

The *Listeria monocytogenes* Virulence Factor InlJ Is Specifically Expressed In Vivo and Behaves as an Adhesin[∇]

Christophe Sabet,^{1,2,3} Alejandro Toledo-Arana,^{1,2,3} Nicolas Personnic,^{1,2,3} Marc Lecuit,^{1,2,3,4}
Sarah Dubrac,⁵ Olivier Poupel,⁵ Edith Gouin,^{1,2,3} Marie-Anne Nahori,^{1,2,3}
Pascale Cossart,^{1,2,3*} and H el ene Bierne^{1,2,3*}

*Institut Pasteur, Unit e des Interactions Bact eries Cellules, Paris F-75015, France*¹; *INSERM, U604, Paris F-75015, France*²;
*INRA, USC2020, Paris F-75015, France*³; *Service des Maladies infectieuses et tropicales, Universit  Paris 5,*
*H pital Necker-Enfants malades, Paris F-75015, France*⁴; and *Institut Pasteur, Unit  de*
*Biologie des Bact eries pathog nes   Gram-positif, Paris F-75015, France*⁵

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The food-borne pathogen *Listeria monocytogenes* is adapted to a diversity of environments, such as soil, food, body fluids, and the cytosol of eukaryotic cells. The transition between saprophytic and pathogenic life is mediated through complex regulatory pathways that modulate the expression of virulence factors. Here we examined the expression of *inlJ*, a recently identified gene encoding a protein of the LPXTG-internalin family and involved in pathogenesis. We show that *inlJ* expression is controlled neither by the major listerial regulator of virulence genes, PrfA, nor by AxyR, a putative AraC regulator encoded by a gene adjacent to *inlJ* and divergently transcribed. The InlJ protein is not produced by bacteria grown in vitro in brain heart infusion medium or replicating in the cytosol of tissue-cultured cells. In contrast, it is efficiently produced and localized at the surface of bacteria present in the liver and blood of infected animals. Strikingly, the expression of *inlJ* by a heterologous promoter in *L. monocytogenes* or *L. innocua* promotes bacterial adherence to human cells in vitro. Taken together, these results strongly suggest that InlJ acts as a novel *L. monocytogenes* sortase-anchored adhesin specifically expressed during infection in vivo.

The food-borne pathogen *Listeria monocytogenes* is associated with severe invasive infections in humans and animals. The clinical features of listeriosis include meningitis, encephalitis, septicemia, abortion, and perinatal infection, with a high fatality rate for immunocompromised individuals, fetuses, and neonates (17). *L. monocytogenes*' ability to invade and multiply in a wide range of mammalian cells and thereby to cross the intestinal, blood-brain, and maternofetal barriers is essential for the development of systemic listeriosis (34, 67).

L. monocytogenes can survive and grow in extreme conditions, such as that encountered in soil, the food chain, the stomach, the gut, body fluids, and the cytosol of mammalian cells (21, 34, 67). This ability to colonize a broad range of ecosystems correlates with an abundance of genes encoding different transport and regulatory proteins (11.6% and 7.3%, respectively, of all predicted genes of *L. monocytogenes* [27]). As is true for many other pathogenic bacteria, *L. monocytogenes* needs to tightly control the expression of virulence genes in order to ensure the production of these effectors at specific sites during bacterial infection. Several studies have focused on the mechanisms by which the listerial effectors involved in the cellular infection process are expressed. These proteins include two invasion proteins, InlA and InlB; the pore-forming toxin listeriolysin O (LLO); two phospholipases, PlcA and PlcB; and ActA, which triggers bacterial intracellular motility (29, 34,

67). These virulence factors are produced under the control of the transcriptional regulator PrfA, which belongs to the Crp/Fnr family of transcriptional activators. PrfA is itself regulated by environmental signals important for the transition between the saprophytic and host cell environments (39). For instance, readily metabolizable sugars that *Listeria* may encounter outside host cells strongly inhibit the activity of PrfA and subsequently repress virulence gene expression (25, 50, 56, 59). Conversely, PrfA-dependent genes are induced when *L. monocytogenes* grows intracellularly, suggesting that the composition of the host cell cytosol provides activating signals for PrfA (10, 19, 32, 36, 54, 59a, 64). Some virulence genes, such as *inlA* and *inlB*, are also controlled by the stress-responsive alternative sigma factor σ B (33, 46). Regulators of two-component systems, such as AgrA (1), VirR (44), and DegU (68), are also associated with *L. monocytogenes* pathogenesis. Finally, recent investigations have shown that the synthesis of at least some virulence factors, including PrfA itself, ActA, InlA, and LLO, is posttranscriptionally regulated. This control relies on the upstream 5' untranslated regions (5'-UTR) of their transcripts, which can form secondary structures affecting the translation efficiency (30, 63, 65, 71).

Besides the well-known virulence factors mentioned above, postgenomics approaches have revealed additional novel proteins that play key roles in *Listeria*'s interaction with its host (3, 29). Among them is InlJ, a protein of the internalin family (5, 61). As is true for InlA, InlJ contains an N-terminal leucine-rich repeat (LRR) domain and a C-terminal LPXTG motif characteristic of surface proteins covalently anchored to the peptidoglycan by a sortase (4, 13, 23, 61). However, InlJ LRRs have a cysteine-containing consensus sequence different from

* Corresponding author. Mailing address: UIBC, Institut Pasteur, Inserm, U604, INRA, USC2020, 25 rue du Dr Roux, Paris F-75015, France. Phone for H. Bierne: 33 1 40 61 31 38. Fax: 33 1 45 68 87 06. E-mail: hbierne@pasteur.fr. Phone for P. Cossart: 33 1 45 68 88 41. Fax: 33 1 45 68 87 06. E-mail: pcossart@pasteur.fr.

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TABLE 1. Bacterial strains

Strain	Property	Reference or source
BUG 1600	<i>L. monocytogenes</i> EGDe wild-type strain	27
BUG 1777	EGDe Δ <i>srtA</i> strain	4
BUG 2159	EGDe Δ <i>inlJ</i> strain	61
BUG 2214	EGDe Δ <i>prfA</i> strain	45
BUG 2354	EGDe/ Δ <i>HTH-axyR</i>	This study
BUG 2350	EGDe/p <i>Tprot-inlJ</i>	This study
BUG 2351	EGDe/ Δ <i>inlJ</i> -pPL2:: <i>prot-inlJ</i>	This study
BUG 2353	EGDe/p <i>PinlJ-gfp</i>	This study
BUG 2364	EGDe/ Δ <i>srtA</i> /p <i>Tprot-inlJ</i>	This study
BUG 2366	<i>E. coli</i> BL21Star/pET101- <i>inlJ</i>	This study
BUG 2293	EGDe/p <i>Tprot</i>	This study
BUG 499	<i>L. innocua</i>	27
BUG 2356	<i>L. innocua</i> expressing p <i>Tprot-inlJ</i>	This study
BUG 1496	<i>L. innocua</i> expressing <i>inlA</i>	41

that of the InlA LRR prototype. In addition, InlJ has four MucBP (MUCin-binding protein) repeats that share similarities with repeats present in MUB, an extracellular mucus-binding protein of *Lactobacillus reuteri* (60). The *inlJ* gene is conserved in the genome of *L. monocytogenes* strains and is absent from nonpathogenic *Listeria* species (5). An *inlJ* deletion mutant is significantly attenuated in virulence after intravenous infections of BALB/c mice or oral inoculations of hEcad mice. However, the function of InlJ is unknown (61).

In this study, we aimed to get insight into the expression of *inlJ* and the function of its product. Our results strongly suggest a mechanism of posttranscriptional regulation, which may silence the expression of *inlJ* gene outside the mammalian host and induce it specifically during infection to facilitate bacterial adherence to host tissues.

MATERIALS AND METHODS

Bacterial strains, mammalian cells, and growth conditions. *Listeria* strains used in this study are listed in Table 1 and were routinely grown in brain heart infusion (BHI) medium (Difco) at 37°C. Erythromycin at 5 μ g/ml was added for the growth of strains carrying plasmids. Experiments involving growth under stress conditions were performed as follows. For iron depletion experiments, bacteria were first cultured at 37°C overnight in BHI, diluted to an optical density at 600 nm (OD₆₀₀) of 0.1, and then grown for 6 h at 37°C in BHI with 100 or 250 μ M of dipyrindyl (Sigma). For acid stress, hypoxia, and temperature shift experiments, the same parameters were used, but growth was at pH 4.5 or pH 5.5 upon the addition of HCl (acid stress), in 6% O₂ atmosphere for 12 h (hypoxia), or in BHI at 16°C, 30°C, 37°C, 39°C, or 42°C (for temperature shift). For bacterial growth in murine blood, bacteria were grown at 37°C in BHI until the OD₆₀₀ was 0.8 and rinsed twice in phosphate-buffered saline (PBS) and once in RPMI (Gibco). Bacteria (10⁶) were then added in 500 μ l of pure or diluted (1:5 in RPMI) murine blood containing 250 units/ml of heparin and incubated for 6 or 24 h at 37°C in 10% CO₂. Eukaryotic cells were JEG-3 placental (ATCC HTB-36) or Lovo and HT29 intestinal (ATCC CCL229 and ATCHTB38) human cells or J774 murine macrophages (ATCC TIB-67) and were grown according to ATCC instructions.

Generation of the EGDe Δ *HTH-axyR* in-frame deletion mutant. Two ~650-bp DNA fragments flanking the sequence encoding the helix-turn-helix (HTH) domain were amplified by PCR from strain EGDe chromosomal DNA. Primers for the HTH 5' flanking fragment were axy-1 (5'-CGCGGATCCGGCGCACT CCTTATTCTCTCTATAATCA-3') and axy-2 (5'-ATCATTGACTTCTTTT CTAGCAGAAGATGGAACCC-3'), and those for the 3' fragment were axy-3 (5'-GGGGTTCCATCTTCTGCTAGGAAAGAAGTCAATGATCATCATGC GGAAACGAATTGTTAATAGAC-3'), which is complementary to axy-2 in 5', and axy-4 (5'-TCCCGGGATCAGCATATGTATTTTTTCGGCTCAAAA AACAT-3'). The amplified 5' and 3' fragments were coligated by PCR using

axy-1 and axy-4 and then cloned in BamHI and SmaI sites of the thermosensitive plasmid pMAD, yielding pMAD- Δ *HTH-axyR*. This plasmid was electroporated into *L. monocytogenes* strain EGDe and gene replacement was performed as described previously (61).

Construction of p*Tprot-inlJ*, pPL2::*prot-inlJ*, p*P_{inlJ}-gfp*, pET101-*inlJ*, and pB29-*inlJ*. (i) p*Tprot-inlJ*. *inlJ* (from ribosome binding site [RBS] to stop codon) was PCR amplified from EGDe chromosomal DNA by using primers inlJcplm-1 (5'-ACGAGCTCAATAAGGAGTGCGCCAAATTG-3') and inlJcplm-2 (5'-TCCCCGGTCTCTGTCGGGCTTTTTACTATTTTTTC-3'). The DNA fragment was digested with SacI and SmaI and inserted into the SacI and SphI sites in the shuttle plasmid pP1 (14) downstream from the promoter *prot*, yielding p*Tprot-inlJ*. This plasmid was electroporated into *L. monocytogenes* strain EGDe.

(ii) pPL2::*prot-inlJ*. The *prot-inlJ* sequence was amplified by PCR by using primers inlJcplm1ppl2 (5'-GGACTAGTGAATTCGATTCGGTCTCCTCT-3') and inlJcplm-2 (5'-TCCCGGGTCTGTCGGGCTTTTTACTATTTTTTC-3'), digested with SpeI and SmaI, and inserted in the integrative vector pPL2, yielding pPL2::*prot-inlJ*, which was integrated in the chromosome as described previously (40).

(iii) p*P_{inlJ}-gfp*. A 297-bp DNA fragment located immediately upstream of the RBS of *inlJ* was amplified by PCR using primers inlJprom-1 (5'-CCGGAATTC GCCCAGAAATAGCAAATACCA-3') and inlJprom-2 (5'-CGCGGATCCAT TCGTTCTCTATAATCATTTATT-3'). The *gfp* gene with an RBS optimized for gram-positive bacteria was PCR amplified from plasmid pNF8 (18) using primers gfp1 (5'-CGGAAAGGAGGTTTATAAATGAGTAAAGGAGAAGAA CTT) and gfp2 (5'-AAACTGCAGTTATTTGTATAGTTCATCCATGCCA-3'). Amplified fragments were digested with BamHI, coligated, and amplified by PCR with inlJprom-1 and gfp2 primers. The resulting P*inlJ*-*gfp* fragment was inserted at EcoRI and PstI sites of the pAT18 vector (66), yielding p*P_{inlJ}-gfp*.

(iv) pET101-*inlJ*. The sequence encoding InlJ (residues 26 to 792) was PCR amplified from EGDe chromosomal DNA by use of primers inlJpet-1 (5'-CAC CATGGCAACGAATGATGTTATTGATAATACG-3') and inlJpet-2 (5'-AGG TTGATCTGTGTTGTGTTTTTC-3') and inserted into the pET101 vector (Invitrogen) according to the manufacturer's instructions.

(v) pB29-*inlJ*. The sequence encoding InlJ (residues 26 to 769) was PCR amplified and cloned in pB29, a LexA N-terminal fusion vector optimized by Hybrigenics. All constructions were verified by sequencing.

InlJ purification. pET101-*inlJ* was used to transform *Escherichia coli* BL21Star (Invitrogen), and recombinant His-tagged InlJ was produced according to the manufacturer's instructions. Briefly, expression of the recombinant protein was induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) during a 2-h period at 37°C. Bacterial pellets were resuspended in buffer A (50 mM Tris-HCl, 150 mM NaCl, pH 7.8) and disrupted with a French press. InlJ was purified from the supernatants by metal affinity chromatography using Ni-nitrilotriacetic acid columns (Novagen). After elution with imidazole, InlJ protein was desalted and concentrated to the desired volume using Centriprep devices (Amicon).

Antibodies, bacterial extracts, and Western blotting techniques. Polyclonal rabbit anti-InlJ (R133) was generated against recombinant His-tagged InlJ protein and affinity purification of the antibodies was done as described previously (20). Polyclonal rabbit anti-*L. monocytogenes* EGDe (R97) was generated against 10⁹ heat-killed bacteria as described previously (28). Anti-*L. monocytogenes* EGDe mouse polyclonal antibodies (MS2) were obtained by immunizing BALB/c mice with 10⁸ heat-killed bacteria in Freund's incomplete adjuvant (Difco). Animals were boosted 3 and 6 weeks after inoculation and bled 10 days after each challenge. We also used rabbit polyclonal antibodies against *L. innocua* (R6 [16]) and mouse monoclonal antibodies against InlA (L7.7 [48]). InlJ and InlA detection at the surface of bacteria was performed as described previously (4) using primary antibodies and goat anti-rabbit Alexa Fluor 488 or goat anti-mouse Cy3-conjugated (Molecular Probes, Jackson) secondary antibodies and 4',6'-diamidino-2-phenylindole (DAPI). Bacterial cell wall extracts were obtained as described previously (31). After separation on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, proteins were detected by Western blotting.

RNA purification, primer extension, and rapid amplification of cDNA ends (RACE). (i) RNA purification. RNA from *L. monocytogenes* bacteria grown to the mid-log phase was extracted with an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Residual DNA on the column was removed with Turbo DNase (Ambion). The absence of DNA from RNA samples was verified by PCR amplification of the genes to be assayed with 5 μ g of RNA as the template.

Primer extension. RNAs were used as templates for primer extension reactions using radiolabeled primer inlJ-ext (5'-TTGGCGCACTCCTTATTCGTTCTCTATAATCATTT-3') as described previously (9).

RACE. 5' RACE was carried out as described previously (24). Reverse transcription of the 5'-UTR of *inlJ* mRNA was performed with the *inlJ*-ext primer, while the *inlJ*-ext2 primer (5'-CTCTATAATCATTTATTATACGCCGTTTTT TTTAC-3') was used for PCR amplification. PCR products were run on a 3% agarose gel in TAE buffer. Bands present only in the tobacco acid pyrophosphatase (TAP)-treated samples were excised from the gel and were purified by use of a Qiagen gel extraction kit. The eluted DNA was cloned using a TOPO TA cloning kit. pCR2.1-TOPO plasmids containing appropriate PCR fragments were sent to sequencing.

Real-time quantitative PCR (qRT-PCR). (i) **cDNA synthesis.** Reverse transcription reaction mixtures (20 μ l) containing 200 ng of random hexamers (Roche, Basel, Switzerland), 0.5 mM deoxynucleoside triphosphate, and 5 μ g of total RNA were incubated at 65°C for 5 min to remove secondary structures and placed on ice. AMV reverse transcriptase (25 U) and its buffer (Roche, Basel, Switzerland) were then added to the mix. After 10 min at 25°C, the mix was incubated at 42°C for 50 min. Reverse transcriptase was then inactivated by heating at 70°C for 15 min.

(ii) **qRT-PCRs.** Primers were selected with the Beacon Designer 4.02 software (Premier Biosoft International, Palo Alto, CA) for *inlJ* (5'-AGTTTGTCTTGGTAAATGC-3' and 5'-CTGGATTGTCTGTGCTGAG-3'), *inlA* (5'-ACACG GTCTCACAAACAG-3' and 5'-TCAAGTATCCACTCCATCG-3'), *gyrA* (5'-GCGATGAGTGTAATTGTTG-3' and 5'-ATCAGAAGTCATACCTAAGTC-3'), and *rpoB* (5'-CACCTGGAGTAAACCAATTAGTACG-3' and 5'-TAGTGGGTTAAGCATGATCAACA-3'). qRT-PCRs were performed in a 25- μ l reaction volume containing 5 μ l of a 1/100 dilution of cDNA, 1 μ l of gene-specific primers (10 μ M), and 12.5 μ l of iQ SYBR green supermix (Bio-Rad, Hercules, CA) with the MyiQ single-color RT iCycler PCR detection system and the MyiQ optical system software (Bio-Rad, Hercules, CA). PCR conditions included an initial denaturation step at 95°C for 3 min followed by a 40-cycle amplification (95°C for 15 s, 55°C for 15 s, 72°C for 15 s). Specificity of the amplified product and primer dimer formation were checked by generating a melting curve with a final step of 80 cycles consisting of a 0.5°C temperature incrementation each 10 s, beginning at 55°C. The absence of contaminating genomic DNA was verified by testing each sample in control reactions without a prior reverse transcription step. The critical threshold cycle (C_T) was defined for each sample. Expression levels of the tested genes were normalized using the *rpoB* or *gyrA* genes of *L. monocytogenes* as an internal standard. Each assay was performed in quadruplicate and repeated with at least two independent RNA samples. The n -fold change of the transcript level was calculated using the following equations: $\Delta C_T = C_{T(\text{test DNA})} - C_{T(\text{reference cDNA})}$; $\Delta\Delta C_T = \Delta C_{T(\text{target gene})} - \Delta C_{T(16S \text{ rRNA})}$; ratio = $2^{-\Delta\Delta C_T}$ (42). Statistical significance between the mean expressions of genes was evaluated by Student's *t* test. A *P* value of <0.05 was considered significant.

Yeast two-hybrid screening. pB29-*inlJ* was transformed into the L40DGAL4 yeast (*Saccharomyces cerevisiae*) strain and yeast two-hybrid screening was performed by Hybrigenics, S.A., Paris, France. Briefly, a human placenta randomly primed cDNA library (RP4) transformed into the Y187 yeast strain and containing 10 million independent fragments was used for mating. The screen was first performed on a small scale to adapt the selective pressure to the intrinsic property of the bait. Neither toxicity nor autoactivation of the bait was observed. Then, the full-scale screen, allowing 104 million interactions to be tested, was performed.

Adherence and invasion assays. *L. innocua* or *L. innocua*/pT_{prot}-*inlJ* grown at an OD₆₀₀ of 0.6 was diluted in modified Eagle's medium and added at an \approx 20:1 ratio to JEG-3 confluent cells or HT29 monolayers of polarized cells that had been differentiated for 3 weeks. After 4 h of incubation, nonadherent bacteria were removed by washing 5 times with modified Eagle's medium. Cells were fixed with 4% paraformaldehyde (wt/vol in PBS) for 20 min and washed three times again with PBS. Bacteria were sequentially labeled with anti-*Listeria innocua* antibodies diluted 1:1,000 in 5% bovine serum albumin-PBS and a 488-labeled goat anti-rabbit immunoglobulin G (Molecular Probes) diluted 1:1,000. Cell nuclei were stained with DAPI. Each assay was carried out in triplicate and repeated two times. Adherent bacteria were counted by fluorescent microscopy, examining \approx 1,000 cells in randomly picked microscopic fields.

Quantification of bacterial entry in JEG-3 cell monolayers was performed with a classical gentamicin assay as described previously (4). To study *inlJ* expression in intracellular bacteria in tissue-cultured cells, EGDe or EGDe/pP_{intr}-*sfp* was incubated with cells for 1 hour then washed twice with cell growth medium and incubated for 1 h, 7 h, or 23 h with cell culture medium containing gentamicin at 20 μ g/ml to kill extracellular bacteria. Cells were then fixed with 4% paraformaldehyde (wt/vol in PBS), permeabilized with 0.4% Triton X-100 in PBS for 5 min at room temperature, and processed for immunofluorescence.

Infections in animals and organ treatments. All animals were treated in accordance with Institut Pasteur guidelines for laboratory animal husbandry. 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, 10⁴ CFU. Seventy-two hours after infection, 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 medium. Blood was resuspended in heparin (250 units/ml) and 100- μ l portions were plated on BHI agar medium. Four animals were used for each experiment. Statistical analyses were performed using the Student *t* test. *P* values of <0.05 were considered statistically different. To study *inlJ* expression in bacteria present in blood or liver, the *L. monocytogenes* wild-type and Δ *inlJ* strains were inoculated intravenously into BALB/c mice with 10⁴ and 10⁵ CFU, respectively. Following 72 h of infection, blood samples and livers were recovered. Blood streams were fixed on coverslips with 4% paraformaldehyde (wt/vol in PBS) for 20 min, and blood cells were permeabilized with 0.3% Triton X-100 in PBS for 5 min and then processed for immunofluorescence. Optimal cutting temperature TissueTek compound (VWR Scientific)-embedded blocks of liver were cryosectioned (7- μ m-thick sections), fixed in ethanol (-20°C for 5 min), and processed for immunofluorescence. Blocking steps, antibody dilutions, and washes were performed in 1% bovine serum albumin in PBS.

Histoimmunocytochemistry. Paraffin-embedded blocks of liver and spleen tissues from mice infected intravenously by *L. monocytogenes* were formalin fixed and sliced. Sections of tissues were stained with anti-EGDe or with anti-*InlJ* primary antibodies followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin. Antigen-antibody complexes were visualized by using reagents supplied in the Envision kit (Dakocytomation, Carpinteria, CA).

RESULTS

***inlJ* transcription occurs independent of AxyR- or PrfA-mediated regulation.** We previously showed by semiquantitative RT-PCR that *inlJ* is transcribed in bacteria grown in BHI rich medium (61). This result was further supported by qRT-PCR experiments indicating that the amount of *inlJ* transcript is approximately one-half the amount of the *inlA* transcript, which allows production of the surface invasion protein InlA (Fig. 1A). Primer extension reactions revealed two *inlJ* transcription start sites upstream of the start codon of *inlJ* (Fig. 1B). However, since the smaller transcript may represent a processed RNA species derived from cleavage of the longer transcript, the transcriptional start sites of *inlJ* were subsequently mapped by 5' RACE (see Materials and Methods). The nucleotide sequences of the 5' RACE PCR products also revealed two transcriptional start sites, P1₊₁ and P2₊₁, located 219 and 158 bp upstream of the *inlJ* translational start codon, respectively. Both are preceded by -35 and -10 regions characteristic of listerial RpoD-dependent promoters (Fig. 1C and D). The *inlJ* P1 promoter is located at the beginning of the coding sequence of the divergently transcribed lmo2820 gene.

lmo2820 encodes a putative bimodular protein with an N-terminal region sharing similarities with transcriptional AraC/XylS regulators (22) and a C-terminal region sharing similarities with β -D-xylosidases (12, 72) that we renamed AxyR (AraC-xylosidase putative regulator). Like other AraC/XylS family members, AxyR contains an \sim 100-amino-acid conserved domain (HTH domain) that comprises two potential HTH DNA-binding motifs. HTH domains, as for other regulatory proteins, are required for transcription regulation by AraC regulators at targeted promoters (22). Some members of the AraC/XylS family regulate virulence genes. It was therefore tempting to examine a role of AxyR in *inlJ* regulation. To avoid alteration of the *inlJ* promoter region that would occur in an *axyR* full deletion mutant, we constructed an *axyR* mutant in which only the sequence encoding the 100-amino-acid HTH

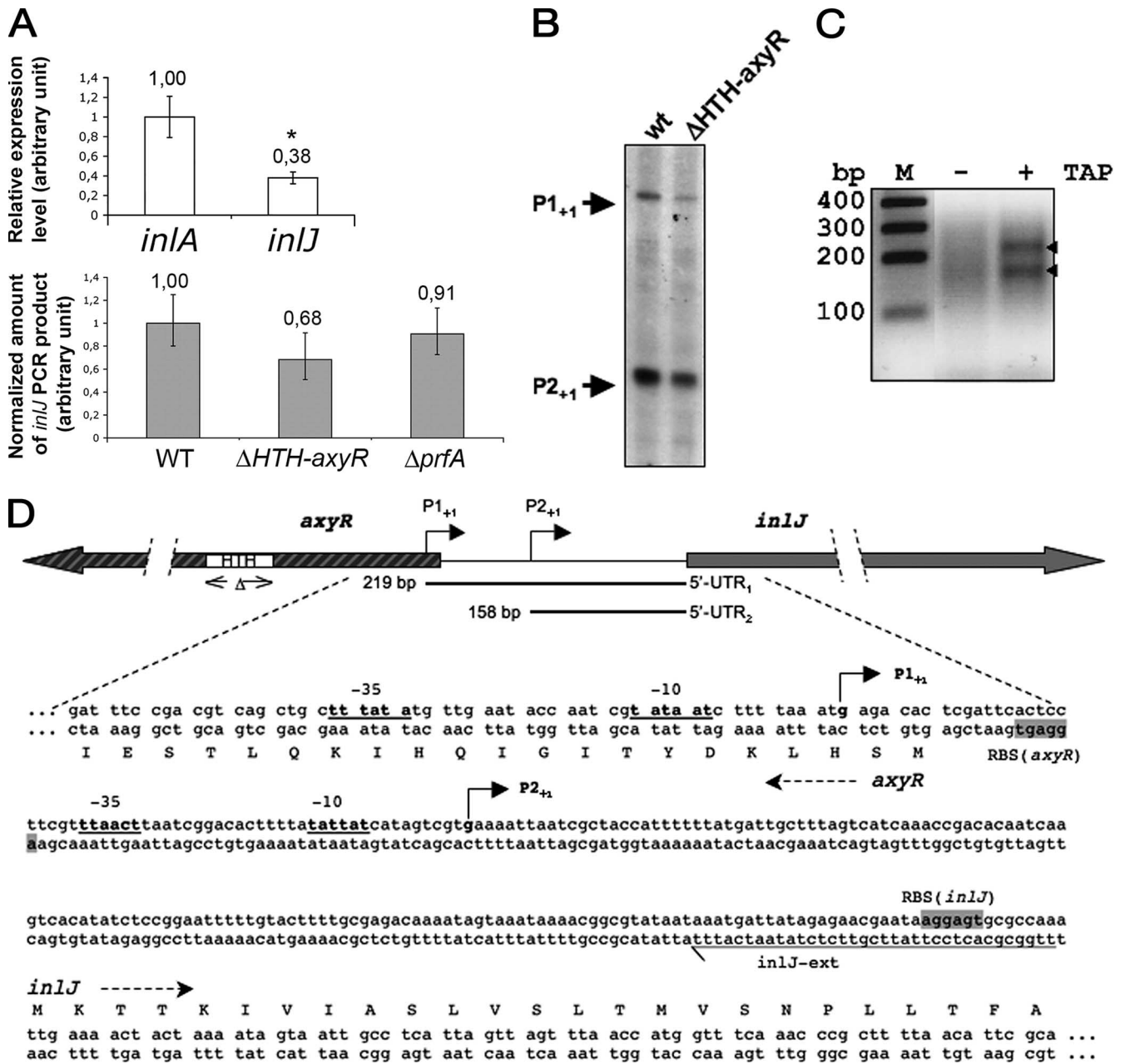


FIG. 1. Studies of *inlJ* promoter region. (A) Studies of *inlJ* transcription by qRT-PCR. (Top) qRT-PCR with *inlA* or *inlJ* internal primers on total RNA extracts from cultures of wild-type *L. monocytogenes* grown in BHI. *inlA* and *inlJ* transcription levels are significantly different (*, $P < 0.05$ by Student's *t* test). (Bottom) qRT-PCR with *inlJ* internal primers on total RNA extracts from cultures of *L. monocytogenes* wild-type, Δ *HTH-axyR*, and Δ *prfA* strains grown in BHI. The amounts of *inlJ* transcripts in Δ *HTH-axyR* and Δ *prfA* mutants are not significantly different from that in the wild-type strain ($P = 0.07$ and $P = 0.3$, respectively, by Student's *t* test). (B) Primer extension analysis of the *inlJ* transcript. Primer extensions were performed on total RNAs extracted from cultures of wild-type and Δ *HTH-axyR* strains grown in BHI to an OD₆₀₀ of 0.8 by use of the *inlJ*-ext primer. The arrows indicate putative transcription starts of the *inlJ* transcript. (C) RACE mapping of *inlJ* transcript 5' end. Total RNA was linked to a 5' adaptor RNA without or after treatment with TAP (TAP- and TAP+ lanes, respectively). TAP converts the 5'-triphosphate of an RNA to a monophosphate. Thus, a comparison can distinguish between transcription start site (carrying a 5'-triphosphate) and processing site (carrying a 5'-monophosphate). *InlJ*-specific cDNA was made and the 5'-UTR of *InlJ* mRNA was amplified using gene- and adaptor-specific primers. PCR products were separated on a 3% agarose gel. Bands indicated by arrows were excised, cloned, and sequenced. Two transcription start sites were mapped and the corresponding position are shown in panel A. (D) Physical map of the *inlJ* and *axyR* gene regions. The locations of the two mapped transcription start sites (P1₊₁ and P2₊₁) of the *inlJ* promoters are shown by arrows, as are the two 5'-UTRs. Deduced -10 and -35 hexamers of putative RpoD-dependent promoters are underlined. The *inlJ* and *axyR* RBS are framed in gray. The HTH domain deleted from the Δ *HTH-axyR* mutant is underlined by double arrows. Primer *inlJ*-ext is underlined.

domain is deleted (Fig. 1). *inlJ* transcription was first examined in primer extension assays on total RNA extracts from the resulting $\Delta HTH\text{-}axyR$ strain grown in BHI at 37°C. The two potential transcription start sites of *inlJ* were still detected in the $\Delta HTH\text{-}axyR$ strain, as in the wild-type strain (Fig. 1B). Moreover, *inlJ* mRNA levels in the $\Delta HTH\text{-}axyR$ strain were not significantly different from those in the wild-type strain, as measured by qRT-PCR ($P > 0.05$) (Fig. 1A).

We then examined whether PrfA, the major regulator of virulence genes in *L. monocytogenes*, regulated *inlJ*. Similar amounts of *inlJ* transcript were detected in RNA extracts from *L. monocytogenes* wild-type and $\Delta prfA$ strains by qRT-PCR (Fig. 1A). This observation is consistent with the absence of a PrfA putative binding site in the *inlJ* promoter region and with data from transcriptome studies that did not reveal *inlJ* as a gene regulated by PrfA (51). Of note was the fact that none of the other regulatory sequences that are known to play a role in *L. monocytogenes* regulation of virulence genes, such as a Fur box (2, 58), a VirR DNA-binding site (44), or a sigma B-specific promoter signature (33), were detected in the *inlJ* promoter region. Taken together, these results indicate that the *inlJ* virulence gene is not part of the PrfA regulon and is apparently not regulated by AxyR, the product of its adjacent gene.

InlJ is not produced by bacteria grown in rich medium unless *inlJ* is expressed from a heterologous promoter. InlJ is an LPXTG surface protein predicted to be anchored to the peptidoglycan by sortase A. To address whether *L. monocytogenes* bacteria produce InlJ, we generated an antibody against a purified recombinant InlJ protein and used it to detect the protein at the bacterial surface by immunofluorescence. Strikingly, bacteria that had been grown in BHI medium were not stained by the InlJ antibody, while they were stained by an InlA antibody, suggesting that InlJ protein was poorly or not produced, in contrast to InlA (Fig. 2A). Furthermore, InlJ was not detected in bacterial total extracts (data not shown) or cell wall extracts (Fig. 2B) of bacteria grown in BHI. These results are in agreement with data indicating that InlJ is not present in the cell wall proteome of *L. monocytogenes* EGDe grown in BHI medium (57). The absence of InlJ protein in *L. monocytogenes* bacteria grown in rich medium, although the *inlJ* gene is transcribed, suggested a mechanism of posttranscriptional regulation.

To verify that the *inlJ* open reading frame encoded a surface protein, *inlJ* was expressed under the control of the promoter region (*prot*) of the protease gene from *Lactococcus lactis* subsp. *cremoris* (38), which is active in *Listeria* (4, 6, 14), in a listerial plasmid (pT*prot-inlJ*). InlJ antibodies efficiently labeled InlJ at the surface of EGDe bacteria expressing *inlJ* fused to the *prot* region (wt pT*prot-inlJ*) and detected the protein in cell wall extracts (Fig. 2B). By contrast, as for InlA, InlJ was not detected by immunofluorescence at the surface of a sortase mutant ($\Delta srtA$ mutant [4]) harboring plasmid pT*prot-inlJ* (Fig. 2A). Furthermore, the amount of InlJ in cell wall extracts from this strain was significantly smaller than that from the wild-type strain (Fig. 2B). We also examined the expression of *inlJ* by the heterologous promoter region when the *prot-inlJ* fusion is present as only one copy in the chromosome. The *prot-inlJ* sequence was inserted into pPL2, a listerial site-specific integration vector (40), and

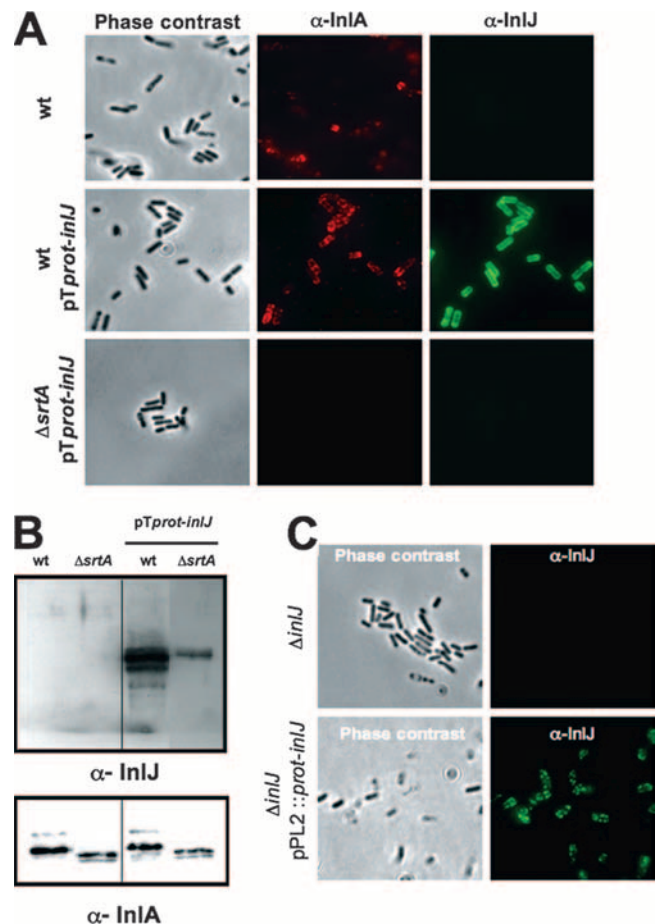


FIG. 2. Bacteria grown in BHI medium do not produce InlJ protein unless *inlJ* is under the control of the constitutive promoter *prot*. Immunofluorescence microscopy (A) or immunoblotting of cell wall extracts (B) of *L. monocytogenes* EGDe wild type (wt), EGDe/pT*prot-inlJ* (wt pT*prot-inlJ*), or the $\Delta srtA$ mutant expressing pT*prot-inlJ* ($\Delta srtA$ pT*prot-inlJ*) grown in BHI medium with anti-InlA- or anti-InlJ-specific antibodies. InlJ is not detected at the surface of wild-type bacteria, while it is anchored at the surface of bacteria expressing pT*prot-inlJ*. As for InlA, this anchoring depends on SrtA. (C) InlJ is produced and present at the surface of $\Delta inlJ$ /pPL2::*prot-inlJ* bacteria carrying the fused sequence *prot-inlJ* in the chromosome. As expected, it is absent at the surface of bacteria of the $\Delta inlJ$ strain.

integrated into the tRNA-Arg site on the chromosome of the $\Delta inlJ$ mutant. InlJ was produced and present at the surface of $\Delta inlJ$ /pPL2::*prot-inlJ* bacteria grown in BHI (Fig. 2C). The labeling was specific, as the InlJ antibody did not label *inlJ* mutant bacteria.

Taken together, these results suggest that the *inlJ* regulatory region lying upstream of the mRNA coding sequence prevents *inlJ* transcript translation in bacteria grown in BHI. When *inlJ* is expressed from the heterologous *prot* promoter region, the InlJ protein is produced, secreted, and anchored at the bacterial surface by a sortase A-dependent process.

***inlJ* expression does not increase upon stress or during infection of cultured cells.** To investigate further the mechanism underlying *inlJ* regulation, we constructed a reporter plasmid, pP_{*inlJ*}-gfp, in which the 300-bp DNA sequence lying upstream of the *inlJ* RBS was fused to *gfp*. The fluorescence of *L.*

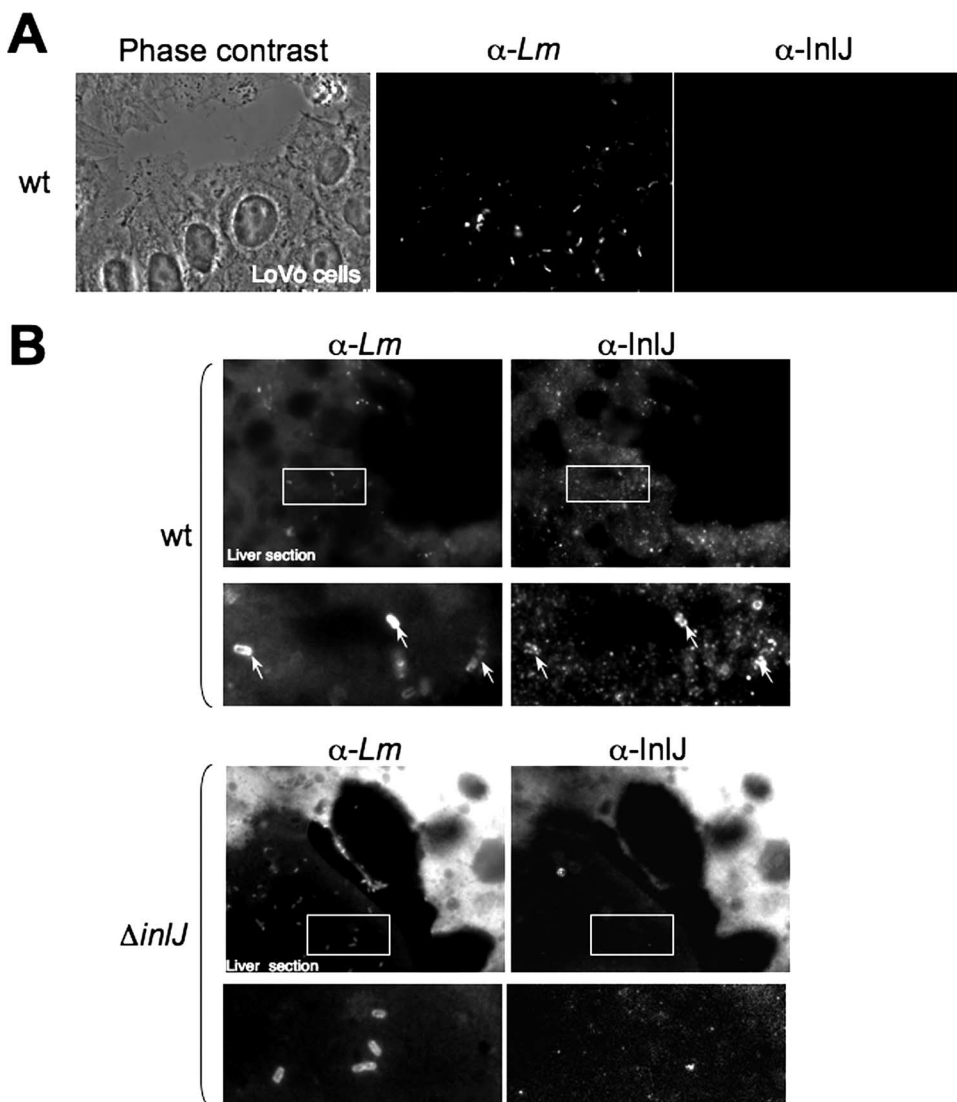


FIG. 3. *inlJ* is not expressed by bacteria in tissue-cultured cells but is efficiently expressed by bacteria present in the livers of infected mice. (A) LoVo cells were infected with *L. monocytogenes* EGDe wild-type bacteria (wt) for 24 h and analyzed by immunofluorescence. InlJ is not detected at the surface of intracellular bacteria. (B) *L. monocytogenes* EGDe wild-type and Δ *inlJ* strains were inoculated intravenously into BALB/c mice with an *inlJ* mutant inoculum 10 times larger than that of the wild-type strain to compensate for the attenuation in the virulence of the Δ *inlJ* strain. Following 72 h of infection, thin liver frozen sections were analyzed by immunofluorescence microscopy. InlJ is present at the surface of wild-type bacteria (indicated by arrows) but not for of the *inlJ* mutant (Δ *inlJ*) within livers of infected mice. The intensity of the fluorescence background varies relative to the thickness and the angle of the section. Bacteria are labeled with the *L. monocytogenes* MS2.1 antibody (α -*Lm*) and with the InlJ antibody (α -InlJ). Framed regions indicate the positions of the fields magnified in the lower images.

monocytogenes live bacteria harboring the pP_{*inlJ*}-*gfp* plasmid and grown in BHI was only 2-fold greater than that of control nonfluorescent bacteria carrying the empty vector and was 10-fold lower than that of bacteria harboring pNF8, a plasmid in which *gfp* is under the control of the strong listerial promoter *Pdlt* (18), indicating that the green fluorescent protein was not efficiently produced (results not shown). This result was consistent with the absence of InlJ production in this growth condition. We then tested the effect of different stresses or growth conditions on the activation of *inlJ* expression. Bacteria were grown in BHI at different temperature (16°C, 30°C, 37°C, 39°C, and 42°C) or with iron depletion or under hypoxia or acidic conditions. None of these stresses stimulated the

fluorescence of *L. monocytogenes*/pP_{*inlJ*}-*gfp* bacteria or allowed the detection of InlJ at the surface of wild-type EGDe bacteria (data not shown). Variation of glucose concentration had also no effect on *inlJ* expression.

We then studied the ability of the InlJ protein to be specifically produced by bacteria during cell infection of tissue-cultured cells, such as human JEG-3 placental cells, LoVo enterocytes, or J774 murine macrophages, or during growth in blood. Cells were infected with *L. monocytogenes* EGDe or EGDe/pP_{*inlJ*}-*gfp* strains for 2, 8, or 24 h. InlJ protein was still not detected at the surface of intracellular wild-type bacteria (Fig. 3A) and none of the intracellular EGDe/pP_{*inlJ*}-*gfp* bacteria appeared fluorescent. Likewise, *inlJ* was not expressed in bac-

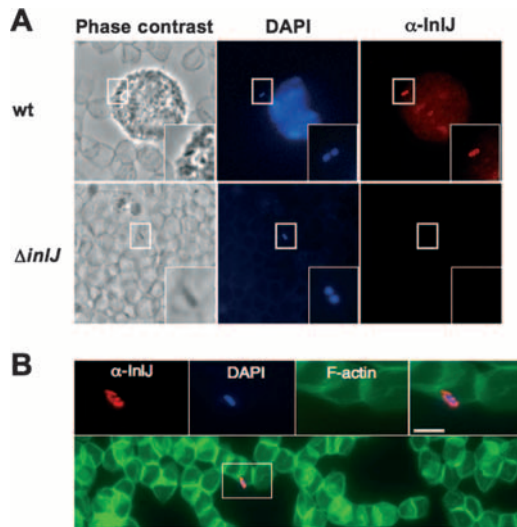


FIG. 4. Bacteria present in the blood of infected mice produce InlJ. *L. monocytogenes* wild-type (wt) or $\Delta inlJ$ strain bacteria were inoculated intravenously into BALB/c mice. Following 72 h of infection, blood was recovered and fixed blood streams were labeled with InlJ antibody (in red) and DAPI, which detects white blood cells and bacterial DNA (in blue). (A) InlJ is present at the surface of a wild-type bacterium associated with a polynuclear cell (labeled with DAPI) and is absent at the surface of an $\Delta inlJ$ mutant bacterium associated with erythrocytes (visible in phase-contrast image). (B) A wild-type *L. monocytogenes* bacterium in contact with erythrocytes expresses InlJ at its surface. Fluorescent phalloidin-488 labels actin in erythrocytes (in green). Scale bar, 2 μ m. Rectangular regions indicate the positions of the magnified inset fields. α , anti-

terial grown in murine blood in vitro (data not shown). These results indicated that the expression of *inlJ*, in contrast to what is seen for many listerial virulence genes, is not dependent on temperature, on entry into host cell cytosol, or on the presence of blood components (7, 30, 36, 64).

InlJ is efficiently produced during *L. monocytogenes* infection of mice. Since *inlJ* plays a role in listerial infections in mice (61), it is likely induced in vivo. To confirm this hypothesis, we searched for the presence of the InlJ protein at the surface of *L. monocytogenes* present in blood or organs of infected mice. Mice were inoculated intravenously either with the wild type or with the $\Delta inlJ$ strain. We used an *inlJ* mutant inoculum 10 times larger than that for the wild-type strain to compensate for the attenuation in the virulence of the $\Delta inlJ$ strain (61). InlJ protein was not produced by wild-type bacteria present in the inoculum (data not shown). By contrast, at 72 h postinfection InlJ was present at the surface of wild-type bacteria in liver (Fig. 3B) or in blood (Fig. 4A and B) from infected animals, while it was not detected at the surface of $\Delta inlJ$ bacteria.

To address the importance of the *inlJ* regulatory region in InlJ function in vivo, the virulence of the $\Delta inlJ/pPL2::prot-inlJ$ strain, in which *inlJ* is expressed under the control of the heterologous *prot* regulatory region, was compared to that of the wild-type and $\Delta inlJ$ strains. It was assessed by determining bacterial counts in liver and spleen of infected mice as well as by histological studies. As expected from our previous studies (61), the *inlJ* mutant strain showed 2 \log_{10} and 1 \log_{10} unit decreases in colonizations of the liver and spleen, respectively, and a 3 \log_{10} unit decrease in bacteremia in blood, compared

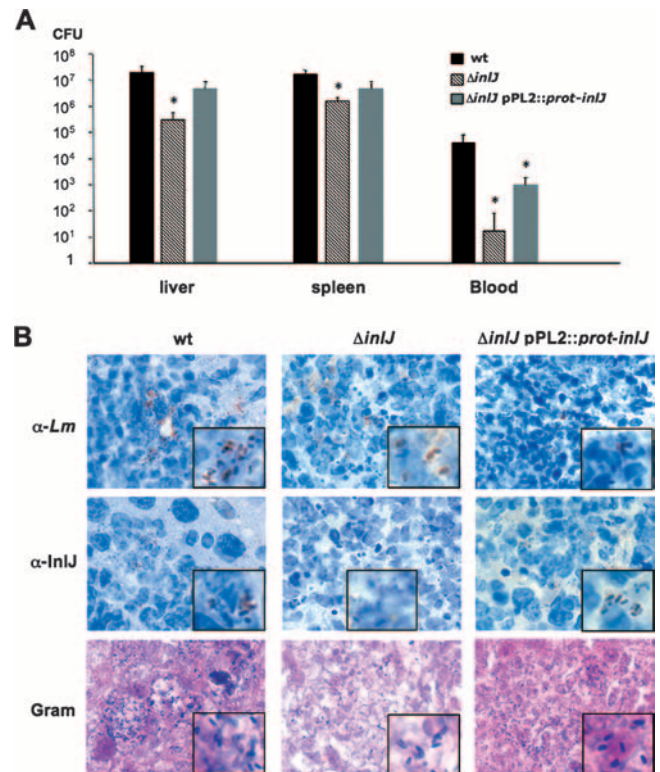


FIG. 5. Importance of *inlJ* regulatory region for InlJ function in vivo. (A) The *L. monocytogenes* wild-type (wt), $\Delta inlJ$, and $\Delta inlJ/pPL2::prot-inlJ$ strains were intravenously inoculated into BALB/c mice at 10^4 CFU. Animals were euthanized 72 h after infection and organs and blood were recovered, homogenized, and plated. The number of bacteria able to colonize liver and spleen or survive in blood is expressed as \log_{10} CFU. $\Delta inlJ/pPL2::prot-inlJ$ bacteria are not as efficient as the wild-type strain in organ colonization. (B) Analysis of InlJ expression at the surface of bacteria present in livers of infected mice by immunohistochemistry. Bacteria were labeled with the *L. monocytogenes* R97 antibody (α -Lm) or with the InlJ antibody (α -InlJ) or were Gram stained. The InlJ antibody stained wild-type and $\Delta inlJ/pPL2::prot-inlJ$ bacteria equally efficiently, whereas $\Delta inlJ$ bacteria were not labeled, as expected. Rectangular inset regions are magnified.

to the wild-type strain (Fig. 5A). Interestingly, the $\Delta inlJ/pPL2::prot-inlJ$ strain showed an phenotype intermediate between those of the wild-type and $\Delta inlJ$ strains in colonizing organs (Fig. 5A) and in terms of abscess induction in liver when examined by histochemistry. The difference between the wild-type and $\Delta inlJ/pPL2::prot-inlJ$ strain phenotypes in vivo was not due to a defect in InlJ production, since InlJ protein was detected at the surface of $\Delta inlJ/pPL2::prot-inlJ$ bacteria as for the surface of wild-type bacteria (Fig. 5B). These results suggest that *inlJ*'s own regulatory region is important for a full-virulence phenotype, enabling the production of InlJ virulence protein at the right time and at the right place during the infection process.

InlJ behaves as an adhesin. We previously reported that an *inlJ* mutant and its parental strain behaved similarly in terms of bacterial adherence, entry, replication, and spreading in tissue-cultured cells (61). However, we show here that bacteria do not express *inlJ* in mammalian cell lines in vitro, thus precluding any function identification. To address InlJ function, we first

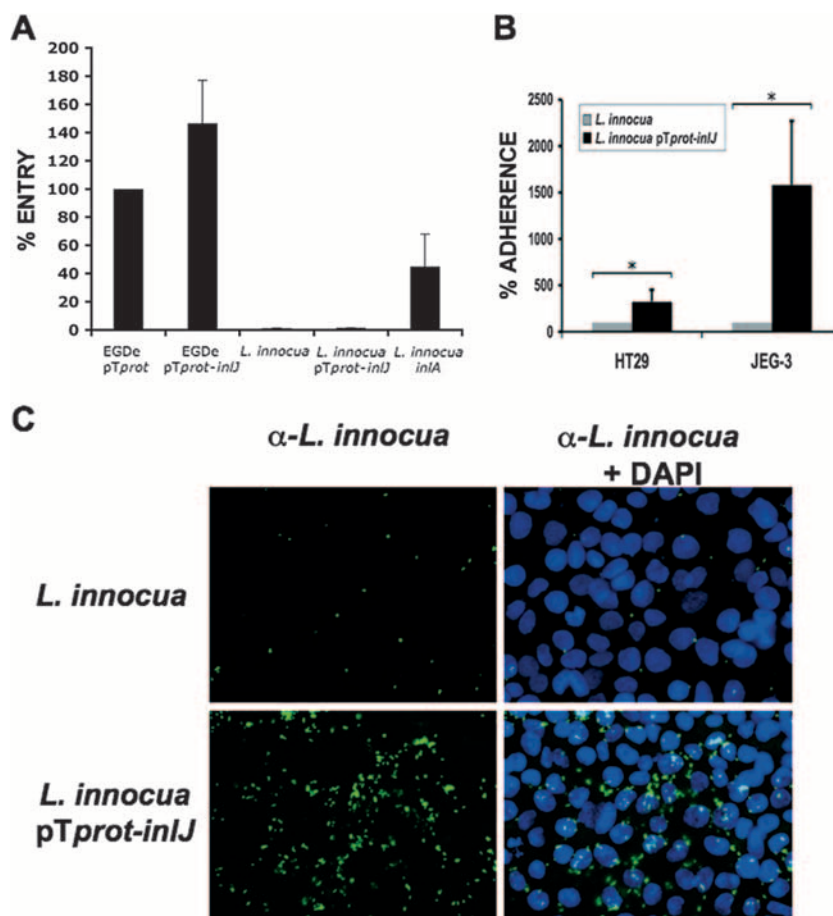


FIG. 6. InI stimulates bacterial adherence to eukaryotic cells. (A) Gentamicin invasion assays in JEG-3 cells of *L. monocytogenes* EGDe/pT_{prot} and EGDe/pT_{prot-inI}J, *L. innocua*, *L. innocua* expressing pT_{prot-inI}J or *inIA*. The level of entry of *L. monocytogenes* EGDe/pT_{prot} was reported as 100. The levels of entry of the other bacteria are given as relative values. For *L. innocua* and *L. innocua* expressing pT_{prot-inI}J, the percentages of entry are $0.29\% \pm 0.09\%$ and $0.77\% \pm 0.2\%$, respectively. Results are means and standard deviations from four different experiments. (B and C) Expression of *inI*J in *L. innocua* stimulates bacterial adherence to JEG-3 cells. Cells were incubated for 4 h at 37°C with *L. innocua* or *L. innocua* expressing *inI*J at a multiplicity of infection of ≈ 20 bacteria per cell, extensively washed, and processed for immunofluorescence and quantification of bacterial adherence. (B) Results are expressed as the mean (\pm standard deviation) number of adherent bacteria per cell. The level of adherence of the *L. innocua* strain has been artificially reported as 100, and the level of adherence of *L. innocua* expressing pT_{prot-inI}J is reported as a relative value. Asterisks indicate that differences are statistically significant ($P < 0.05$). (C) An immunofluorescence experiment is shown, with bacteria labeled with an anti-*L. innocua* antibody (in green) and cell nuclei detected with DAPI (in blue). α -, anti-

searched for a mammalian interactor by a two-hybrid screen against a human cDNA library, since InI as other LRR-containing proteins is likely to interact with a protein partner (37). However, among 104 million interactions tested, no partner was found.

To further investigate a role of InI in the cellular infection process, we quantified entry into placental JEG-3 cells of *L. monocytogenes* expressing *inI*J from the *prot* promoter, in which InI is efficiently produced and anchored at the bacterial surface. Interestingly, entry of EGDe/pT_{prot-inI}J increased by $46\% \pm 30\%$ from that of control bacteria carrying the empty vector (EGDe/pT_{prot}) (Fig. 6A). To address whether this stimulation of invasion resulted from increased adhesion to host cells or increased internalization per se, we then expressed *inI*J in the noninvasive species *L. innocua*. Like in *L. monocytogenes*, InI was efficiently produced and anchored at the surface of *L. innocua* carrying plasmid pT_{prot-inI}J (data not shown). *inI*J expression in *L. innocua* did not promote bacterial entry

into JEG-3 cells (Fig. 6A) or HT29 cells (data not shown), in contrast to expression of the invasion gene *inIA* (Fig. 6A) (40). However, *inI*J expression in *L. innocua* stimulated adherence 16-fold in JEG-3 cells and 3-fold in HT29 cells (Fig. 6B and C). Taken together, these results indicate that InI stimulates bacterial adherence to host cells, which then favors bacterial internalization.

DISCUSSION

We report here the characterization of InI, an *L. monocytogenes* surface protein of the internalin family involved in virulence. We show that InI is exposed on the listerial surface by a sortase A-mediated process and contributes to bacterial adherence to host cells. Furthermore, we show that InI is specifically produced and anchored at the bacterial surface during the infection process in vivo, whereas it is not produced

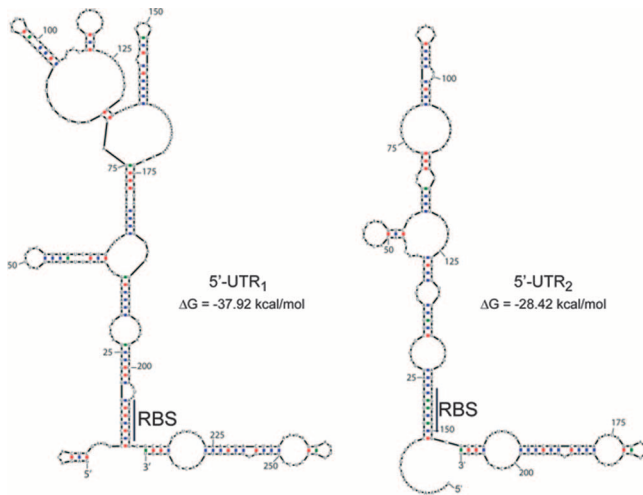


FIG. 7. Predictive folding of *inlJ* 5'-UTR RNAs. *inlJ* 5'-UTR₁ and 5'-UTR₂ RNAs predicted from the two transcriptional starts (Fig. 1) may fold into structures in which the RBS could be sequestered by a sequence present at the 5' extremity of the UTR. The energetically most stable structure is shown. The RNA sequence includes the 5'-UTR and 50 bases inside the mRNA coding sequence. Watson-Crick base pairing is indicated in red for cytosine and guanine and in blue for adenine and uracil. Wobble base pairing (guanine with uracil) is indicated in green. (Pairing was done according to Mfold [http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi].).

by bacteria grown in BHI medium, within the cytosol of tissue-cultured cells, or in blood in vitro.

This observation contrasts with the situation for other major listerial virulence factors, such as InlA, InlB, LLO, ActA, PlcA, and PlcB. First, all these proteins are detectable in extracts of *L. monocytogenes* strain EGDe grown in BHI medium. Second, they are all upregulated during bacterial infection of the host cell cytosol in vitro (7, 10, 36, 64), whereas InlJ is not.

inlJ expression may be regulated at the transcriptional level by as-yet-unknown regulators. Signatures for major listerial regulatory proteins such as PrfA, VirR, Fur, and sigma B are not present in the *inlJ* regulatory region, and the inactivation of PrfA has no effect on *inlJ* transcription. Furthermore, no role in *inlJ* regulation could be attributed to AxyR, encoded by a putative regulator gene adjacent to *inlJ*. The only signal that appears to regulate *inlJ* transcription is temperature, as revealed by a recent study examining temperature-dependent expression of some listerial internalins which shows that the level of *inlJ* transcript increases upon a shift from 16°C to 30°C (47). However, as we show here, increasing temperature does not lead to a stimulation of InlJ protein synthesis. The *inlJ* mRNA is detected in bacteria grown in vitro, while the InlJ protein is not produced. Taken together, all results converge to establish that *inlJ* is submitted to a posttranscriptional control.

Promoter mapping indicates the existence of two *inlJ* transcriptional starts, generating 219-bp and 158-bp 5'-UTRs that each could fold into a structure sequestering the *inlJ* RBS (Fig. 7). Such structures could mediate *inlJ* posttranscriptional regulation, as described previously in the cases of *prfA*, *actA*, *inlA*, and *hly* (30, 63, 65, 71). Changes in RNA secondary structures can occur upon the binding of a specific component, as in the case of riboswitches (11, 53, 55, 69, 70). It is possible that

during host infection by *Listeria* a metabolite or another molecule could be specifically released from the *inlJ* 5'-UTR, providing a quick switch-on of InlJ synthesis. Another possibility is that the regulation of *inlJ* expression could be coupled to *axyR* transcription because of the overlap between *inlJ* and *axyR* promoter regions. Future work will address this hypothesis.

We show here that when *inlJ* is expressed from a heterologous promoter, the InlJ protein is clearly exposed at the surface of bacteria and acts as an adhesin. This result suggests that InlJ interacts with a receptor present at the surface of the host cell. A two-hybrid screening approach did not succeed in finding an InlJ human receptor. This may be explained by several hypothesis: (i) InlJ may not interact with a protein; (ii) the cDNA encoding the InlJ receptor may not be present in the cDNA library; (iii) the interaction may require posttranslational modifications of the receptor, such as glycosylations; (iv) the receptor may be a membrane protein, as the two-hybrid screen is known to fail in detecting membrane-interacting proteins; and (v) the function of InlJ in adherence may be indirect, for example in stabilizing or activating another adhesin on the cell surface. Techniques other than two-hybrid screening will be used to search for an InlJ mammalian or bacterial partner.

Adhesion and invasion are crucial in initiating *L. monocytogenes* infection in intestinal, uteroplacental, hepatic, and neurological phases of infection. There are only a few adhesion-associated genes known for *L. monocytogenes*, although genome annotation suggests the existence of several other candidates (3). These genes encode the autolysin Ami (52), the fibronectin binding protein (FbpA) (15, 26), and the *Listeria* adhesion protein LAP (35). Furthermore, by interacting with the adhesion molecule E-cadherin, the well-characterized internalin InlA acts as an adhesin before playing a role in invasion (49). An LRR protein from group B streptococci, LrrG, was also shown to adhere to epithelial cells, suggesting that it may function as an adhesion factor (62). InlJ is thus a novel example of an LRR bacterial protein with adhesive properties produced by a pathogen. *L. innocua* cell lines expressing *inlJ* were found to bind eukaryotic cells with efficiencies that differed between cell lines and were high in placental cells, suggesting that InlJ binds to receptor molecules that are variably expressed by eukaryotic cells. This issue warrants further studies with a larger panel of cell lines, as well as investigation of the role of InlJ as a colonizing factor for placental infection in vivo. Of note is the fact that an InlJ-like protein recently identified in the oral pathogen *Porphyromonas gingivalis* is involved in biofilm formation (8). Whether listerial InlJ plays a role in biofilm formation on tissues or on abiotic surfaces is still unknown.

In conclusion, our report is the first description of a listerial virulence protein detected at the surface of bacteria present in infected organs and undetectable on bacteria infecting tissue-cultured cells. A strict regulation is likely to enable bacteria to rapidly adjust *inlJ* gene expression to environmental changes, especially during the infection process in vivo to stimulate adhesion to tissues. This regulation may involve the *inlJ* 5'-UTR. Given the large number of listerial genes with long 5'-UTRs (43), *inlJ* may be the first member of a list of genes specifically expressed by *L. monocytogenes* in vivo.

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ADDENDUM IN PROOF

The X-ray structure of the InIj internalin domain has recently been determined (M. Bublitz, C. Holland, C. Sabet, J. Reichelt, P. Cossart, D. W. Heinz, H. Bierre, and W.-D. Schubert, *J. Mol. Biol.*, in press).

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