is not critical for its surface pre-

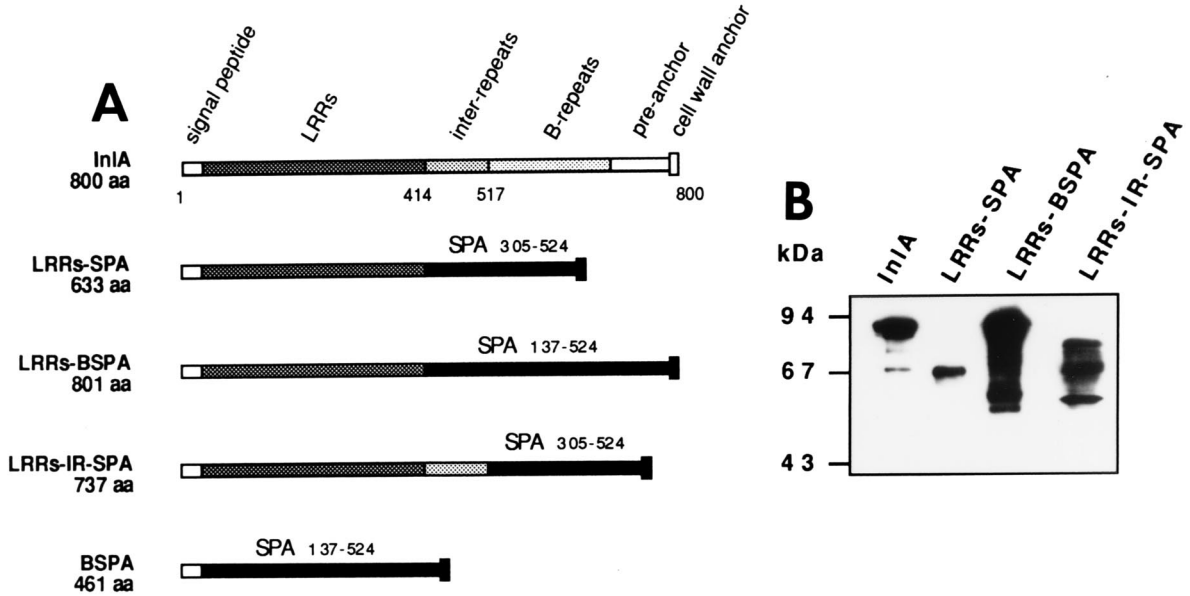

FIG. 6. Schematic representation of internalin-protein A chimeric proteins and Western blot analysis of these chimeric proteins. (A) Wild-type InlA is represented as in Fig. 4. Each chimeric protein (LRRs-SPA, LRRs-BSPA, LRRs-IR-SPA, and BSPA) is also represented. The carboxy-terminal part of SPA fused to internalin regions is indicated by a black box. The numbers indicate the amino acid position in wild-type protein A, and the total length of the chimeric proteins is indicated. (B) Immunoblotting analysis of InlA-protein A chimeric proteins expressed in *L. innocua*. Western blot analysis was performed as described for Fig. 4. Full-length InlA and chimeric proteins were all detected with the G6.1 anti-LRR antibody. The intense signal observed with LRRs-BSPA is probably due to the presence of Fc receptors on the protein A part fused to the internalin LRRs.

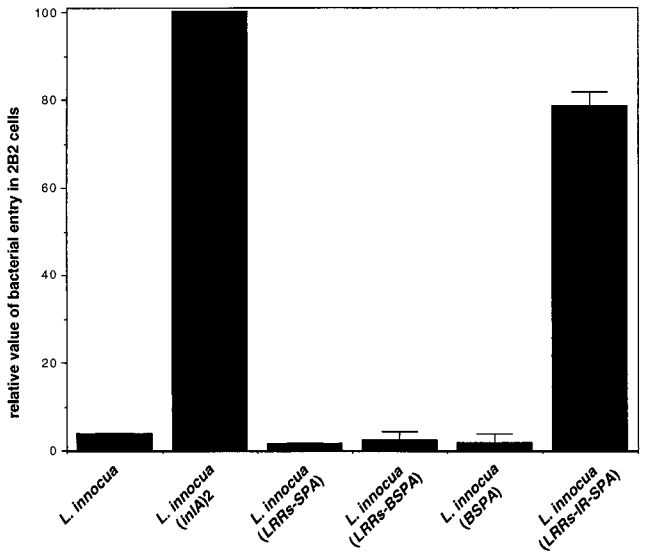

FIG. 7. Invasiveness conferred by InlA-protein A chimeric proteins. Invasiveness of *L. innocua*, *L. innocua* (*LRRs-SPA*), *L. innocua* (*LRRs-BSPA*), *L. innocua* (*LRRs-IR-SPA*) and *L. innocua* (*BSPA*) is given as a relative value of the level of entry of *L. innocua* (*inlA*)2, which has been reported as 100. The level of entry of the other bacteria is given as a relative value. Results are means ± standard deviations of three different experiments done each time in duplicate.

sentation or its internalizing function. The absence of effect of the deletion of the PA region, situated directly upstream of the anchoring motif LPXTG, demonstrates that this region is not critical for internalin export and anchoring to the cell wall. Considering the functional homology between internalin–E-cadherin and invasin-β1 integrin interactions, the nonrequirement of the B-repeat region for internalin-mediated entry is noteworthy. Indeed, the B-repeat region of internalin encompasses an FATDK motif (as well as an FATSK motif and an FSTDK motif), which is also present on *Yersinia* invasin (27). This FATDK invasin motif is situated in the carboxy-terminal region of invasin, known to be sufficient for invasin-mediated entry and to interact directly with β1 integrin (25, 27). Both the phenylalanine and aspartic acid residues of this FATDK motif were shown to be critical for invasin function (30). Even if the B-repeat region of internalin is not required for internalin interaction with E-cadherin, one cannot rule out the possibility that the B-repeat region of internalin would be able to interact with β1 integrin, even without leading to entry.

The LRRs-IR part of internalin is necessary and sufficient to promote entry. LRR motifs are classically involved in protein-protein interactions, and LRR structures have been shown to be critical for various ligand-receptor interactions involving a protein with LRRs (11, 37). Thus, the LRR region of internalin is a good candidate to interact directly with its cellular receptor E-cadherin. Our present finding that deletion of part or all of the internalin LRRs abolishes internalin's function is in agreement with our previous experiments showing that monoclonal antibodies directed against internalin LRRs had an inhibitory effect on entry (28). Taken together, these data establish that the internalin LRR region is crucial for the entry process and suggest that this region participates directly in the interaction of internalin with its receptor, as has been described for many other LRR proteins (24, 37). Interestingly, preliminary results suggest that InlB LRRs are also crucial for InlB-mediated entry (1).

However, surface expression of only internalin LRRs is not sufficient to promote entry, and the purified internalin LRR region does not interact with cells expressing internalin receptor. In fact, a second region, situated directly downstream of the LRRs, the IR region, is required for internalin-dependent entry. Whether this IR region participates directly in internalin interaction with its receptor or whether it has only an indirect role, participating in the correct folding of the LRR region required for receptor recognition, is not known. We presently favor the second hypothesis. Indeed, the IR region is the most conserved one among all of the Inl proteins identified to date in *L. monocytogenes* (10). If the function of five of these Inl proteins remains to be determined, internalin and InlB clearly have different receptors and functions (16). For instance, InlB-mediated entry occurs in cells nonpermissive for InlA (9, 17), and InlB stimulates phosphatidylinositol 3-kinase (17). The carboxy-terminal region of InlB located downstream of the IR region has been shown to be involved in the bacterial surface association of this protein and not to be required for InlB functionality (1, 2), and so it is likely that InlB function also relies on its LRRs-IR regions. It has been proposed that the specificity of protein-protein interactions of LRR-containing proteins and, therefore, the basis of their functions are due to the specific composition of nonconsensus residues of the repeats and might be influenced by the flanking domains of the LRR region (21). Since InlA and InlB LRR regions differ in the numbers of their LRRs (15 and 7 repeats, respectively) and in the internal compositions of their repeats, whereas their IR regions are very conserved (77.5% similarity and 50.5% identity), it is possible that the divergence of their LRR regions accounts for their functional differences, whereas the similarity of their IR region accounts for the structural similarity of their LRR regions. Thus, we propose that the IR region stabilizes the structure of the LRRs present in the amino-terminal part of *Listeria* LRR proteins, LRRs being specifically involved in the target recognition of these proteins. The structure of internalin is currently being investigated and should help to

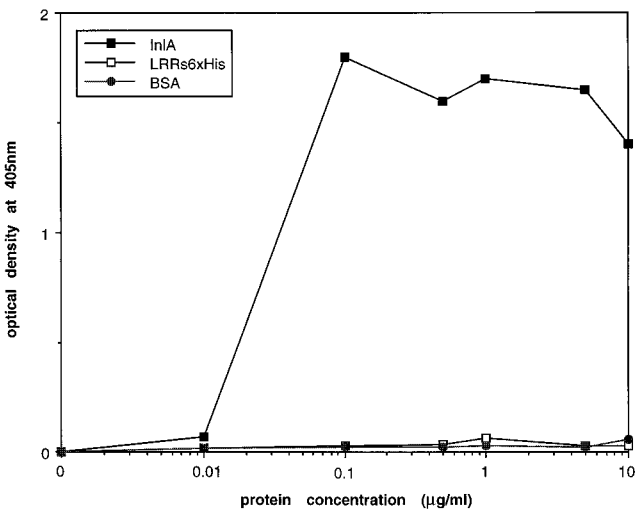

FIG. 8. Cell binding assay of LRRs6×His. To study Caco-2 cell binding to LRRs6×His and compare it to what is observed with the positive control protein InlA and the negative control protein BSA, serial dilutions of these three proteins were used to coat 96-well microtiter dishes. After incubation with a 100-μl suspension of Caco-2 cells (10^6/ml), wells coated with either LRRs×6His, InlA, or BSA were washed, and the number of bound cells was determined by assaying for lysosomal hexosaminidase. The optical density at 405 nm is directly proportional to the number of cells per well. A curve is presented that links the optical density at 405 nm observed in LRRs6×His-coated wells, InlA-coated wells, and BSA-coated wells to the protein concentrations used for coating the wells.

elucidate the functional and structural characteristics of the LRR and IR regions of internalin.

ACKNOWLEDGMENTS

We thank R. Brackenbury for the gift of the L2071 and LE6 cell lines, P. Courvalin for the gift of *E. faecalis* OG1X and advice about the electroporation protocol, O. Chesneau for the gift of *S. epidermidis* BM3302 and plasmid pRB474, O. Schneewind for the gift of plasmid pRIT5:*spa* and advice about the electroporation protocol, G. Tran Van Nhieu for discussions concerning experiments with latex beads, P. Gounon for discussion and help in electron microscopy, R. Hellio for assistance with the confocal laser-scanning microscope, and R. Hurme for critical reading of the manuscript.

M. Lecuit was a recipient of a fellowship from the Fondation pour la Recherche Médicale and the Ministère de l'Education Nationale, de l'Enseignement Superieur et de la Recherche.

REFERENCES

1. **Braun, L.** Unpublished data.
2. **Braun, L., S. Dramsi, P. Dehoux, H. Bierne, G. Lindahl, and P. Cossart.** 1997. InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. Mol. Microbiol. **25:**284–294.
3. **Brückner, R.** 1992. A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. Gene **122:**187–192.
4. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
5. **Chen, H., N. E. Paradies, M. Fedor-Chaiken, and R. Brackenbury.** 1997. E-cadherin mediates adhesion and suppresses cell motility via distinct mechanisms. J. Cell Sci. **110:**345–356.
6. **Cossart, P.** 1997. Subversion of the mammalian cell cytoskeleton by invasive bacteria. J. Clin. Invest. **99:**2307–2311.
7. **Cruz-Rodz, A. L., and M. S. Gilmore.** 1990. High efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. Mol. Gen. Genet. **224:**152–154.
8. **Devriese, L. A., M. D. Collins, and R. Wirth.** 1992. The genus *Enterococcus*, p. 1465–1481. *In* A. Balows (ed.), The prokaryotes. Springer-Verlag, New York, N.Y.
9. **Dramsi, S., I. Biswas, E. Maguin, L. Braun, P. Mastroeni, and P. Cossart.** 1995. Entry of *L. monocytogenes* into hepatocytes requires expression of InlB, a surface protein of the internalin multigene family. Mol. Microbiol. **16:**251–261.
10. **Dramsi, S., P. Dehoux, M. Lebrun, P. L. Goossens, and P. Cossart.** 1997. Identification of four new members of the internalin multigene family in *Listeria monocytogenes* EGD. Infect. Immun. **65:**1615–1625.
11. **El Tayar, N.** 1996. Advances in the molecular understanding of gonadotropins-receptors interactions. Mol. Cell. Endocrinol. **125:**65–70.
12. **Finlay, B. B., and P. Cossart.** 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science **276:**718–725.
13. **Gaillard, J.-L., P. Berche, C. Frehel, E. Gouin, and P. Cossart.** 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell **65:**1127–1141.
14. **Gaillard, J. L., P. Berche, J. Mounier, S. Richard, and P. 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.
15. **Gaillard, J.-L., and B. B. Finlay.** 1996. Effect of cell polarization and differentiation on entry of *Listeria monocytogenes* into the enterocyte-like Caco-2 cell line. Infect. Immun. **64:**1299–1308.
16. **Ireton, K., and P. Cossart.** 1997. Host pathogen interactions during entry and actin-based movement of *Listeria monocytogenes*. Annu. Rev. Genet. **31:**113–138.
17. **Ireton, K., B. Payrastre, H. Chap, W. Ogawa, H. Sakaue, M. Kasuga, and P. Cossart.** 1996. A role for phosphoinositide 3-kinase in bacterial invasion. Science **274:**780–782.
18. **Isberg, R. R., and J. M. Leong.** 1990. Multiple $\beta_1$chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell **60:**861–871.
19. **Isberg, R. R., and G. Tran Van Nhieu.** 1994. Two mammalian cell internalization strategies used by pathogenic bacteria. Annu. Rev. Genet. **27:**395–422.
20. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
21. **Kobe, B., and J. Deisenhofer.** 1994. The leucine-rich repeat: a versatile binding motif. Trends Biochem. Sci. **19:**415–420.
22. **Lasa, I., V. David, E. Gouin, J. Marchand, and P. Cossart.** 1995. The amino-terminal part of ActA is critical for the actin based motility of *Listeria monocytogenes*: the central proline-rich region acts as a stimulator. Mol. Microbiol. **18:**425–436.
23. **Lebrun, M., J. Mengaud, H. Ohayon, F. Nato, and P. Cossart.** 1996. Internalin must be on the bacterial surface to mediate entry of *Listeria monocytogenes* into epithelial cells. Mol. Microbiol. **21:**579–592.
24. **Lee, F. S., and B. L. Vallee.** 1990. Modular mutagenesis of human placental ribonuclease inhibitor, a protein with leucine-rich repeats. Proc. Natl. Acad. Sci. USA **87:**1879–1883.
25. **Leong, J. M., P. E. Morrisey, and R. R. Isberg.** 1993. A 76-amino acid disulfide loop in the *Yersinia pseudotuberculosis* invasin protein is required for integrin receptor recognition. J. Biol. Chem. **268:**20524–20532.
26. **Lorber, B.** 1996. Listeriosis. Clin. Infect. Dis. **24:**1–11.
27. **Marra, A., and R. R. Isberg.** 1996. Bacterial pathogenesis: common entry mechanisms. Curr. Biol. **6:**1084–1086.
28. **Mengaud, J., M. Lecuit, M. Lebrun, F. Nato, J.-C. Mazié, and P. Cossart.** 1996. Antibodies to the leucine-rich repeat region of internalin block entry of *Listeria monocytogenes* into cells expressing E-cadherin. Infect. Immun. **64:**5430–5433.
29. **Mengaud, J., H. Ohayon, P. Gounon, R. M. Mège, and P. Cossart.** 1996. E-cadherin is the receptor for internalin, a surface protein required for entry of *Listeria monocytogenes* into epithelial cells. Cell **84:**923–932.
30. **Saltman, L. H., Y. Lu, E. M. Zaharias, and R. R. Isberg.** 1996. A region of the *Yersinia pseudotuberculosis* invasin protein that contributes to high affinity to integrin receptors. J. Biol. Chem. **271:**23438–23444.
31. **Schneewind, O., D. Mihaylova-Petkov, and P. Model.** 1993. Cell wall sorting signals in surface proteins of Gram positive bacteria. EMBO J. **12:**4803–4811.
32. **Schneewind, O., P. Model, and V. A. Fischetti.** 1992. Sorting of protein A to the staphylococcal cell wall. Cell **70:**267–281.
33. **Swanson, J. A., and S. C. Baer.** 1995. Phagocytosis by zippers and triggers. Trends Cell Biol. **5:**89–93.
34. **Trieu-Cuot, P., C. Carlier, P. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to Gram positive bacteria. FEMS Microbiol. Lett. **48:**289–294.
35. **Trieu-Cuot, P., C. Carlier, C. Poyart-Salmeron, and P. Courvalin.** 1991. Shuttle vectors containing a multiple cloning site and a *lacZ*α gene for conjugal transfer of DNA from *Escherichia coli* to gram-positive bacteria. Gene **102:**99–104.
36. **Van Putten, J. P. M., J. F. L. Weel, and H. U. C. Grassmé.** 1994. Measurements of invasion by antibody labeling and electron microscopy. Methods Enzymol. **236:**420–437.
37. **Ware, J., S. R. Russel, P. Marchese, M. Murata, M. Mazzucato, L. De Marco, and Z. M. Ruggeri.** 1993. Point mutation in a leucine-rich repeat of platelet glycoprotein Ibα resulting in the Bernard-Soulier syndrome. J. Clin. Invest. **92:**1213–1220.
38. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

Editor: V. A. Fischetti