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Identification of LpeA, a PsaA-Like Membrane Protein That Promotes Cell Entry by *Listeria monocytogenes*

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The intracellular life of *Listeria monocytogenes* starts by a complex process of entry involving several bacterial ligands and eukaryotic receptors. In this work, we identified in silico from the sequence of the genome of *L. monocytogenes* a previously unknown gene designated *lpeA* (for lipoprotein promoting entry) encoding a 35-kDa protein homologous to PsaA, a lipoprotein belonging to the LraI family and implicated in the cell adherence of *Streptococcus pneumoniae* and related species. By constructing a mutant of *L. monocytogenes* in which *lpeA* is deleted (*lpeA* mutant), we show that the PsaA-like protein LpeA is not involved in bacterial adherence but is required for entry of *L. monocytogenes* in eukaryotic cells. In contrast to wild-type bacteria, mutant bacteria failed to invade the epithelial Caco-2 and hepatocyte TIB73 cell lines, as confirmed by confocal microscopy. The mutant bacteria rapidly penetrated in mouse bone marrow-derived macrophages. Surprisingly, *lpeA* mutant bacteria survive better in macrophages than do wild-type bacteria. This was correlated with a weak exacerbation of virulence of the *lpeA* mutant in the mouse. LpeA is therefore a novel invasin favoring the entry of *L. monocytogenes* into nonprofessional phagocytes but not its invasion of macrophages. This is the first report of a lipoprotein promoting cell invasion of an intracellular pathogen.

The gram-positive, nonsporulating bacterium *Listeria monocytogenes* is a food-borne pathogen widely spread in the environment and responsible for severe infections in humans and most animals. The virulence of this facultative intracellular pathogen is due to its capacity to invade and multiply within host cells, including macrophages, hepatocytes, and epithelial and endothelial cells (51). During the intracellular infectious cycle of *L. monocytogenes*, bacteria first adhere to the surface of eukaryotic cells and subsequently penetrate into these cells, a process involving a zipper-type mechanism. Bacterial entry is a complex multistep molecular mechanism involving several eukaryotic receptors and bacterial ligands. The bacterial uptake by macrophages implicates receptors such as the C3bi and the C1q complement receptors and the macrophage scavenger receptor of phagocytic cells (1, 12, 16). Entry in nonprofessional phagocytes is mediated by the transmembrane glycoprotein E-cadherin (36), the gC1q-binding protein (7), and the Met receptor or hepatocyte growth factor (47). Other receptors including components of the extracellular matrix such as heparan sulfate proteoglycans (2) and fibronectin (22) have been described. The bacterial ligands are surface proteins behaving as adhesins or invasins. So far, several adhesins have been identified such as the autolysin Ami with a C-terminal domain similar to that of InlB (8, 35, 37), the 104-kDa Lap (45), and lectins (18, 40). The invasins such as the internalins InlA and InlB (20), the actin-polymerizing protein ActA (14, 29), and Iap (33) facilitate the process of penetration into eukaryotic cells. Invasion might be promoted through signaling cascades triggered by the interaction ligands-receptors, but the exact mechanisms are poorly understood. It is known that InlB is an agonist of the lipid phosphatidylinositol 3-kinase, the InlB-mediated uptake being associated with activation of the phosphatidylinositol 3-kinase (26, 27). The bacterial cell wall polymer lipoteichoic acid induces the expression of the proinflammatory cytokines interleukin 1α/β, interleukin 6, and tumor necrosis factor alpha in murine and human monocytes (4, 32). During the intimate contact of bacteria with endothelial cells, listeriolysine O and phospholipases can also elicit host cell responses, including the generation of lipid second messengers (48, 49), NF-κB activation (29, 46), stimulation of cytokines and chemokines, and induction of cell adhesion molecule expression (17, 24, 29). During invasion, *Listeria* bacteria are engulfed within phagocytic vacuoles and disrupt the phagosome membranes to be free in the cytoplasm. Listeriolysine O and phospholipases are involved in the escape from the phagosomal compartment. Through ActA, a bacterial membrane-anchored protein, intracytoplasmic bacteria become surrounded with actin filaments that form an actin tail allowing bacteria to move inside the cytoplasm and to spread from cell to cell (reviewed in reference 51).

In this work, we searched for new virulence factors implicated in the entry of *L. monocytogenes* into eukaryotic cells. We identified in silico from the recently completed sequence of the genome of *L. monocytogenes* (23) a gene encoding a PsaA-like protein belonging to the LraI family. PsaA is a lipoprotein previously implicated in the cell adherence of *Streptococcus pneumoniae* (44) and related species (10). By constructing a mutant in which this gene is deleted, we show that this PsaA-like protein is required for entry of *L. monocytogenes* into eukaryotic cells, not into macrophages. This protein, designated LpeA (for lipoprotein promoting entry), promotes entry and facilitates intracellular survival in infected macrophages.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. We used the reference strain of *L. monocytogenes* EGD-e belonging to serovar 1/2a (recently sequenced [23]) and the *inlAB*-defective mutant BUG8, previously described (20). Brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich.) and Luria-Bertani (Difco) broth and agar were used as media in which to grow *L. monocytogenes* and *Escherichia coli* strains, respectively. We also used the following 17 wild-type *Listeria* strains previously described (5): *L. monocytogenes* (ATCC 19115, ATCC 19111, CNL880203, CHUT861141, CNL895793, CNL895795, INRA119, INRA85), *L. ivanovii* (ATCC 19119, SLCC2379), *L. innocua (*ATCC 33090, CHUT861158, INRA86), *L. seeligeri* (CHUT860478, CHUT861166, CHUT861167), and *L. welshimeri* (CHUT860477). Strains harboring plasmids were grown in the presence of the following antibiotics: for pCR derivates, kanamycin (Km) (50 μg/ml), and for pAUL-A derivates, erythromycin (Em) (150 μg/ml for *E. coli* and 5 μg/ml for *L. monocytogenes*). To analyze mutant bacteria, we studied 50 metabolic characters on API-50 strips (Biomerieux, Marcy l'Etoile, France). Bacterial growth assays were also performed with the previously defined synthetic medium F70 (41).

Genetic manipulations. Chromosomal DNA, plasmid extraction, electrophoresis, restriction enzyme analysis, and amplification by PCR were performed according to standard protocols (43). Restriction enzymes and ligase were purchased from New England Biolabs and used as recommended by the manufacturer. DNA was amplified with the ampliTaq DNA polymerase of *Thermus aquaticus* from Perkin-Elmer (Branchburg, N.J.) in a Gene Amp System 9600 thermal cycler (Perkin-Elmer). Nucleotide sequencing was carried out with *Taq* DiDeoxy terminators and by the DyePrimer cycling sequence protocol developed by Applied Biosystems (Perkin-Elmer) with fluorescently labeled dideoxynucleotides and primers, respectively (Life Technologies). Labeled extension products were analyzed on an ABI Prism 310 apparatus (Applied Biosystems).

Construction of a mutant of *L. monocytogenes* lacking the *lpeA* gene. An *lpeA* mutant was constructed by deletion of a 270-bp internal fragment of *lpeA* and insertion of a promoterless *aphA-3* gene conferring resistance to Km by double recombination, as previously described (6). The deletion replacement mutant of *lpeA* was constructed by inserting a 1,067-bp *Eco*RI-*Bam*HI EGD DNA fragment (−38 to +1,017), an 855-bp *Bam*HI *Enterococcus faecalis* DNA fragment carrying *aphA-3*, and a 1,062-bp *Bam*HI-*Hin*dIII EGD DNA fragment (+1,293 to +2,336) between the *Eco*RI and *Hin*dIII sites of the thermosensitive shuttle vector pAUL-A, as previously described (6), to yield pAUL-*lpeA* Ω*aphA3*. These three DNA fragments were amplified by PCR from *L. monocytogenes* (EGD-e) genomic DNA by using the following primers pairs: papEco (5′-GGAATTCCG CAGCGGGGGTGTAAGAGTTGTTGTTTTTA-3′) and papBamas (5′-CGCG GATCCACGCCAACCAGGGGTACAATAC-3′); km1 (5′-CGGGATCCCGA CTAACTAGGAGGAATA-3′) and km2 (5′-CGGGATCCCGGGTCATTATT CCCTCC-3′); and papBams (5′-CTTGTAAAAGCGGATCCAGACAATGCG GA-3′) and papHind (5′CCCAAGCTTGGGGGGGGCCTTTGGGACGGAG ACAATTGCGGC-3′). Oligonucleotides were synthesized by Genset (Paris, France). The two amplified double-stranded DNA fragments were first cloned into the pCRTM cloning vector by using the TA cloningTM kit (Invitrogen Corporation, San Diego, Calif.). Plasmid pAUL-*lpeA* Ω *aphA3* was electroporated into EGD, and transformants were selected for Em resistance at 30°C. Allelic exchange was obtained by homologous recombination by using a two-step procedure: at 40°C, a single crossing-over event integrated the entire plasmid into the chromosome; the plasmid was then excised by subculture at 30°C. The deletion was confirmed by PCR sequence analysis of chromosomal DNA from the mutant.

RNA isolation and Northern blot analysis. Hybridizations of total RNA extracted during exponential phase at 37°C from wild-type EGD were done as described previously (41). A specific probe (length, 1,062 bp) used for hybridization was generated with the following primers pair: psa1 (5′-CGCACCGAA ACAAGGCTTGCTATTTTC-3′) and pap2 (5′-CCGGGTTCGTAAAACGGA GCAAAAAC-3′).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Proteins from extracts were prepared from cultures of bacteria grown in BHI broth at 37°C (optical density at 600 nm, 0.6). The bacterial pellets were suspended in 10 mM Tris–1 mM EDTA, and bacteria were disrupted with a Fastprep FP120 apparatus (BIO101; Ozyme) by three pulses of 30 s at a speed of 6.5 m/s. Bacterial debris were removed by centrifugation, and the resulting supernatant consisted of the cytoplasmic proteins. Electrophoresis and Western blotting were carried out as described previously in 10% sodium dodecyl sulfate-polyacrylamide minigels (Mini Protean II; Bio-Rad) (38). Nitrocellulose sheets were probed with anti-ScaA monoclonal antibody kindly provided by P. E. Kolenbrander (Bethesda) and antirabbit horseradish peroxidase-conjugated secondary antibody. Mouse monoclonal antibodies directed against InlA, InlB, or ActA obtained from P. Cossart (Institut Pasteur, Paris) were also used as previously described (38). Antibodies were used at a final dilution of 1:1,000. Antibody binding was revealed by adding 0.05% diaminobenzidine-tetrahydrochloride (Sigma) and 0.03% hydrogen peroxide (Sigma).

Infection of macrophages and cell lines. Bone marrow-derived macrophages from C57/BL6 mice (IFFA-CREDO, Grenoble, France) were cultured and infected for growth curves at a cell-to-bacterium ratio of 1 to 1, as previously described (6). After 15 min of bacterial adherence on ice, macrophages were exposed for 15 min at 37°C (time zero). The numbers of intracellular bacteria were estimated in cell lysates at selected intervals (from time zero to 8 h postinfection). We also used the human colon carcinoma cell line Caco-2 (ATCC HTB37) and the murine embryonic hepatocyte cell line TIB73 (ATCC TIB73) from the American Type Culture Collection (Manassas, Va.). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing *N*-acetyl-L-alanyl-L-glutamine (Gibco Laboratories, Grand Island, N.Y.) and supplemented with 10% fetal bovine serum (Gibco Laboratories). Cells were maintained in 10% CO_2 at 37°C without antibiotics. Cells were seeded at a density of $10^5/cm^2$ in 24-well tissue culture plates (Falcon Labware; Becton Dickinson & Co., Lincoln Park, N.J.). Monolayers were used 24 to 48 h after seeding. The invasion assays were carried out as described previously (37). Briefly, cells were inoculated with bacteria at a multiplicity of infection of approximately 100 bacteria/cell. They were incubated for 1 h and then washed three times with DMEM and overlaid with fresh DMEM containing gentamicin (10 mg/liter) to kill extracellular bacteria. At 2 and 4 h, cells were washed and lysed by addition of cold water. Viable bacteria released from the cells were plated onto BHI plates. Each experiment was carried out in triplicate and repeated three times, and results were expressed as means ± standard deviations. We compared the differences between the curves by Student's *t* test.

Confocal microscopy. Infected cells were examined at progressive times by confocal microscopy. Double fluorescence labeling of F-actin and bacteria was performed as described previously (30) by using phalloidin coupled to Oregon Green 488 (Molecular Probes, Eugene, Oreg.) and a rabbit anti-*Listeria* serum (J. Rocourt, Institut Pasteur, Paris, France) revealed with an anti-immunoglobulin G antibody coupled to Alexa 546 (Molecular Probes). Images were scanned on a Zeiss LSM 510 confocal microscope.

Immunogold labeling. Bacteria were grown overnight in BHI broth and processed as previously described (20). The grids were incubated for 1 h with rabbit anti-*Listeria* or rabbit anti-ScaA antibodies and further incubated with goat anti-rabbit immunoglobulin G conjugated to 10-nm gold particles.

Mouse virulence assay. Six- to 8-week-old female Swiss mice (Janvier, Le Geneset St-Isle, France) were inoculated intravenously (i.v.) with various doses of bacteria. Mortality was monitored over a 14-day period for groups of five mice. The 50% lethal doses (LD_{50}) were determined by the probit method. Bacterial growth in organs (spleen and liver) of mice infected i.v. with 10^5 bacteria was monitored as previously described (38). For growth in organs, we compared the data obtained with mutants to the data obtained with wild-type strains by a multifactorial variance analysis.

RESULTS

Identification of a gene encoding a PsaA-like protein in *L. monocytogenes*. In a search for new virulence factors in *L. monocytogenes*, we used the sequence of the adhesin PsaA of *S. pneumoniae* to perform a Blast search (NCBI Blastp 2 version) of the entire sequence of the genome of *L. monocytogenes* EGD-e serovar 1/2a (23). We thus identified an ORF (open reading frame) encoding a PsaA-like protein of 310 residues (*orf 3387.1*), which we then designated *lpeA*. The deduced protein shares 50 to 60% identity with the sequences of various proteins encoding streptococcal adhesins, including PsaA of *S. pneumoniae*, FimA of *Streptococcus parasanguis*, ScaA of *Streptococcus gordonii*, SsaB of *Streptococcus sanguis*, and EfmA of *Enterococcus faecium*. These proteins constitute a group of streptococcal adhesins involved in adhesion to components of the oral cavity. All these proteins possessed a Leu-X-X-Cys sequence near the N-terminal end, corresponding to a lipoprotein consensus sequence and to the site for cleavage of the signal peptide (28). In addition, LpeA displays a metal-binding

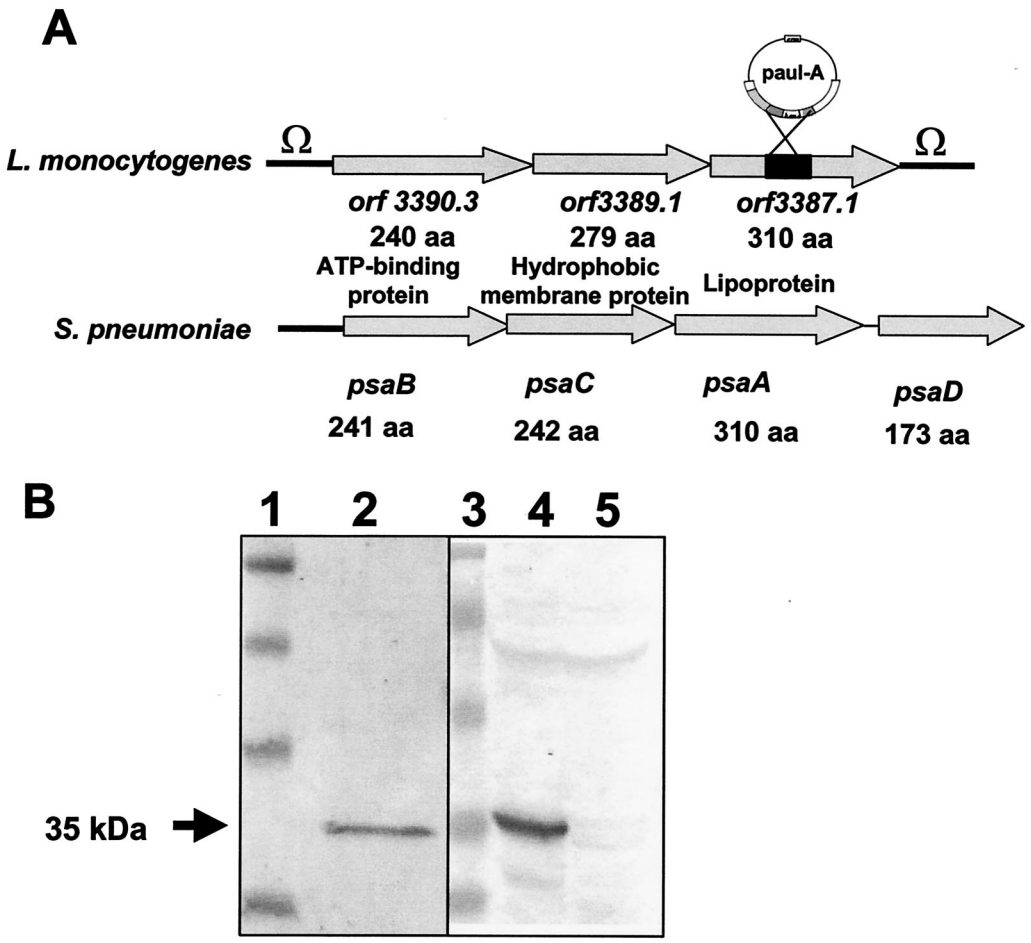

FIG. 1. (A) Comparison of the genetic organization of the *lpeA* operon of *L. monocytogenes* with that of the *psaA* operon of *S. pneumoniae*. The *lpeA* operon is framed by two terminators (Ω). The *lpeA* gene is the third of the operon (*orf3387.1*), and there is no homologue for the regulator *psaD* in the genome of *L. monocytogenes. lpeA* encodes a lipoprotein homologous to PsaA of *S. pneumoniae*. Shown is the insertion of the promoterless *aphA*-3 cassette on plasmid pAUL-A into the *lpeA* gene. (B) Western blot analysis of the mutant of *L. monocytogenes* lacking *lpeA*. Bacterial extracts from EGD-e or *lpeA* mutant were tested with a monoclonal antibody (MAb) anti-ScaA, a PsaA homologue of *S. gordonii*. (Left panel) The MAb recognizes a 35-kDa band from a bacterial extract of *S. gordonii*. (Right panel) The MAb recognizes a 35-kDa band in wild-type EGD-e and does not react against the extracts from the *lpeA* mutant of *L. monocytogenes*. Lanes: 1 and 3, molecular markers; 2, *S. gordonii*; 4, EGD-e; 5, *lpeA* mutant.

site formed by His-67, His-139, Glu-205, and Asp-280, as described for PsaA of *S. pneumoniae* (34).

By BLAST search, we also found a PsaA-like homologue in *L. innocua*, sharing 94% identity with LpeA (*orf 3387.1*) (23). Using primers specific for *lpeA* of *L. monocytogenes,* we showed by Southern blotting that these genes were present in all 17 tested strains of *L. monocytogenes* and other *Listeria* species (*L. ivanovii*, *L. innocua*, and *L. seeligeri*) (data not shown), suggesting that *lpeA* is highly conserved in the genus *Listeria.*

***lpeA* of *L. monocytogenes* belongs to an operon resembling those of a putative ATP-binding cassette (ABC) transporter family.** Analysis of the region of the *L. monocytogenes* genome comprising *lpeA* revealed the presence of three overlapping ORFs in the same orientation (*orf 3390.3*, *orf 3389.1*, and *orf 3387.1*), surrounded by two terminators (Fig. 1 A). The first *orf* encodes a putative ATP-binding protein of 240 amino acids, with a consensus nucleotide-binding site for ATP (GPNGAGKST) starting at position 33, corresponding to the consensus sequence (GXXGXGKS/T) in the glycine-rich loop of ATP-binding enzymes (52). In addition, a glutamine-glycine-rich motif (LSGGQLQR) could function as a peptide linker joining different domains of the protein. The second *orf* encodes a putative transmembrane protein of 279 residues, with a 16-amino-acid sequence (ALQTVGIILVVAMLITP) in position 182, also present in several hydrophobic membrane proteins involved in the transport of peptides and other small molecule transports (39).

A transcriptional analysis of the locus was performed by Northern blotting during exponential growth of *L. monocytogenes* in BHI medium. With an *lpeA*-specific probe, we detected by Northern blotting a single 2.3-kb transcript in the wild-type strain (data not shown), suggesting that *lpeA* is part of an operon of three genes. The genetic organization of the *lpeA* operon is similar to that of *S. pneumoniae* (Fig. 1A) and of the other *psaA*-like operons of *E. faecium*, *E. faecalis*, *S.*

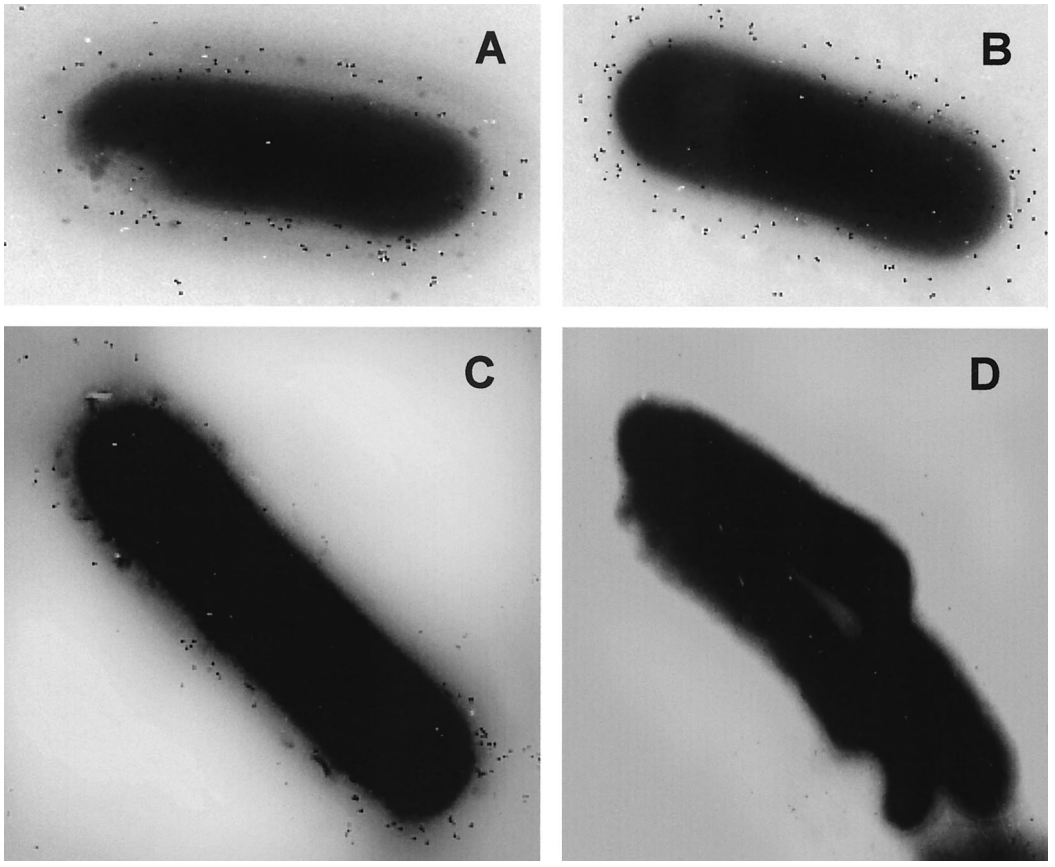

FIG. 2. Immunogold labeling of the wild-type strain (A and C) and the *lpeA* mutant (B and D) of *L. monocytogenes*, using as control a polyclonal rabbit anti-*Listeria* antiserum (A and B) and a monoclonal anti-ScaA from *S. gordonii* directed against LpeA (C and D). Both strains are strongly labeled by the polyclonal anti-*Listeria* serum. Only the wild-type bacteria were labeled by the MAb anti-ScaA, contrasting with the absence of labeling of the *lpeA* mutant.

gordonii, *S. sanguis*, and *S. parasanguis*. However, we did not find a *psaD*-like regulator gene, which is usually located downstream *pleA*, in contrast to the other operons described. This gene was absent in the genome of *L. monocytogenes*, suggesting that the regulation of the *lpeA* operon notably differs from that of the other gram-positive species. This organization is reminiscent of that of the ABC-lipoprotein-dependent transporter systems, which is similar to the periplasmic binding-protein-dependent transport systems of gram-negative bacteria (bacterial permeases) (11).

Construction and phenotypic analysis of an *lpeA* mutant of *L. monocytogenes*. We constructed an *lpeA* mutant of *L. monocytogenes* strain EGD-e by deletion of an internal fragment of *lpeA* gene (270 bp) and chromosomal integration by allelic replacement (EGDΔ*lpeA*). Inactivation of *lpeA* was confirmed by Western blot analysis of bacterial extracts from the wild-type EGD-e or from the *lpeA* mutant by using antibodies directed against ScaA of *S. gordonii*, a protein sharing 57% of peptide identity with LpeA of *L. monocytogenes*. We found that this antiserum recognizes LpeA as a single 35-kDa band in bacterial extracts from the wild-type strain, in contrast to the mutant (Fig. 1B).

We did not find any difference between the mutant and the wild-type strain EGD-e with respect to microscopic morphology, motility, colony aspect, hemolysis on blood agar plates, metabolic profiles on API strips, and growth in BHI broth at 4, 37, or 42°C (data not shown). In contrast to previous data on the phenotype of a *psaA* mutant of *S. pneumoniae* (13), growth of the *lpeA* mutant of *L. monocytogenes* was not impaired on previously defined synthetic medium F70 (41). Since LpeA might be a putative factor involved in bacterial invasion, we confirmed by Western blot analysis that the proteins InlA, InlB, and ActA were produced at the same levels in the mutant and the wild-type bacteria (data not shown). Using the anti-ScaA serum, we also detected LpeA on the bacterial surface of the wild-type strain by immunogold labeling, in contrast to the absence of such labeling with the *lpeA* mutant, showing that LpeA is a surface-exposed protein (Fig. 2).

LpeA promotes cell invasiveness of *L. monocytogenes*. We then studied the capacity of the *lpeA* mutant to penetrate into and to replicate within cells in vitro. Using a multiplicity of infection of 100 bacteria/cell, we tested two different types of cell lines: the human enterocyte-like cell line Caco-2 and the murine embryonic hepatocyte cell line TIB73, which have been extensively used to study the in vitro infection by *L. monocytogenes* (15). Cell lines were exposed to strain EGD-e, EGDΔ*lpeA*, or an *inlAB* mutant as a control for invasiveness. Cells were then washed and incubated in the presence of gen-

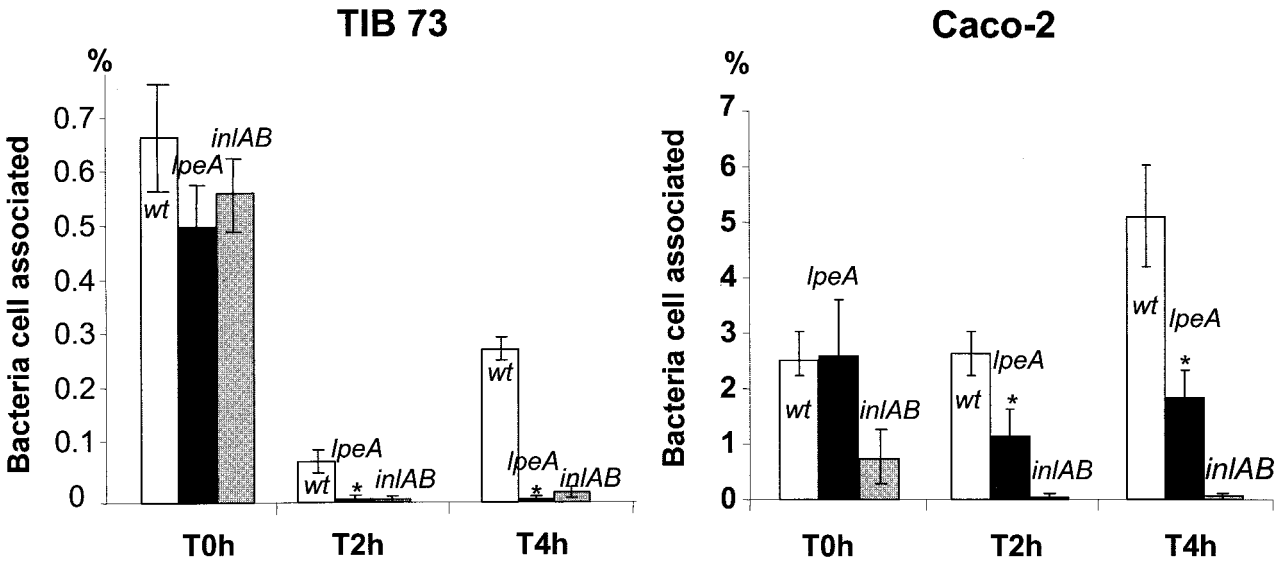

FIG. 3. Role of LpeA in the entry of *L. monocytogenes* in epithelial and hepatocyte cell lines. TIB73 or Caco-2 cells were exposed to 100 bacteria of EGD-e, *inlAB* mutant (control), or *lpeA* mutant per cell. The initial numbers of bacteria associated to cells were similar at time zero for wild-type and *lpeA* mutant in both cell lines. As expected, the percentage of cells infected by wild-type bacteria after 2 and 4 h of incubation was significantly higher in Caco-2 than in TIB73 cells. The invasive capacity of both mutants was strongly impaired. Compared to that of wild-type bacteria, the invasion rate of the *lpeA* mutant was significantly reduced, although to a lesser extent than that of the *inlAB* mutant. Experiments were repeated three times for both cell lines. *, $P < 0.01$ by Student's *t* test.

tamicin (10 mg/liter) to eliminate extracellular bacteria. Bacteria were counted at time zero and after 2 and 4 h of incubation. No significant difference between EGD-e and EGDΔ*lpeA* was observed by time zero in either cell line, indicating that LpeA does not influence adherence to cells (Fig. 3). As previously described (21), the rate of infection of Caco-2 cells by wild-type bacteria reached about 5% after 4 h, compared to a low infection rate of TIB73 cells (<0.3%) exposed to the same conditions. We found that the invasion by the *lpeA* mutant was significantly reduced in both cell lines, compared to that by wild-type EGD-e ($P < 0.01$; Student's *t* test). This defect of invasion was less pronounced in Caco-2 cells than that of the *inlAB* mutant (Fig. 3).

These results were confirmed by confocal electron microscopy of TIB73 cells exposed to bacteria under the same conditions (Fig. 4). By time zero, the numbers of cell-associated bacteria were similar for both strains (Fig. 4A and D). After 2 and 4 h of incubation, wild-type bacteria formed actin comets and massively invaded the cell monolayers as expected (Fig. 4B and C). In contrast, by time 2 h, most mutant bacteria remained located inside the phagosomal compartment without forming comets (Fig. 4E). After 4 h of incubation, the majority of TIB73 cells remained noninfected. However, we observed rare clusters of spreading mutant bacteria with polymerized actin (Fig. 4F). These results clearly indicate that LpeA is involved in cell invasion of *L. monocytogenes*.

Intramacrophage growth and virulence of the *lpeA* mutant. We then tested the capacities of *lpeA* mutant and of wild-type bacteria to survive in mouse bone marrow-derived macrophages. We observed that mutant *lpeA* bacteria invade and rapidly grow in macrophages. We repeatedly found that there was no initial drop of intramacrophage bacteria (2 h) for the mutant, in contrast to what was observed with wild-type bacteria (Fig. 5A). Then, both strains grew at a similar rate, with a higher final bacterial load for the *lpeA* mutant (Fig. 5A). This difference in growth between mutant and wild-type strains was statistically significant ($P < 0.001$).

Finally, we studied the role of LpeA in the virulence of *L. monocytogenes* by determining the LD_{50} after i.v. inoculation of Swiss mice. The LD_{50} of the mutant was estimated at $10^{4.3}$, compared to $10^{4.6}$ for the wild-type strain. After i.v. infection by the same inoculum (10^5 bacteria), the growth rate of mutant bacteria was greater in organs, especially in the spleen, than that of the wild-type bacteria, with an early mortality, beginning as soon as day 3 (Fig. 5B and C). This difference (days 1 to 2) was statistically significant ($P < 0.007$). Mice infected with wild-type bacteria died between days 4 and 6. These results indicate that the bacterial virulence of *L. monocytogenes* was weakly exacerbated in the absence of LpeA.

DISCUSSION

In this work, we identified in the genome of *L. monocytogenes* a previously unknown gene, *lpeA*, encoding a putative membrane lipoprotein homologous to PsaA, a structural adhesin of *S. pneumoniae* (42), and to other PsaA-like proteins produced by *Streptococcus* spp. implicated in cell adhesion and virulence of certain species (9, 19, 31). By transcriptional analysis, we found that *lpeA* is the third gene of an operon encoding an ATP-binding protein and a transmembrane protein homologous to several hydrophobic membrane proteins involved in the transport of peptides and other small-molecule transports (39). The genetic organization of the *lpeA* operon of *L. monocytogenes* is similar to that of the *psaA* operon of *S. pneumoniae* and reminiscent of that of the ABC lipoprotein-dependent transporter systems similar to the periplasmic-binding-protein-

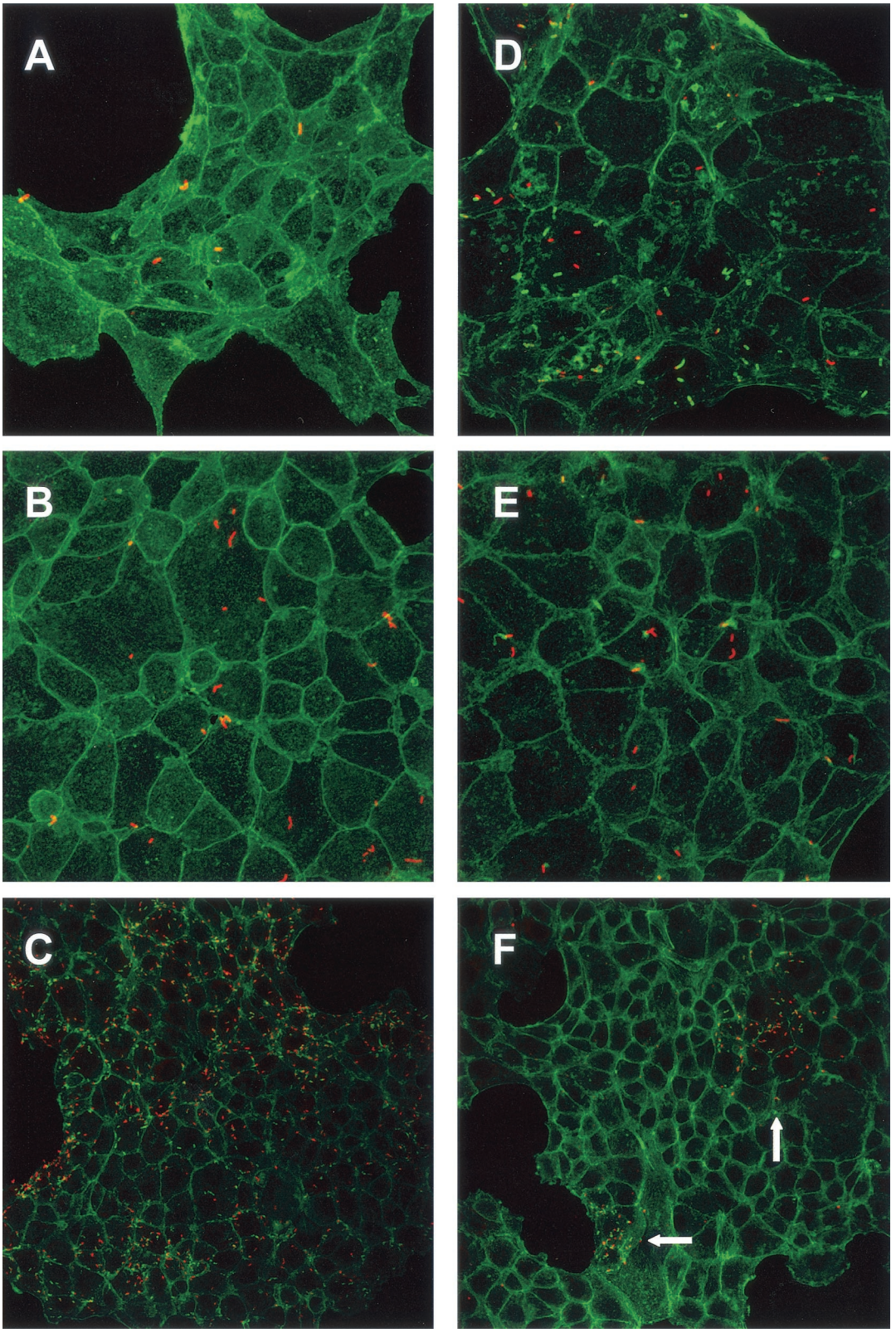

FIG. 4. Confocal microscopy of TIB-73 cells infected (100 bacteria/cell) with EGD-e (A, B, and C) or *lpeA* mutant (D, E, and F). Cells were observed at time zero (A and D) and 2 h (B and E) and 4 h (C and F) postinfection. F-actin was stained with phalloidin (green). Bacteria were labeled with an anti-*Listeria* serum (red). At time zero, no difference between the two strains was observed. After 2 h, many wild-type bacteria polymerized actin and started forming comets (B), whereas the majority of mutant bacteria were not associated with actin (E). After 4 h, wild-type bacteria massively spread to the entire cell monolayers (C). In contrast, the cell monolayers remained poorly infected by the mutant, except for rare clusters shown in panel F (indicated by arrows), in which mutant bacteria polymerized actin and multiplied inside cells. Magnifications, ×480 (A, B, D, and E) and ×320 (C and F).

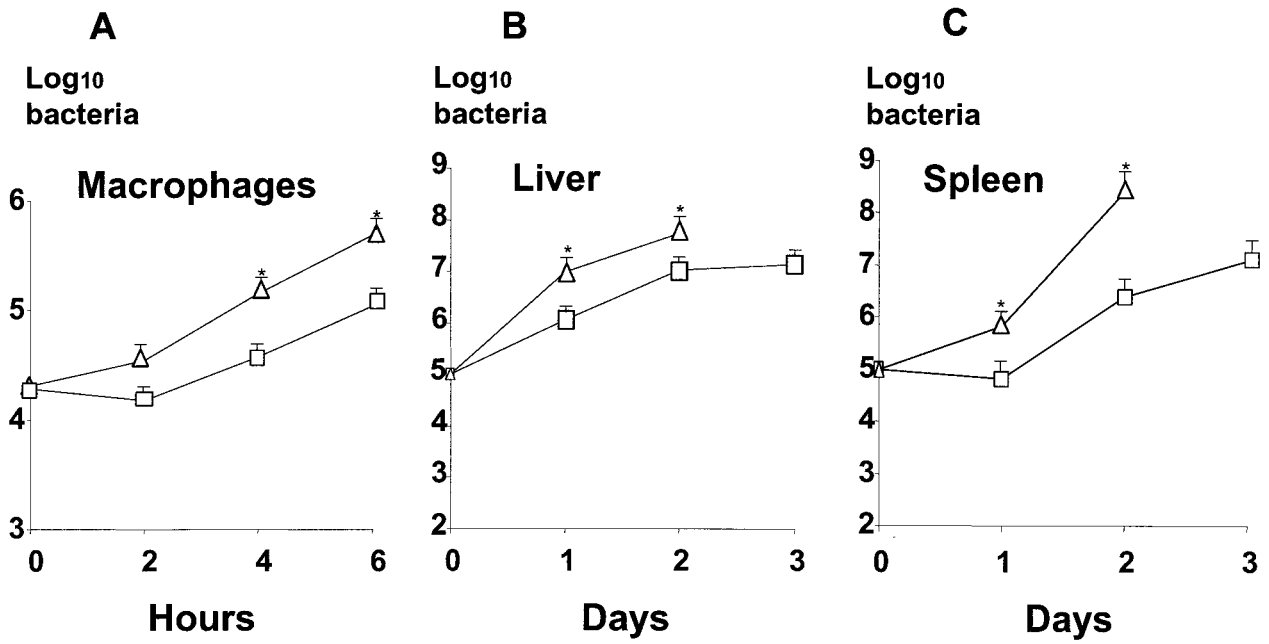

FIG. 5. Intramacrophage growth and virulence of the *lpeA* mutant of *L. monocytogenes*. □, wild-type EGD-e; △, *lpeA* mutant. (A) Growth of *L. monocytogenes* in bone marrow-derived macrophages from C57/BL6 mice. The cells were exposed for 15 min (time zero) to bacteria (1 bacterium per cell), and bacterial survival was monitored for 6 h after the infection. Entry was very efficient in both strains, and mutant bacteria repeatedly grew faster in macrophages (*, $P < 0.001$). (B and C) Growth of *L. monocytogenes* in the spleen (B) and the liver (C) of Swiss mice inoculated i.v. with 10^5 bacteria. Mutant bacteria grew faster in organs (*, $P < 0.007$) than did wild-type bacteria, and they induced an early mortality in mice by day 3.

dependent transport systems of gram-negative bacteria (bacterial permeases) (10). As PsaA of *S. pneumoniae*, LpeA belongs to a family of surface-associated proteins designated lipoprotein receptor-associated antigen I (LraI), identified in at least seven species of *Streptococcus* and in two species of *Enterococcus* (3, 28). For example, they are implicated in the adherence of *Streptococcus sanguis* to platelet fibrin matrix (9), of *S. pneumoniae* to type II pneumocytes (3), of *Streptococcus agalactiae* to laminin (50), and of various *Streptococcus* species to salivary glycoproteins, fibrin, or epithelial cells (25), and also in the coaggregation of *S. gordonii* with *Actinomyces* (31). Some of them have been shown to be essential for the virulence of bacteria, such as *S. pneumoniae*, in experimental animal models. These polypeptides play a dual role in adhesion and transport. Indeed, the LraI polypeptides are components of ABC-type membrane transport systems implicated in the uptake of polypeptides, oligopeptides, and multiple sugars (10). Recently, it has been proposed that the members of the LraI family constitute a new family of extracellular solute-binding proteins specific for metal ions (Zn or Mn) (10, 39). The role of LpeA of *L. monocytogenes* as an ABC transporter remains unknown.

The main finding of this work is that LpeA is a surface-exposed protein promoting cell invasion by *L. monocytogenes*. Using a mutant lacking *lpeA*, we observed that LpeA-defective bacteria efficiently adhere to eukaryotic cells but fail to penetrate in vitro into epithelial and hepatocyte cells, as confirmed by confocal microscopy showing that very few *lpeA* mutant bacteria penetrate inside cells and then rapidly grow in the cytoplasm (Fig. 4). In contrast, an *S. pneumoniae psaA* mutant expresses a reduced adherence to A549 lung cells in vitro (3). These results demonstrate that (i) *L. monocytogenes* LpeA is not an adhesin and behaves as an invasin and that (ii) LpeA is not required to escape from the phagosomal compartment. This is the first demonstration that a putative lipoprotein promotes invasion by the pathogen *L. monocytogenes*.

The LpeA-dependent reduced invasion of epithelial and hepatocyte cell lines contrasts with the rapid invasion of bone marrow macrophages by *lpeA* mutant bacteria, resulting in a moderately higher bacterial intracellular load than with wild-type bacteria (Fig. 5A). This was confirmed by the finding of a moderate exacerbation of virulence in the absence of LpeA. Indeed, the mutant was fully virulent in the mouse with an LD_{50} estimated at $10^{4.3}$, moderately lower than the LD_{50} value of the wild-type strain ($10^{4.6}$). Under the same infecting-challenge conditions, we observed higher bacterial loads in organs, associated with an early mortality (Fig. 5B and C). This result is surprising, since internalin-defective *Listeria* mutants express a weakly reduced level of virulence after i.v. inoculation (21). Moreover, an *S. pneumoniae psaA* mutant displays reduced virulence in mice infected by intranasal and intraperitoneal routes (3). Our results suggest that virulent bacteria at the initial phase of i.v. infection do not require invasins to directly spread in vivo from cell to cell. Circulating bacteria might mainly target resident or mobile macrophages, including dendritic cells, and then spread to adjacent cells, ultimately resulting in rapid growth in organs. This also suggests that direct invasion of hepatocytes or epithelial cells is not required for the expression of virulence in this model. To our knowledge, the exacerbation of virulence has never yet been observed with

a deletion mutant of *L. monocytogenes.* The reasons for this virulence phenotype remain unknown. LpeA might act directly through a cell receptor or indirectly through its hypothetical function of the ABC transporter, which might influence the cell sensing or an intracellular cascade associated to cell entry through the uptake of polypeptides, oligopeptides, and multiple sugars. Our results clearly show that the putative lipoprotein LpeA of *L. monocytogenes* is a novel invasin involved in the entry process, but not in intracellular survival.

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REFERENCES

1. **Alvarez-Dominguez, C., E. Carrasco-Marin, and F. Leyva-Cobian.** 1993. Role of complement component C1q in phagocytosis of *Listeria monocytogenes* by murine macrophage-like cell line. Infect. Immun. **61:**3664–3672.
2. **Alvarez-Dominguez, F., J. A. Vazquez-Boland, E. Carrasco-Marin, P. Lopez-Mato, and F. Leyva-Cobian.** 1997. Host cell heparan sulfate proteoglycans mediate attachment and entry of *Listeria monocytogenes*, and the listerial surface protein ActA is involved in heparan sulfate receptor recognition. Infect. Immun. **65:**78–88.
3. **Berry, A. M., and J. C. Paton.** 1996. Sequence heterogeneity of PsaA, a 37-kDa putative adhesin essential for virulence of *Streptococcus pneumoniae*. Infect. Immun. **64:**5255–5262.
4. **Bhakdi, S., T. Klonisch, P. Nuber, and W. Fischer.** 1991. Stimulation of monokine production by lipoteichoic acids. Infect. Immun. **59:**4614–4620.
5. **Borezée, E., T. Msadek, L. Durant, and P. Berche.** 2000. Identification in *Listeria monocytogenes* of MecA, a homologue of the *Bacillus subtilis* competence regulatory protein. J. Bacteriol. **182:**5931–5934.
6. **Borezée, E., E. Pellegrini, and P. Berche.** 2000. OppA of *Listeria monocytogenes*, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. Infect. Immun. **68:**7069–7077.
7. **Braun, L., B. Ghebrehiwet, and P. Cossart.** 2000. gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*. EMBO J. **19:**1458–1466.
8. **Braun, L., H. Ohayon, and P. Cossart.** 1998. The InlB protein of *Listeria monocytogenes* is sufficient to promote entry into mammalian cells. Mol. Microbiol. **27:**1077–1087.
9. **Burnette-Curley, D., V. Wells, H. Viscount, C. L. Munro, J. C. Fenno, P. Fives-Taylor, and F. L. Macrina.** 1995. FimA, a major virulence factor associated with *Streptococcus parasanguis* endocarditis. Infect. Immun. **63:**4669–4674.
10. **Claverys, J. P.** 2001. A new family of high-affinity ABC manganese and zinc permeases. Res. Microbiol. **152:**231–243.
11. **Claverys, J. R., C. Granadel, A. M. Berry, and J. C. Paton.** 1999. Penicillin tolerance in *Streptococcus pneumoniae*, autolysis and the Psa ABC Mn-permease. Mol. Microbiol. **32:**881–883.
12. **Croizé, J., J. Arvieux, P. Berche, and M. Colomb.** 1993. Activation of the human complement alternative pathway by *Listeria monocytogenes*: evidence for direct binding and proteolysis of the C3 component on bacteria. Infect. Immun. **61:**5134–5139.
13. **Dinthilhac, A., G. Alling, C. Granadel, and J. R. Claverys.** 1997. Competence and virulence of *Streptococcus pneumoniae*: *adc* and *psaA* mutant exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Mol. Microbiol. **25:**727–740.
14. **Domann, E., J. Wehland, M. Rohde, S. Pistor, M. Hartl, W. Goebel, M. Leimeister-Wächter, M. Wuenster, and T. Chakraborty.** 1992. A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J. **11:**1981–1990.
15. **Dramsi, S., I. Biswas, E. Maguin, L. Braun, P. Mastroeni, and P. Cossart.** 1995. Entry of *Listeria monocytogenes* into hepatocytes requires expression of InIB, a surface protein of the internalin multigene family. Mol. Microbiol. **16:**251–261.
16. **Drevets, D. A., J. M. Leenen, and P. A. Campbell.** 1993. Complement receptor type 3 (CD11b/CD18) involvement is essential for killing of *Listeria monocytogenes* by mouse macrophages. J. Immunol. **151:**5431–5439.
17. **Drevets, D. A.** 1998. *Listeria monocytogenes* virulence factors that stimulate endothelial cells. Infect. Immun. **66:**232–238.
18. **Facinelli, B., E. Giovanetti, G. Magi, F. Biavasco, and P. E. Varaldo.** 1998. Lectin reactivity and virulence among strains of *Listeria monocytogenes* determined in vitro using enterocyte-like cell line Caco-2. Microbiology **144:**109–118.
19. **Fenno, J. C., A. Shaikh, G. Spatafora, and R. Fives-Taylor.** 1995. The *t7mA* locus of *Streptococcus parasanguis* encodes an ATP-binding membrane transport system. Mol. Microbiol. **15:**849–863.
20. **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.
21. **Gaillard, J. L., F. Jaubert, and P. Berche.** 1996. The *inlAB* locus mediates the entry of *Listeria monocytogenes* into hepatocytes *in vivo*. J. Exp. Med. **183:**359–369.
22. **Gilot, P., P. André, and J. Content.** 1999. *Listeria monocytogenes* possesses adhesins for fibronectin. Infect. Immun. **67:**6698–6701.
23. **Glaser, P., L. Frangeul, C. Buchriesser, A. Amend, F. Baquero Mochales, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chétaouni, E. Couvé, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durand, O. Dusurget, K. D. Entian, H. Fsihi, F. Garcia Del-Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, J. Kreft, F. Junst, G. Kurapkat, E. Madueno, A. Maitournam, J. Mata Vicente, E. Ng, G. Nordsiek, B. de Pabolos, J. C. Perez-Dias, B. Remmel, M. Rose, C. Rusniok, T. Schluerer, N. Simoes, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of *Listeria* species. Science **294:**849–852.
24. **Greiffenberg, L., Z. Sokolovic, H. J. Schnittler, A. Spory, R. Böckmann, W. Goebel, and M. Kuhn.** 1997. *Listeria monocytogenes*-infected human umbilical vein endothelial cells: internalin-independent invasion, intracellular growth, movement, and host cell responses. FEMS Microbiol. Lett. **157:**163–170.
25. **Harrington, D. J., J. S. Greated, N. Chanter, and I. C. Sutcliffe.** 2000. Identification of lipoprotein homologues of pneumococcal PsaA in the equine pathogens *Streptococcus equi* and *Streptococcus zooepidemicus*. Infect. Immun. **68:**6048–6051.
26. **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.
27. **Ireton, K., B. Payrastre, and P. Cossart.** 1999. The *Listeria monocytogenes* protein InlB is an agonist of mammalian phosphoinositide 3-kinase. J. Biol. Chem. **274:**17025–17032.
28. **Jenkinson, H. F.** 1994. Cell surface protein receptors in oral streptococci. FEMS Microbiol. Lett. **121:**133–140.
29. **Kayal, S., A. Lilienbaum, C. Poyart, S. Memet, A. Israel, and P. Berche.** 1999. Listeriolysin O-dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-kB and upregulation of adhesion molecules and chemokines. Mol. Microbiol. **31:**1709–1722.
30. **Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart.** 1992. *Listeria monocytogenes* induced actin assembly requires the *actA* gene product, a surface protein. Cell **68:**521–531.
31. **Kolenbrander, P. E., R. N. Andersen, R. A. Baker, and H. F. Jenkinson.** 1998. The adhesion-associated *sca* operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for Mn^{2+} uptake. J. Bacteriol. **180:**290–295.
32. **Kuhn, M., and W. Goebel.** 1997. Responses by murine macrophages infected with *Listeria monocytogenes* crucial for the development of immunity to this pathogen. Immunol. Rev. **158:**57–67.
33. **Kuhn, M., and W. Goebel.** 1989. Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. Immun. **57:**55–61.
34. **Lawrence, M. C., P. A. Pilling, V. C. Epa, A. D. Ogunniyi, and J. C. Paton.** 1998. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. Structure **6:**1553–1561.
35. **McLaughlan, A. M., and S. J. Foster.** 1998. Molecular characterization of an autolytic amidase of *Listeria monocytogenes* EGD. Microbiology **144:**1359–1367.
36. **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.
37. **Milohanic, E., R. Jonquières, P. Cossart, P. Berche, and J. L. Gaillard.** 2001. The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eucaryotic cells via its cell wall anchor. Mol. Microbiol. **39:**1212–1224.
38. **Nair, S., E. Milohanic, and P. Berche.** 2000. The ClpC ATPase is required for cell adhesion and invasion of *Listeria monocytogenes*. Infect. Immun. **68:**7061–7068.
39. **Novak, R., J. S. Braun, E. Charpentier, and E. Tuomanen.** 1998. Penicillin tolerance genes of *Streptococcus pneumoniae*: the ABC-type manganese permease complex Psa. Mol. Microbiol. **29:**1285–1296.
40. **Ofek, I., and N. Sharon.** 1988. Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. Infect. Immun. **56:**539–547.
41. **Rouquette, C., R. Tascon, E. Pellegrini, J. M. Bolla, M. T. Ripio, J. A. Vazquez-Boland, and P. Berche.** 1996. Identification of a ClpC ATPase

required for stress tolerance and *in vivo* survival of *Listeria monocytogenes*. Mol. Microbiol. **21:**977–987.

42. **Russell, H., J. A. Tharpe, D. E. Wells, E. H. White, and J. E. Johnson.** 1990. Monoclonal antibody recognizing a species-specific protein from *Streptococcus pneumoniae*. J. Clin. Microbiol. **28:**2191–2195.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Sampson, J. S., S. P. O'Connor, A. R. Stinson, J. A. Tharpe, and H. Russel.** 1994. Cloning and nucleotide sequence analysis of *psaA*, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus* sp. adhesins. Infect. Immun. **62:**319–324.
45. **Santiago, N. I., A. Zipf, and A. K. Bhunia.** 1999. Influence of temperature and growth phase on expression of a 104-kilodalton *Listeria* adhesion protein in *Listeria monocytogenes*. Appl. Environ. Microbiol. **65:**2765–2769.
46. **Schwarzer, N., R. Nost, J. Seybold, S. K. Parida, O. Fuhrmann, M. Krüll, R. Schmidt, R. Newton, S. Hippenstiel, E. Domann, T. Chakraborty, and N. Suttorp.** 1998. Two distinct phospholipases C of *Listeria monocytogenes* induce ceramide generation, nuclear factor-kB activation, and E-selectin expression in human endothelial cells. J. Immunol. **161:**3010–3018.
47. **Shen, Y., M. Naujokas, M. Park, and K. Ireton.** 2000. InlB-dependent internalization of *Listeria* is mediated by the met receptor tyrosine kinase. Cell **103:**501–510.
48. **Sibelius, U., T. Chakraborty, B. Krögel, J. Wolf, F. Rose, R. Schmidt, J. Wehland, W. Seeger, and F. Grimminger.** 1996. The listerial exotoxins listeriolysin and phosphatydilinositol-specific phospholipase C synergize to elicit endothelial cell phosphoinositide metabolism. J. Immunol. **157:**4055–4060.
49. **Sibelius, U., F. Rose, T. Chakraborty, A. Darji, J. Wehland, S. Weiss, W. Seeger, and F. Grimminger.** 1996. Listeriolysin is a potent inducer of the phosphatidylinositol response and lipid mediator generation in human endothelial cells. Infect. Immun. **64:**674–676.
50. **Spellerberg, B., E. Rozdzinski, S. Martin, J. Weber-Heynemann, N. Schnitzler, R. Lutticken, and A. Podbielski.** 1999. Lmb, a protein with similarities to the Lral adhesin family, mediates attachment of *Streptococcus agalactiae* to human laminin. Infect. Immun. **67:**871–878.
51. **Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehlan, and J. Kreft.** 2001. *Listeria* pathogenesis and molecular determinants. Clin. Microbiol. Rev. **14:**584–640.
52. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase myosin kinases and other ATP requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.

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