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LPXTG Protein InlJ, a Newly Identified Internalin Involved in *Listeria monocytogenes* Virulence

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Listeria monocytogenes **expresses surface proteins covalently anchored to the peptidoglycan by sortase enzymes. Inactivation of *srtA* attenuates *Listeria* virulence in mice (H. Bierne, S. K. Mazmanian, M. Trost, M. G. Pucciarelli, G. Liu, P. Dehoux, L. Jansch, F. Garcia-del Portillo, O. Schneewind, and P. Cossart, Mol. Microbiol. 43:869–881, 2002). We show here that an *srtA* mutant is more attenuated than an internalin mutant in orally infected guinea pigs and transgenic mice expressing human E-cadherin (hEcad mice), indicating the involvement of other SrtA substrates, LPXTG proteins, in food-borne listeriosis. Data recently generated with a listerial DNA macroarray identified two LPXTG protein-encoding genes present in the genomes of *L. monocytogenes* strains and absent from all other *Listeria* species, *inlI* (lmo0333) and *inlJ* (lmo2821). They also revealed two other LPXTG protein-encoding genes, ORF29 and ORF2568, present only in a subclass of *L. monocytogenes* serovars, including the epidemic serovar 4b. We report here that an *inlJ* deletion mutant, in contrast to *inlI* and ORF29 mutants, is significantly attenuated in virulence after intravenous infection of mice or oral inoculation of hEcad mice. Interestingly, a ΔORF2568 strain showed a slight increase in virulence. *inlJ* encodes a leucine-rich repeat (LRR) protein that is structurally related to the listerial invasion factor internalin. However, the consensus sequence of the InlJ LRR defines a novel subfamily of cysteine-containing LRRs in bacteria. In conclusion, this postgenomic approach identified InlJ as a new virulence factor among the proteins belonging to the internalin family in *L. monocytogenes*.**

Listeria monocytogenes is a bacterial pathogen that causes listeriosis, a severe food-borne disease in humans (21). It is a facultative intracellular pathogen that is able to enter and multiply in both professional phagocytes (45) and nonphagocytic cells (24, 35). Following ingestion of contaminated food, some bacteria cross the intestinal barrier and gain access to the liver and spleen via the bloodstream. In these two organs the majority of bacteria are eliminated by the immune system. In immunocompromised individuals and pregnant women bacteria can cross the tight blood-brain barrier and the maternofetal barrier, respectively, and reach the central nervous system and the placenta.

Several listerial surface proteins play key roles in listerial interactions with mammalian host cells (8). One of these proteins, internalin (InlA), promotes *Listeria* internalization in epithelial cells by interacting with the host adhesion protein E-cadherin (51) and is required for invasion of intestinal and placental villi in vivo (38, 40). InlA possesses an N-terminal leucine-rich repeat (LRR) domain (59) and a C-terminal sorting signal, including an LPXTG motif. Anchoring of InlA to the bacterial surface is achieved by sortase A (SrtA) (6), a transpeptidase that cleaves the LPXTG motif between the threonine and glycine residues and covalently links the threonine to the peptidoglycan (12, 16, 48, 61). Inactivation of *srtA* in *L. monocytogenes* inhibits anchoring of LPXTG proteins to the cell wall and attenuates virulence following intravenous or oral infection in mice (6, 27). The *Listeria* genome encodes another sortase, SrtB, which seems to have only two substrates carrying an NXZTN motif instead of LPXTG and which is not required for the infectious process in mice (5).

A better understanding of listeriosis pathophysiology relies on identification of new bacterial virulence factors and their characterization in adapted animal models. Intravenous inoculation of mice has proven to be a good route to study the virulence factors involved in the intracellular life of *L. monocytogenes* (18, 35). However, bacterial translocation across the intestinal barrier is inefficient in the mouse (40). In contrast, in guinea pigs and in transgenic mice expressing human E-cadherin, the internalin receptor (40), in enterocytes (hEcad mice), bacteria can efficiently cross the intestinal epithelial barrier and gain access to deeper tissues. These two models thus appear to be more appropriate than a normal mouse model for studying listeriosis acquired via the oral route (37), which is the natural route for infection in humans (57).

In the past few years, major listerial virulence factors have been identified by using classical genetic approaches, such as generation of transposition mutants and characterization of these mutants in infection experiments in cultured cells (18, 35). Determination and comparison of the genome sequences of *L. monocytogenes* and the nonpathogenic closely related species *Listeria innocua* (28) have now paved the way for identifying new virulence factors (8, 14, 28, 53). Such a comparative

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Property	Reference
Strains		
BUG 1600	*L. monocytogenes* EGDe wild-type strain	28
BUG 1777	Isogenic Δ*srtA* strain	6
BUG 1877	Isogenic Δ*srtB* strain	5
BUG 1454	Isogenic Δ*inlA* strain	42
BUG 2158	Isogenic Δ*inlI* strain	This study
BUG 2159	Isogenic Δ*inlJ* strain	This study
BUG 2099	*L. monocytogenes* CLIP80459 seravor 4b wild-type strain	14
BUG 2194	Isogenic ΔORF29 strain	This study
BUG 2195	Isogenic ΔORF2568 strain	This study
Plasmids		
pMAD		1
pMAD-Δ*inlI*	Thermosensitive plasmid pMAD carrying coligated 650-bp fragments flanking *inlI*	This study
pMAD-Δ*inlJ*	Thermosensitive plasmid pMAD carrying coligated 650-bp fragments flanking *inlJ*	This study
pMAD-ΔORF29	Thermosensitive plasmid pMAD carrying coligated 650-bp fragments flanking ORF29	This study
pMAD-ΔORF2568	Thermosensitive plasmid pMAD carrying coligated 650-bp fragments flanking ORF2568	This study

approach has, for instance, led to the identification of a bile salt hydrolase (17) and an autolysin (9) as new factors involved in *Listeria* infections. Besides whole-genome comparisons, a *Listeria* DNA array has recently been generated to study inter- and intraspecies diversity (14). This macroarray, which harbors specific genes from three sequenced strains, two pathogenic *L. monocytogenes* strains (serovars 1/2a and 4b) and a nonpathogenic *L. innocua* strain, was used to analyze the DNA content and genomic biodiversity of 113 *Listeria* strains of different species and serovars. Hybridization results identified *L. monocytogenes*-specific marker genes, including *inlA* and other previously identified virulence factors, such as *hly*, *actA*, or *inlB*, as well as serovar-specific marker genes. It is now well established that *L. monocytogenes* strains do not appear to be equally able to generate disease in humans (30). Only 4 of the 13 serovars identified in this species, serovars 1/2a, 1/2c, 1/2b, and 4b, are responsible for the reported human listeriosis cases (31). Notably, serotype 4b strains are overrepresented compared with the strains of other serotypes among the organisms responsible for outbreaks and sporadic cases of listeriosis (63).

In this work, we first confirmed that *srtA* inactivation strongly attenuates *Listeria* virulence in orally acquired listeriosis, supporting the role of LPXTG surface proteins in the infectious process. Then, by exploiting the genome and biodiversity array data, we addressed the role of four previously uncharacterized LPXTG protein-encoding genes, two *L. monocytogenes*-specific genes and two genes present in a subset of serovars, including serovar 4b. We generated deletion mutants and analyzed them in vitro and in vivo. This work revealed the role of the lmo2821 gene product, InlJ, an LRR cysteine-containing protein, in orally acquired listeriosis. In addition, inactivation of the ORF2568 gene in *L. monocytogenes* serovar 4b slightly increased bacterial virulence, suggesting that the gene product has a role in virulence modulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cell lines. The strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in brain heart infusion (BHI) medium (Difco) supplemented with erythromycin (5 μg ml^{-1}) when they carried pMAD derivatives. The cell lines used were as follows: human choriocarcinoma JEG-3 cells (ATCC HTB-36), colon carcinoma Caco-2 cells (ATCC HTB-37) and LoVo cells (ATCC CCL-229), hepatocarcinoma HepG2 cells (ATCC HB-8065), cervix carcinoma HeLa cells (ATCC CCL-2), human embryonic kidney HEK 293 epithelial cells (ATCC CRL-1573), umbilical vein HUVEC endothelial cells (ATCC CRL-1730), and RAW 264.7 (ATCC TIB-71) and J774 (ATCC TIB-67) murine macrophages. Cell lines were cultured in modified Eagle medium (Gibco), Dulbecco modified Eagle medium (DMEM), or Ham's F12K medium supplemented with fetal bovine serum (10% or 20%), 1 mM pyruvate, 2 mM glutamine, and 1% nonessential amino acids (Gibco) according to American Type Culture Collection recommendations. HUVEC cells were cultured with 0.1 mg · ml^{-1} of heparin (Sigma) and 0.05 mg · ml^{-1} of endothelial growth factor. Macrophages were cultured with decomplemented fetal bovine serum. Cells were incubated at 37°C in the presence of 10% CO_2.

Chromosomal inactivation of the *inlI*, *inlJ*, ORF29, and ORF2568 genes. (i) Construction of the in-frame Δ*inlI* and Δ*inlJ* *L. monocytogenes* EGDe deletion mutants. Two ~650-bp fragments flanking the target gene (*inlI* or *inlJ*) were amplified by PCR from EGDe chromosomal DNA with primers inside and outside the *inlI* and *inlJ* loci. The primers used for the *inlI* 5′ flanking fragment were i1 (5′-CGCGGATCCTGCGGTATCAAGAAGTTTATC-3′) and i2 (5′-AAAAGGCCTCTTTTTCAAGACTTTCCCTCT-3′), and the primers used for the 3′ fragment were i3 (5′-AAAAGGCCTACGAACCAATAAATGGATAAAC-3′) and i4 (5′-TCCCCCGGGCGGAGTTGCTGTGTATCTAGT-3′). The primers used for the *inlJ* 5′ flanking fragment were j1 (5′-CGCGGATCCTAGCAGAAGATGGAACCC-3′) and j2 (5′-AAAAGGCCTAGTTTTCAATTTGGCGCACTC-3′), and the primers used for the 3′ fragment were j3 (5′-AAAAGGCCTAAATAGTAAAAAAGCCGGACA-3′) and j4 (5′-CCGGAATTCCAAATGATGTTATCGGTCAGC-3′). After restriction of the amplified 5′ and 3′ fragments with BamHI and StuI or StuI and SmaI (for Δ*inlI* construction) and with BamHI and StuI or StuI and EcoRI (for Δ*inlJ* construction), 5′ and 3′ fragments were coligated in the thermosensitive plasmid pMAD digested by BamHI and SmaI or by BamHI and EcoRI, yielding the pMAD-Δ*inlI* and pMAD-Δ*inlJ* plasmids. These plasmids were electroporated into *L. monocytogenes* strain EGDe, and gene replacement was performed as described previously (1) but at a nonpermissive temperature growth, 43.5°C, resulting in in-frame deletions of the *inlI* or *inlJ* genes.

(ii) Construction of the in-frame ΔORF29 and ΔORF2568 deletion mutants in *L. monocytogenes* serovar 4b strain. ΔORF29 and ΔORF2568 were constructed as described above for the Δ*inlI* and Δ*inlJ* strains, with the following modifications. The primers used for ΔORF29 construction were k1 (5′-CGCGGATCCGTGAGCCTATGTACGGTC-3′), k2 (5′-AAAAGGCCTCACAGAAAAGCCCCCTTA-3′), k3 (5′-AAAAGGCCTAGAAGTTAAACCACTCCAT-3′) for the 5′ and 3′ flanking fragments, respectively, and k4 (5′-CCGGAATTCCCATTAAAGAAGCAGAACA-3′), and the primers used for ΔORF2568 construction were l1 (5′-CGCGGATCCCGTCATATATCAATCTCCAT-3′), l2 (5′-AAAAGGCCTCATTTTTTTTGCCCCTTCAA-3′), l3 (5′-AAAAGGCCTTAAAAAAACGCCCAGTATTTG-3′), and l4 (5′-TCCCCCGGGTCTCCAAGCCACAATCAC-3′) for the 5′ and 3′ flanking fragments, respectively. The enzymes used for the construction of pMAD-ΔORF29 and pMAD-ΔORF2568 were BamHI, StuI, and EcoRI for pMAD-ΔORF29 and BamHI, StuI, and SmaI for pMAD-ΔORF2568.

Mutant verification by PCR and sequencing. Mutants were verified by PCR analysis of chromosomal DNA using pairs of oligonucleotide primers inside genes, including i7 (5′-CGGAGCGCCTGTCATTTC-3′) and i8 (5′-TACGCCG TCTTCATTCGT-3′) for Δ*inlI*, j7 (5′-GAGGCTGAAGGGCAAACAATC-3′) and j8 (5′-CATCCGCCAATTTTTCTCCTT-3′) for Δ*inlJ*, k7 (5′-GGGAGGA ACTCTTTTAGTGTC-3′) and k8 (5′-GCTTGGTACAATGAGTGTACT-3′) for ΔORF29, and l7 (5′-CATCTTCAAGATCAAGCATTA-3′) and l8 (5′-GCT AACTTATTTCCATCACCA-3′) for ΔORF2568, and pairs of oligonucleotide primers in regions flanking the genes, including i5 (5′-GCGTACTCATTTAAG ACGAAT-3′) and i6 (5′-AAGCCTCTCTTTAATGGACAG-3′) for Δ*inlI*, j5 (5′-ATAAATACGCCTCGCTTA-3′) and j6 (5′-GACTAGTTTTGATGTGGA-3′) for Δ*inlJ*, k5 (5′-TCTAGCGGGAATACTTGGGTT-3′) and k6 (5′-GCAG GAGCAACATTCGGTTGG-3′) for ΔORF29, and l5 (5′-AGGCGAGCTAGA ACATAATAC-3′) and l6 (5′-CTGCTTCCAAAAGCAACAATC-3′) for ΔORF2568. Amplified fragments from Δ*inlI*, Δ*inlJ*, ΔORF29, and ΔORF2568 strain chromosomal DNA, obtained by using primers i5 and i6, primers j5 and j6, primers k5 and k6, and primers l5 and l6, respectively, were verified by sequencing.

RNA techniques. RNA from *Listeria* was isolated and purified with an RNAeasy kit (QIAGEN). Briefly, cultures of *Listeria* were centrifuged at 4,000 × *g* for 6 min. The pellets were resuspended in 100 μl of Tris-EDTA and 250 μl of RNAeasy lysis buffer and transferred to a Bead Beater tube containing 0.4 g of mini glass beads (Sigma). Bacteria were broken mechanically using a Fast Prep apparatus (Bio 101) and centrifuged (13,000 × *g*, 1 min), and the supernatants were transferred and treated according to the manufacturer's procedure. DNA contaminants were eliminated with a DNase kit (Ambion). The presence of the *inlI*, *inlJ*, ORF29, and ORF2568 genes in wild-type and mutants strains was assed by amplification of internal fragments by reverse transcriptase (RT)-PCR, using Superscript one-step RT-PCR (Invitrogen) and the internal primers described above. The absence of DNA contamination of the RNA was checked by PCR. For mutant verification, bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.6. Primers iap-F (5′-AAAGCAACTATCGCGGCTAC-3′) and iap-R (5′-TCTTGAACAGAAACACCGTA-3′), which amplify the *iap* gene encoding the p60 protein, were used as a control for RNA amplification. In the semiquantitative PCR assay, bacteria were grown to an OD_{600} of 0.8, as in the gentamicin survival assay (see below). Primers inlA-1 (5′-CATCACCTTATAT GCCCAATATAGC-3′) and inlA-2 (5′-GATTTTTCGTAAATTGAGCGTAC AG-3′) and primers gyrA-1 (5′-AACTTTGGTTCGGTTGATGG-3′) and gyrA-2 (5′-TGGCTCACGTTCAGAACC-3′), which amplify the *inlA* and *gyrA* genes, respectively, were used as controls for known expressed genes.

Gentamicin survival assay. A gentamicin survival assay was performed as described previously (25, 51). Briefly, the *Listeria* strains were grown in BHI medium to an OD_{600} of 0.8 to 1, washed in phosphate-buffered saline, and diluted in DMEM, so that the multiplicity of infection was about 50 bacteria per cell. Bacterial suspensions were added to mammalian cells for 1 h, the cells were washed, and the noninvasive bacteria were killed by adding 10 μg/ml gentamicin for 2 h. After washing, the cells were lysed in 0.2% Triton X-100, and the number of viable bacteria released from the cells was assessed by plating on agar plates.

In vivo experiments. Four animals were used for each experiment, and all experiments were reproduced at least once and gave similar results. Prior to oral infection, the animals were starved to prevent variations linked to gastric repletion, which may influence intragastric bacterial survival. Statistical analyses were performed using the Student *t* test. P values of <0.05 were considered statistically significant. All animals were treated in accordance with Institut Pasteur guidelines for laboratory animal husbandry.

(i) Guinea pig oral infections. Experiments were performed as described previously (40). Briefly, 300-g male Hartley guinea pigs (Charles River) were starved for 2 days and anesthetized (15 mg/ml ketamine injected intramuscularly). Five milliliters of a 25-mg/ml $CaCO_3$ solution in phosphate-buffered saline was injected intragastrically, followed by 1 ml of a sublethal bacterial inoculum (2.5×10^{10} CFU). After 96 h of infection, spleens, livers, and the whole mesenteric lymph nodes were sterilely dissected. The central 20-cm portion of the guinea pig small intestine was rinsed in DMEM (Gibco) to remove the intestinal contents, incubated at 20°C for 2 h in DMEM containing 100 mg/liter gentamicin (Gibco) to kill the extracellular bacteria from the intestinal lumen, and rinsed three times in DMEM. For bacterial enumeration, the numbers of CFU were determined by plating serial dilutions of organ (intestine, mesenteric lymph node, liver, and spleen) homogenates on BHI agar.

iFABP-hEcad transgenic mouse oral infections. iFABP-hEcad transgenic mice (40) were starved for 1 day and anesthetized. Three hundred microliters of a 50-mg/ml $CaCO_3$ solution was injected intragastrically along with 200 μl of a sublethal bacterial inoculum (10^9 CFU). After 72 h of infection, spleens, livers, and the whole mesenteric lymph nodes were sterilely dissected. The central long portion of the mouse small intestine was rinsed in DMEM (Gibco) to remove the intestinal contents, incubated at 20°C for 2 h in DMEM containing 100 mg/liter gentamicin (Gibco) to kill the extracellular bacteria from the intestinal lumen, and rinsed three times in DMEM. For bacterial enumeration, the numbers of CFU were determined by plating serial dilutions of organ (intestine, mesenteric lymph node, liver, and spleen) homogenates on BHI agar.

Mouse intravenous infections. Bacterial growth in mice was studied by injecting 4- to 6-week-old specific-pathogen-free female BALB/c mice (Charles River) intravenously with a sublethal bacterial inoculum (10^4 CFU). At 24, 48, and 72 h after infection, the livers and spleens were sterilely dissected, and the numbers of CFU were determined by plating serial dilutions of organ (liver and spleen) homogenates on BHI agar.

RESULTS

Role of sortase A substrates in *L. monocytogenes* oral infections in guinea pigs. *L. monocytogenes* translocation across the intestinal barrier occurs efficiently by an InlA-mediated process in guinea pigs (37). To address the global role of LPXTG proteins in the crossing of the intestinal barrier, we analyzed the virulence of an *srtA* deletion mutant in this animal model and compared it to that of a Δ*inlA* strain. We also addressed the effect of inactivating the second sortase gene, *srtB*. Quantification of the level of infection in the small intestine, mesenteric lymph nodes, liver, and spleen was used as a measure of virulence. Guinea pigs were orally infected with the *L. monocytogenes* wild-type reference strain EGDe or with *srtA*, *inlA*, or *srtB* isogenic deletion mutants. Bacterial counts in the organs were determined 96 h postinfection, which corresponded to the peak for the bacterial load in this animal model (40). For the Δ*srtA* strain there were decreases in the bacterial counts of 3 $\log_{10}$ in the intestine and liver and 2 $\log_{10}$ in the mesenteric lymph nodes compared to the wild-type strain and decreases of 1 $\log_{10}$ in both organs compared to the Δ*inlA* strain (Fig. 1). No Δ*srtA* or Δ*inlA* bacteria were recovered from spleens. In contrast to the Δ*srt*A strain, the Δ*srtB* strain did not exhibit a virulence defect. These results confirmed that at least one SrtA substrate other than InlA plays a role in the intestinal and hepatic phases of orally acquired listeriosis, which is consistant with our previous results for mice (6). Moreover, they strongly suggest that some LPXTG proteins play an additive role along with InlA in the crossing of the intestinal barrier.

Search for new *L. monocytogenes* virulence factors in the LPXTG protein family. In order to identify new LPXTG proteins implicated in *L. monocytogenes* virulence, we exploited the data resulting from a *Listeria* DNA macroarray analysis (14) to find genes conserved in pathogenic species. Seven genes encoding surface proteins were found as markers for the *L. monocytogenes* species; these genes were conserved in the genomes of all *L. monocytogenes* isolates and were absent from all other *Listeria* species (14). Six of the genes encode proteins belonging to the internalin family (*inlA*, *inlB*, *inlH*, *inlE*, lmo0333, and lmo2821). All these genes are present in the five *L. monocytogenes* strains that have been sequenced (14, 28, 53). Δ*inlA*, Δ*inlB*, Δ*inlH*, and Δ*inlGHE* strains have been shown to be attenuated in virulence (18, 35, 38, 40, 55, 58), while the contributions of the lmo0333 and lmo2821 genes have never been studied. Therefore, we focused on the lmo0333 and lmo2821 genes in this study.

Predicted structures of the internalin-like proteins Lmo0333 (InlI) and Lmo2821 (InlJ). lmo0333 (5,337 bp) and lmo2821 (2,556 bp) are predicted to encode structurally re-

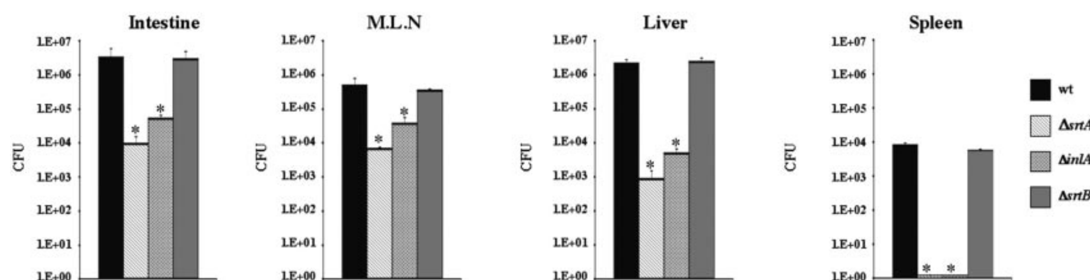

FIG. 1. *srtA* is involved in *L. monocytogenes* oral infections in guinea pigs. The wild-type EGDe strain (wt) and isogenic Δ*srtA*, Δ*inlA*, and Δ*srtB* strains were orally inoculated into guinea pigs, as described in Materials and Methods. Animals were euthanized 96 h after infection, and organs were recovered, homonogenized, and plated. The numbers of bacteria able to colonize the intestine, mesenteric lymph nodes (M.L.N), liver, and spleen are expressed in $\log_{10}$ CFU. In each experiment four animal were used for each bacterial strain. The results are representative of two independent experiments. An asterisk indicates a significant difference ($P < 0.05$) between a mutant strain and the wild-type strain.

lated proteins consisting of 1,778 and 851 amino acids, respectively. The two proteins possess an N-terminal signal sequence followed by an LRR domain consisting of 28 and 15 LRRs, respectively, a domain characteristic of internalin family members (8, 15). Given this structural characteristic, Lmo0333 and Lmo2821 were desiganted InlI and InlJ. InlI is the largest internalin among the 19 internalin-like proteins present in the EGDe strain (8). As in InlA and in other described internalins (58), the LRR domains of both InlI and InlJ are followed by a conserved immunoglobulin-like domain, a variable region, and a C-terminal sorting signal that includes an LPXTG motif. The variable regions of InlI and InlJ contain specific repeats that are ~70 residues long reported to be MucBP (*muc*in *b*inding *p*rotein) domains (previously DUF1085; accession number PF06458) (Fig. 2A). MucBP domains are found in conjunction with an LRR domain and an LPXTG sorting signal in several bacterial peptidoglycan-bound proteins, especially in *Listeria* and *Lactobacillis* species. The InlI and InlJ proteins contain three and four MucBP domains, respectively. The InlI variable region also contains a PKD (*p*olycystin *k*idney *d*isease) repeat region (8) related to that found in the human membrane polycystin kidney disease protein (4).

Interestingly, while the LRR consensus in InlI is similar to that in InlA and other previously described internalins (58), the consensus in InlJ is slightly different (Fig. 2B). The internalin LRR prototype motif forms a structural unit consisting of a β strand and a 3_{10}-helix, which occurs several times in tandem arrrays, and most repeats are 22 residues long (46). InlJ LRRs are in most cases 21 residues long and contain a conserved cysteine at position 7 (or position 9 in repeat 12) in place of a leucine (Fig. 2B). Furthermore, the expected 3_{10}-helix region appears to be shorter than that in InlA, with only one residue (usually an aspartate) between the two leucines instead of the two residues in InlA. Lastly, an asparagine residue usually flanks the 3_{10}-helix.

Only one other internalin-like protein in *L. monocytogenes*, Lmo0331, and its ortholog in *L. innocua*, Lin0354, contain LRRs of the InlJ type. We also found LRRs in proteins from five other bacterial species (Fig. 2C). Two proteins from entomopoxviruses may be related to this subfamily. Finally, several eukaryotic LRRs contain cysteines, but they are not at the same position as in the InlJ LRR consensus. This is the case for the RNase inhibitor LRR type and several proteins of the CC (*c*ysteine-*c*ontaining) subfamily (Fig. 2C) (33, 36). Thus, InlJ defines a new type of LRR in the internalin family in bacteria.

Effect of *inlI* or *inlJ* gene inactivation in the *L. monocytogenes* cellular infectious process in vitro. In the genome of *L. monocytogenes* EGDe, *inlI* is the first gene of a putative operon comprising seven other genes (28). Six of these genes are predicted to encode proteins with unknown functions, while one, lmo0339, exhibits similarities with a pyrophosphatase-encoding gene. Four genes in this operon, including *inlI*, are absent in the genome of *L. innocua* CLIP11262, a sequenced strain. The *inlJ* gene is also absent from the *L. innocua* genome, but the surrounding regions are identical in *L. innocua* and *L. monocytogenes*. *inlJ* is in the orientation opposite that of lmo2820, a gene encoding a protein with an N-terminal domain exhibiting similarities with the transcriptional AraC/XylS family of regulators (26). No specific recognition sequence for the *Listeria* virulence regulator PrfA precedes the *inlI* and *inlJ* genes. The expression of *inlI* and *inlJ* in bacteria grown in BHI medium in the late exponential phase at 37°C was assessed by RT-PCR and compared to that of *inlA* and *gyrA* genes, which encode the invasion protein InlA and gyrase, respectively. Expression products were detected in all cases, indicating that *inlI* and *inlJ* are transcribed in these conditions (Fig. 3A).

In order to study their potential role in virulence, the *inlI* and *inlJ* genes were inactivated by in-frame deletion to prevent polar effects. Deletion mutants were obtained by allelic exchange in *L. monocytogenes* EGDe, as previously described (1). As expected, no RT-PCR products were detected from the mutant strains (Fig. 3B). The Δ*inlI* and Δ*inlJ* strains displayed no growth defect in BHI medium at 37°C compared to the wild-type strain (data not shown). We then examined the ability of these strains to adhere to two epithelial cell lines, intestinal Caco-2 and placental JEG-3 cells, and to J774 macrophages, using previously described techniques (7, 15). After 1 h of infection at an initial multiplicity of infection of 50 bacteria per cell, the Δ*inlI* and Δ*inlJ* strains displayed no noticeable difference from the wild-type strain in adherence to host cells (data not shown). Then their ability to enter cells was examined by using nine different cell types, including epithelial and endothelial cells, hepatocytes, and macrophages. As shown in Table 2, the entry of the Δ*inlI* or Δ*inlJ* strain after 1 h of infection was similar to that of the wild-type strain for all cell types, suggesting that these genes are not involved in the *L.*

A

Signal peptide

MKTTKIVIASLVSLTMVSNPLLTFAATNDVIDNTTEITTDKETSSTQP
TIKNTLKAGQTQSFNDWFPDDNFASEVAAAFEMQATDTISEEQLA

Sequence	
TLTSLDCHNSSITDMTGIEKLT	LRR1
GLTKLICTSNNITTLDLSQNT	LRR2
NLTYLACDSNKLTNLDVTPLT	LRR3
KLTYLNCDTNKLTKLDVSQNP	LRR4
LLTYLNCARNTLTEIDVSHNT	LRR5
QLTELDCHLNKKITKLDVTPQT	LRR6
QLTTLDCSFNKITELDVSQNK	LRR7
LLNRLNCDTNNITKLDLNQNI	LRR8
QLTFLDCSSNKLTELDVTPLT	LRR9
QLTYFDCSVNPLTELDVSTLS	LRR10
KLTTLHCIQTDLLELDLTHNTQ	LRR11
LIYFQAEGCRKIKELDVTHNT	LRR12
QLYLLDCQAAGITELDLSQNP	LRR13
KLVYLYLNNTELTELDVSHNT	LRR14
KLKSLSCVNAHIQDFSSVGKI	LRR15

InlJ

PALNNNFEAEG
QTITMPKETLTNNSLTIAVSPDLLDQFGNPMNIEPGDGGVYDQATNTIT Ig-like
WENLSTDNPAVTYTFTSENGAIVGTVTTPFEA
PQPIKGED
VTVHYLDDKGEKLADDEVLSGNLDDPYTSSAKDIPDYTLTTTPDNATGTFTTTSQSVTYVYTKNIVAAEP MucBP
VTVNYVDDTGKTLSPSEILNGNVGDTYNATAKQIDGYTLSAEPTNATGQFTSSAQTVNYIYTKNPAPEKGV MucBP
VEIHYVDEDNKQLNSTTEISGTIGDNYTTEPKTIEGYTLTTTPGNATGTFTTGSQTVTYVYTKNIEAAEP MucBP
ITVNYVDANGKTLAPSETLNGNVGDTYKATAKQIDGYTLSAEPTNATGQFTSSAQTVNYIYTKNTNTDQPL MucBP
PTKKPTNTTPTKPSNLKTTEVKKASDTLPKTGDSAPWKSA*LLGVFLSSTALVI*WKKKK

B

β strand (positions 3–7); 3_{10}–helix (positions 15–19)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
InlA	X	L	X	X	L	X	L	X	X	N	X	I	S/T	D	L	X	X	L	X	X	L	X
InlJ	X	L	T	X	L	X	C	X	X	N	X	I/L	T	X	L	D	V/L	X	X	N	X	

C

Name	Length	Consensus sequence	Organism	Accession Number
InlJ	21-22	X L T X L X C X X N X I/L T X L D L/V S X N X	*Listeria monocytogenes*	NP_466343
Lmo0331	21	X L X X L X C X X N Q L T X L D V S K/Q N L	*Listeria monocytogenes*	NP_463861
Lin0354	21	A L X X L X C X X N Q L T N L D V S X N L	*Listeria innocua*	NP_469699
EF2250	21-22	X L X X L D/S C S X N X L T/D X L D V/I T/S X N X	*Enterococcus faecalis*	NP_815909
COG4886	21	X L X X L X C X X N X L T X L/I D V S V/I N L	*Cytophaga hutchinsonii*	ZP_00310106
TDE0593	22	A L Q X L X C X X N X L T S L D/N V/I Q G L X	*Treponema denticola*	NP_971207
BT0284	20-21	X L X X L/I X C X X X X L X X L/I D L/I S X N X	*Bacteroides thetaiotaomicron*	NP_809197
PG0350	21	X L X X L X C X X N X L T X L D/N V/L S A N T	*Porphyromonas gingivalis*	NP_904662
ORFMSV251	19	X L X X L X C X N N X I X X L/I X I N X	*Melanoplus sanguinipes entomopoxvirus*	AAC97727
AMVITR01	22	N L K/E K/E L X C S/Y X T X I X S L K G I/L X N L I	*Amsacta moorei entomopoxvirus*	NP_065061

Name	Length	Consensus sequence	Organism	Reference
InlA	21-22	X L X X L X L X X N X I T/S D L X X L X X L X	Bacteria	(33, 36)
InlJ (bCC)	19-21	X L X X I/L X C X X N X I/L T X I/L D/N V/L X X N/L X	Bacteria	(This study)
RI	28-29	X L X X L X L X X N/C X L X X X G O X X L X X O L X X X X X	Animals	(33, 36)
CC	25-27	X L X X L X L X X C X X I T D X X O X X L A/G X X L/C X	Animals, plants and fungi	(33, 36)

FIG. 2. InlJ LRR defines a new family of bacterial cysteine-containing LRRs. (A) Amino acid sequence of the *inlJ* gene product. The signal peptide is indicated by boldface letters. The 15 LRRs are aligned, with leucines, isoleucines, and valines indicated by a red background and cysteines indicated by a blue background. The other conserved residues are indicated by colored letters. The immunoglobulin (Ig)-like domain is indicated by a gray background. The four MucBP repeats are aligned with conserved residues in boldface letters. The LPXTG motif in the C-terminal sorting signal is underlined, and the hydrophobic residues are indicated by italics. (B) Comparison of InlA and InlJ LRR consensus sequences. Residue positions are numbered as described by Schubert et al. (58). The positions of the expected β strand and 3_{10}-helix are indicated. Conserved residues are indicated by colored letters (hydrophobic core, red; cysteines, blue). (C) Alignment of LRR consensus sequences of InlJ and proteins from different species. (Top) Alignment of InlJ LRR with LRRs of bacterial or viral proteins. (Bottom) Alignment of InlA and InlJ LRR consensus sequences with LRRs of RNase inhibitor (RI) and cysteine-containing (CC) types from eukaryotes. Conserved residues are indicated by colored letters (leucines, valines, and isoleucines, red; cysteines, dark blue; threonine, light blue; asparagine, green; aspartic acid, orange). O, hydrophobic residues; bCC, bacterial cysteine containing.

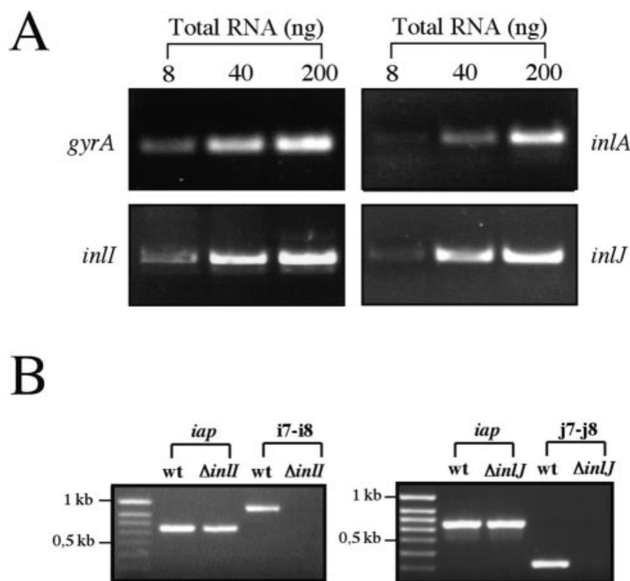

FIG. 3. Expression of *inlI* and *inlJ*. (A) RT-PCR for total RNAs from the end of the exponential phase (OD_{600}, 0.8) for cultures in BHI medium at 37°C of *L. monocytogenes* wild-type strain. Serial dilutions of the RNA templates were used. The *gyrA* and *inlA* genes were used as controls of known expressed genes. (B) RT-PCR for RNAs from *L. monocyogenes* wild-type and Δ*inlI* or Δ*inlJ* strains. Amplification of the *iap* gene was used to control RNA amounts. wt, wild type.

monocytogenes internalization process. Finally, these mutants did not appear to be altered in intracellular multiplication, actin-based motility, and cell-to-cell spreading (data not shown). Taken together, these results indicate that the Δ*inlI* and Δ*inlJ* strains displayed no detectable defect in infection of tissue-cultured cells.

Effect of *inlI* and *inlJ* inactivation on organ colonization. The contribution of the *inlI* and *inlJ* genes in the *L. monocytogenes* infectious process in vivo was examined first by using an intravenous mouse model of infection. BALB/c mice were infected intravenously with the wild type or with the Δ*inlI* or Δ*inlJ* strain. Bacterial counts in the liver and spleen were determined 24, 48, and 72 h postinfection. As shown in Fig. 4A, at 72 h postinfection a 1-$\log_{10}$ decrease in the liver and spleen

TABLE 2. Determination of internalization efficiencies

Cell line[a]	Relative % of entry into cell		
	Wild type	Δ*inlI*	Δ*inlJ*
Caco-2	100	115 ± 27	110 ± 32
JEG-3	100	100 ± 3	102 ± 3
Hep-G2	100	102 ± 8	96 ± 11
HUVEC	100	86 ± 8	102 ± 8
LoVo	100	115 ± 9	93 ± 4
HeLa	100	115 ± 6	102 ± 5
HEK293	100	104 ± 5	101 ± 4
J774	100	86 ± 9	127 ± 14
RAW 264.7	100	125 ± 9	107 ± 5

[a] Cells were infected with the bacterial strains for 1 h, and invasion frequencies were calculated from the number of bacteria that survived gentamicin treatment compared with the total number of inoculated bacteria. The level of entry of the wild-type strain was arbitrarily defined as 100%, and the levels of entry of the different mutants are relative values.

was observed for the *inlJ* mutant strain. In contrast, no difference was observed between Δ*inlI* and the wild-type strain. These results strongly suggest that InlJ contributes to *Listeria* virulence.

To address the contribution of these internalins during oral infection, we then performed oral infection experiments with hEcad mice expressing human E-cadherin in enterocytes. hEcad mice are a better model than wild-type mice for studying orally acquired listeriosis as *L. monocytogenes* efficiently crosses their intestinal barrier by an InlA-mediated process (40). The virulence phenotypes of Δ*inlI* and Δ*inlJ* strains were compared to those of wild-type, Δ*inlA*, and Δ*srtA* strains. After 72 h of infection, the Δ*inlJ* strain displayed 1- and 1.5-$\log_{10}$ decreases in colonization of the mesenteric lymph nodes and spleen, respectively, compared to the wild-type strain (Fig. 4B). This attenuation was comparable to that of Δ*inlA* and Δ*srtA* strains. The bacterial counts of the Δ*inlJ* strain were reduced 2 $\log_{10}$ in the liver, as observed for Δ*inlA*, while the Δ*srtA* strain displayed a more severe attenuation defect. Finally, Δ*inlJ* bacteria were recovered from intestines at a level of 3×10^3 CFU, a level intermediate between that of the wild-type strain (3×10^4 CFU) and that of Δ*inlA* and Δ*srtA* strains (3×10^2 CFU), which is at the limit of bacterial detection in this tissue. No significant attenuation in virulence was detected with the Δ*inlI* strain.

Taken together, these results indicate that the *inlJ* gene product is involved in several steps of *L. monocytogenes* infection and validate the hypothesis that LPXTG-anchored proteins other than InlA are implicated in virulence when the oral route is used.

Inactivation of two other LPXTG protein-encoding gene markers for *L. monocytogenes* serovar 4b. *L. monocytogenes* serovar 4b strains are responsible for the majority of listeriosis epidemic cases, suggesting that this serovar expresses specific factors favoring infection in human. However, data generated with the biodiversity array did not reveal any gene that was present only in serovar 4b strains (14). Nevertheless, three genes encoding LPXTG proteins, ORF29, ORF1761, and ORF2568, were specifically detected in lineage II (serovars 1/2b, 4a, 4b, and 4c) and not in the other serovars (14). We therefore investigated whether these genes could be involved in *L. monocytogenes* serovar 4b infection. Reexamination of the sequence of ORF1761 revealed that this gene fused with ORF2017 and is present in serovar 1/2a. Therefore, we focused on the ORF29 and ORF2568 genes in this study.

Both ORF29 and ORF2568 are present in the three *L. monocytogenes* serovar 4b strains that have been sequenced (14, 53). ORF29 (1,470 bp) and ORF2568 (1,617 bp) are predicted to encode LPXTG proteins consisting of 489 and 538 amino acids, respectively, that do not possess an LRR domain but contain MucBP domains (Fig. 5A). Notably, the amino-terminal region encoded by ORF29 contains five hydrophobic repeats consisting of 22 amino acids of unknown structure and two repeated MucBP domains. The protein encoded by ORF2568 exhibits no similarity with known proteins except that it contains two MucBP domains separated by 156 amino acids.

To address the contribution of ORF29 and ORF2568 to virulence, the genes were inactivated in the *L. monocytogenes* serovar 4b CLIP 80459 sequenced strain, and deletion mutants

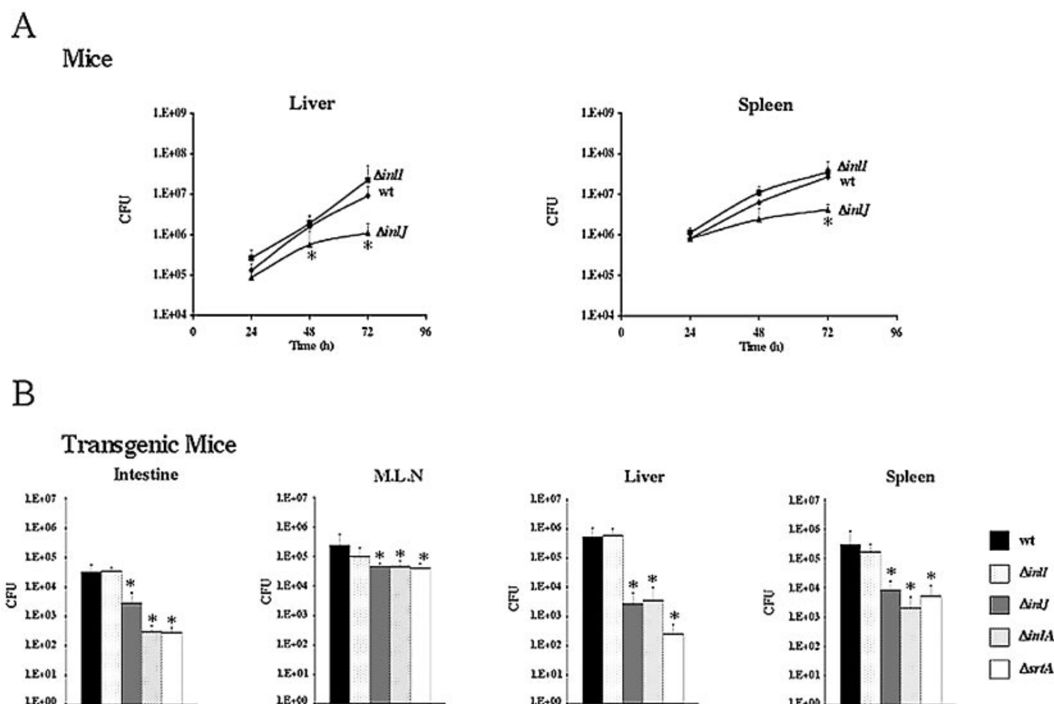

FIG. 4. *inlJ* is involved in *L. monocytogenes* virulence. (A) Mice were inoculated intravenously with 10^4 CFU of the wild-type EGDe strain or Δ*inlI* or Δ*inlJ* strain. Bacterial growth was monitored in the liver and the spleen at 24 h, 48 h, and 72 h. The results are the means of two independent experiments. (B) hEcad mice were orally infected with 10^9 CFU of the wild-type strain or the Δ*inlI*, Δ*inlJ*, Δ*inlA*, or Δ*srtA* strain. The numbers of bacteria able to colonize the intestine, mesenteric lymph nodes (M.L.N), liver, and spleen were determined at 72 h. The data are expressed in $\log_{10}$ CFU (see Materials and Methods). In each experiment four animals were used for each bacterial strain. The results are the means of two (Δ*inlI* strain) or three (wild-type, Δ*inlJ*, Δ*inlA*, and Δ*srtA* strains) independent experiments. An asterisk indicates a significant difference ($P < 0.05$) between a mutant strain and the wild-type strain. wt, wild type.

were verified by PCR and RT-PCR, as described previously for the *inlI* and *inlJ* genes (data not shown). When tested in vitro in invasion assays for entry, intracellular replication, and motility, ΔORF29 and ΔORF2568 strains behaved like the wild-type strain (data not shown). These mutants were then tested in vivo by oral infection of transgenic hEcad mice, as described above for Δ*inlI* and Δ*inlJ* strains. As shown in Fig. 5A, the colonization of the intestine, mesenteric lymph nodes, liver, and spleen by the ΔORF29 strain was similar to the colonization by the wild-type serovar 4b strain. In contrast, strikingly, for the ΔORF2568 strain there was a reproducible and statistically significant slight increase in the number of CFU in the intestine, mesenteric lymph nodes, and spleen (Fig. 5B). Thus, the ΔORF2568 bacteria displayed levels of colonization higher than those of the wild-type serovar 4b strain (0.5 $\log_{10}$ in the intestine, mesenteric lymph nodes, and liver and 1 $\log_{10}$ in the spleen). Together, these results indicate that ORF29 is not required for efficient *L. monocytogenes* serovar 4b oral infection, at least in the experimental conditions tested here. Moreover, inactivation of ORF2568 increased bacteriemia in tissues, suggesting that expression of this gene may affect *L. monocytogenes* virulence during host infection. The ORF2568 gene and the adjacent ORF3840 and ORF2763 genes are absent from the *L. monocytogenes* EGDe genome (Fig. 5C). These genes, whose functions are not known, are located in a chromosomal region which has not previously been associated with virulence or regulation.

DISCUSSION

Role of sortases and LPXTG proteins in orally acquired listeriosis. In this study we investigated and demonstrated the contribution of sortase A to orally acquired listeriosis, using guinea pigs and hEcad mice in which *L. monocytogenes* can efficiently cross the intestinal barrier. The invasion protein InlA was the first LPXTG protein reported to be required for efficient *L. monocytogenes* invasion of the intestinal epithelium (40). We show here that a Δ*srtA* strain is significantly more attenuated in virulence than a Δ*inlA* strain after oral infection in guinea pigs or hEcad mice, confirming the critical role of SrtA in bacterial invasion and/or persistence in deeper organs following oral infection (6). Therefore, it is likely that SrtA substrates other than InlA participate in different steps of the infectious process, from the crossing of the intestinal barrier to the hepatic phase of infection.

The second sortase of *L. monocytogenes*, SrtB, which does not play any detectable role in virulence in the mouse (5), is not required for *L. monocytogenes* oral infection in guinea pigs. This suggests that the two SrtB substrates (5, 54) do not play major roles in food-borne listeriosis. In *Staphylococcus aureus srtB* inactivation moderately attenuates virulence, and detection of this effect requires long-term infection (32, 49).

Identification of a new virulence factor among internalin-LPXTG proteins. Consistant with the *srtA* attenuation phenotype, we identified a new virulence factor among the 41

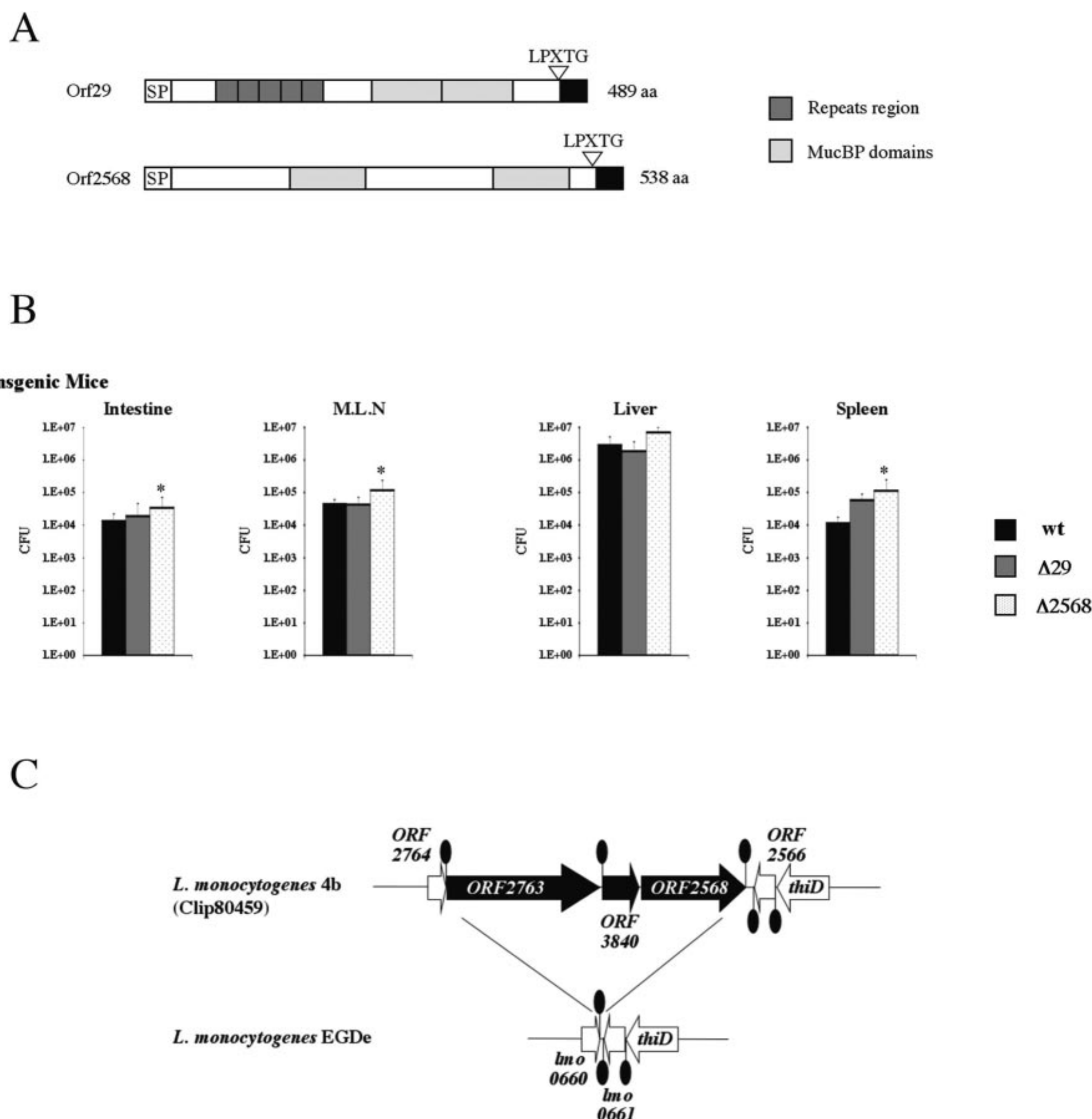

FIG. 5. (A) Schematic representation of ORF29 and ORF2568 LPXTG proteins specific for serovar 4b. The signal peptide (SP), repeat region, MucBP domains, and C-terminal sorting signal (solid box) are indicated. (B) Effect of ORF29 and ORF2568 inactivation on *L. monocytogenes* serovar 4b virulence. The wild-type *L. monocytogenes* serovar 4b strain or ΔORF29 and ΔORF2568 isogenic mutants were orally inoculated into hEcad mice (10^9 CFU). The numbers of bacteria able to colonize the intestine, mesenteric lymph nodes (M.L.N), liver, and spleen were determined at 72 h and expressed in $\log_{10}$ CFU (see Materials and Methods). In each experiment four animals were used for each bacterial strain. The results are the means of two (ΔORF29 strain) or three (wild-type and ΔORF2568 strains) independent experiments. An asterisk indicates a significant difference ($P < 0.05$) between a mutant strain and the wild-type strain. wt, wild type. (C) Genetic organization of the ORF2568 region in *L. monocytogenes* serovar 4b strain CLIP80459 and in *L. monocytogenes* serovar 1/2a strain EGDe. Open reading frames are indicated according to the genome nomenclature described previously (ORF [14] and lmo [28]). Open reading frames present only in *L. monocytogenes* serovar 4b are indicated by solid arrows. Other open reading frames are indicated by open arrows. The ovals indicate putative transcription terminators.

LPXTG proteins encoded by *L. monocytogenes* EGDe (8, 28), the InlJ protein. Recent work in our laboratory has identified another LPXTG protein involved in orally acquired listeriosis, VIP, encoded by the lmo0320 gene (10). InlJ, in contrast to VIP, belongs to the internalin multigene family, which encompasses 25 genes in EGDe (8, 28). Internalins are characterized by the presence of an N-terminal LRR domain. Nineteen of these proteins, including InlA, have the LPXTG motif that is expected to anchor the protein to the peptidoglycan. One, InlB, is loosely attached to lipoteichoic acids at the bacterial surface. The last five, including InlC (13, 19), do not display any surface-targeting domain and are therefore expected to be secreted into the extracellular medium. InlA, InlH, InlB, and InlC are established virulence factors (18, 35, 58). Thus, InlJ extends the list of internalins involved in virulence.

inlJ is present only in *L. monocytogenes* among the *Listeria* species, as shown by PCR analysis of a small pool of virulent and avirulent strains (43) and confirmed on a larger scale by the use of DNA arrays (14). The *inlJ* mutant displays a virulence defect in both wild-type mice after intravenous infection

and hEcad mice after oral infection, suggesting that it may be required at successive steps of listeriosis. Interestingly, Δ*inlJ* exhibits a 10-fold defect in intestinal colonization of hEcad mice, whereas Δ*inlA* has at least a 100-fold defect, which is at the limit of detection of *L. monocytogenes* in this tissue. In contrast, both mutants are similarly attenuated in the liver. InlA is probably the limiting factor among LPXTG proteins for efficient crossing of the intestinal epithelium by bacteria in hEcad mice, as it promotes *Listeria* internalization into enterocytes (40).

No phenotype could be attributed to the Δ*inlJ* strain in the cell culture system in vitro, making the function of *inlJ* elusive. As observed for *inlJ*, deletions of some other internalin genes, such as *inlC* or *inlGHE*, decrease the virulence in a mouse model but do not affect entry into epithelial cells, intracellular multiplication, or cell-to-cell spread (13, 15, 55). The various internalins may cooperate for efficient cell invasion, as proposed previously (3), or may have a totally different role in pathogenicity, such as interaction with the immune system.

Transcription of the *inlJ* gene is detectable by RT-PCR, similar to the *inlA* gene in bacteria grown in BHI medium at 37°C at the growth phase used for infection. However, we do not know yet at what level the encoded protein is produced. Using a highly sensitive gel-less method, Calvo et al. recently identified a pool of polypeptides linked to the *L. monocytogenes* EGDe peptidoglycan from bacteria grown in the same conditions (11). It is interesting that the InlJ protein was not detected in that pool, suggesting that its abundance at the bacterial surface is low. Of the 19 internalin-LPXTG proteins, only 5 were detected in cell wall extracts by this method (InlA, InlG, InlH, Lmo0327, and Lmo0610). This raises the possibility that InlJ synthesis, exportation, or anchoring at the bacterial surface might be low when bacteria are grown in BHI medium at 37°C and used to infect cultured cells. Interestingly, *inlJ* is adjacent to a gene encoding a putative transcriptional regulator, Lmo2820. Work is in progress to investigate where and when the InlJ protein is expressed and to address its possible regulation by the Lmo2820 protein.

InlJ defines a new subclass family of cysteine-containing LRR proteins. Sequence comparisons for the large group of LRR proteins suggest that there are several different subfamilies of LRR, which are characterized by different lengths and consensus sequences (33, 36). An LRR in internalins typically contains 22 residues. The LRR domain forms a curved solenoid with conserved leucines and isoleucines forming the hydrophobic core, as described for InlB, InlH, and InlA (44, 47, 58, 59). The InlJ consensus type described here is unusual in that it comprises 21 residues, possibly shortening the 3_{10}-helix, and displays a conserved cysteine at position 7 in place of a leucine (Fig. 2C). Cysteines are presumably part of the hydrophobic core of the molecule and may be protected from oxidation and therefore unlikely to form disulfide bonds. Only one cysteine in repeat 12 is not in the conserved position and could be accessible to an external ligand. Other internalin-like proteins containing LRRs of the InlJ type are found in *Listeria* species and in proteins from only five other bacterial species (Fig. 2C). Interestingly, all of them interact with mammalian hosts and are either commensals or pathogens.

A wide range of functions have been ascribed to LRR proteins, and these functions are related to the ability to bind structurally unrelated protein ligands (34, 36). This is exemplified by the specific interaction of the LRRs of InlA and InlB with at least two different cellular receptors, E-cadherin (39, 51, 59) and the hepatocyte growth factor receptor (60), respectively. In prokaryotes, several LRR proteins of pathogenic bacteria, such as secreted internalins in *Listeria ivanovii* (20), YopM in *Yersinia* (41, 50), IpaH in *Shigella* (22, 62), SspH in *Salmonella* (52), and Slr in *Streptococcus* (56), are known to play roles in host-pathogen interactions. LRRs in microorganisms may have been selected during evolution as a consequence of their structural similarities with mammalian LRR proteins, especially those involved in recognition of bacterial pathogens, such as TLR and NOD proteins (2, 29). It will be very interesting to identify the eukaryotic binding partner of InlJ.

Another putative structural feature of InlJ is the presence of MucBP domains, which are repeated four times in the C-terminal part of the protein (Fig. 2A). Identical motifs are present in InlI, ORF29, and ORF2568 and in several proteins bound to peptidoglycan of gram-positive bacteria (accession number PF06458). The function of these domains has not been elucidated.

Inactivation of the ORF2568 gene in *L. monocytogenes* serovar 4b increases virulence. Inactivation of two genes, ORF29 and ORF2568, encoding LPXTG proteins present in a subset of *L. monocytogenes* serovars, including epidemic serovar 4b, does not alter the listerial infectious process in vitro and does not attenuate *Listeria* virulence following oral infection. However, the bacterial loads of the ORF2568 deletion mutant in hEcad mice organs following oral inoculation, especially spleens, were increased compared to the loads of the wild-type serovar 4b strain. Inactivation of this gene may somehow affect the expression or function of other virulence factors and enhance the bacterial fitness in organs. Future work will address these possibilities.

In conclusion, this study showed the power of the post-genomic approach for successful identification of new virulence determinants. However, this candidate-based approach has its limitations. In contrast to InlA and InlB, whose role in *Listeria*-induced phagocytosis was identified following screening for noninvasive mutants in tissue-cultured cells (23), the InlJ function has not been determined. The challenge for the future is to identify the eukaryotic binding ligand and the signaling pathways triggered by the interaction with InlJ. In addition, in this study we could not identify any role for *inlI* or the serovar 4b LPXTG ORF29 gene in vitro or in virulence assays. These genes may not be required for pathogenesis but may function in another host or in specific infection conditions. Finally, the presence of multiple surface LPXTG proteins suggests that some of them may be important for virulence, while others may play a role in survival in food or other environments, which is critical for contamination.

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