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Identification of Four New Members of the Internalin Multigene Family of *Listeria monocytogenes* EGD

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Listeria monocytogenes **is a bacterial pathogen that is able to invade nonphagocytic cells. Two surface proteins, internalin, the *inlA* gene product, and InlB, play important roles in the entry into cultured mammalian cells. These proteins also have extensive sequence similarities. Previously, Southern hybridization predicted the existence of an internalin multigene family. Recently, InlC, a secreted protein of 30 kDa homologous to InlA and InlB, was identified. In this work, we identified and characterized four new members of the internalin multigene family, *inlC2*, *inlD*, *inlE*, and *inlF* which encode proteins of 548, 567, 499, and 821 amino acids respectively. *inlC2*, *inlD*, and *inlE* are contiguous on the chromosome of *L. monocytogenes* EGD, whereas *inlF* is located in a different chromosomal region. These four *inl* gene products display the principal features of internalin, namely, a signal sequence, two regions of repeats (or LRR and B repeats), and a putative cell wall anchor sequence containing the sorting motif LPXTG. The four *inl* genes were maximally expressed albeit at a low level during early exponential growth in bacterial medium at 37°C. The role of these *inl* genes in *L. monocytogenes* invasion was assessed by constructing isogenic chromosomal deletion mutants and testing them for entry into various nonphagocytic cells. Unexpectedly, the *inlC2*, *inlD*, *inlE*, and *inlF* null mutants were not affected for entry into any of the cell lines tested, raising the possibility that these genes are needed for an aspect of pathogenicity other than invasion. The identity of such an aspect remains to be determined.**

Listeria monocytogenes is a ubiquitous gram-positive bacterium that is responsible for severe opportunistic infections in both humans and animals. Entry into the host normally occurs in the gut after ingestion of contaminated foodstuffs. Bacteria pass through the gastrointestinal barrier and spread via the lymph and the blood to distant tissues. In murine infection, bacteria accumulate predominantly in the liver, where replication takes place until the host develops a protective cellular immune response. Depending on the immune response of the host, bacteria either are eliminated or undergo further hematogenous dissemination to the brain and/or placenta (for a review, see reference 42). A key aspect of the pathogenicity of this bacterium is its ability to invade and multiply in phagocytic and nonphagocytic cells. We identified the first genetic locus involved in *L. monocytogenes* invasiveness (15). This locus is composed of two genes, *inlA* and *inlB*, which are organized in an operon. These genes are homologous and encode the surface proteins necessary for invasion of different cell lines (11, 15–17, 19, 28). Internalin (or InlA), encoded by *inlA*, is required for entry into the human enterocyte-like cell line Caco-2, while InlB is required for entry into cultured hepatocytes and into some epithelial cell lines including HeLa, HEp-2, and Vero. At the sequence level, InlA displays some characteristic features (see Fig. 3): a signal sequence, two regions of repeats, and a C-terminal cell wall anchor. The carboxy-terminal region of InlA contains an LPXTG motif preceding the hydrophobic membrane-spanning region—a signature sequence that is necessary for sorting some surface proteins of gram-positive bacteria to the cell wall (35, 40).

The cellular receptor for InlA has recently been shown to be the adhesion molecule E-cadherin (33). How interaction between InlA and E-cadherin leads to bacterial uptake by the host cell is not yet understood.

Although InlB contains homology to the LRR, interrepeat, and B regions of InlA (see Fig. 3), it has some different features: a third region of repeats (region C) and no hydrophobic C-terminal region. The cellular receptor for InlB is unknown.

A third member of the internalin family, named IrpA or InlC, was recently identified (14, 27). It encodes a secreted protein of 297 amino acids that is homologous to InlA and InlB. Expression of *inlC* is completely dependent on the *prfA* gene, which encodes a transcriptional activator of virulence gene expression. In contrast, the expression of *inlA* and *inlB* is only partially dependent on PrfA (12, 28). In addition, *inlC* is strongly transcribed in the cytoplasm of phagocytic J774 cells whereas *inlA* is poorly transcribed under these conditions, suggesting that InlC may play a role in a late stage of infection rather than in the uptake of *L. monocytogenes* by nonphagocytic cells. The function of *inlC* is currently unknown. However, an *inlC* deletion mutant showed 50-fold-reduced virulence when tested in a mouse model by an intravenous route (14).

In this work, we report the identification of four new members of the internalin multigene family in *L. monocytogenes* EGD: InlC2, InlD, InlE, and InlF. In contrast to InlB and InlC, these internalins display all of the characteristic features of internalin, i.e., a signal sequence, two regions of repeats (LRR and B), and a putative C-terminal cell wall anchor. These four genes are contained in two chromosomal loci with *inlC2*, *inlD*, and *inlE* grouped together. All four genes are transcribed during growth in bacterial medium at 37°C. The striking homology between these *inl* proteins and InlA and the existence

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TABLE 1. Bacterial strains used in this work

Strain	Genotype, relevant properties	Source or reference
EGD	*L. monocytogenes*, serotype 1/2a	29
BUG 947	EGD Δ*inlA*	11
BUG 1047	EGD Δ*inlB*	11
BUG 949	EGD Δ*inlAB*	11
BUG 948	EGD Δ*inlC2*	This work
BUG 1077	EGD Δ*inlD*	This work
BUG 950	EGD Δ*inlE*	This work
BUG 1078	EGD Δ*inlF*	This work
BUG 1472	EGD Δ*inlC2DE*	This work
BUG 1065	EGD Δ*inlABC2DE*	This work
BUG 1080	EGD Δ*inlABC2DEF*	This work

of an *inlAB*-independent pathway for the invasion of mammalian cells (11, 33) prompted us to examine whether the newly identified proteins might be involved in the entry of *L. monocytogenes* into nonphagocytic cells. A series of isogenic—single or multiple—deletion mutant strains were constructed and tested for entry into the enterocyte, hepatocyte and fibroblast cell lines. Finally, the *inl* null mutants were tested for virulence in the mouse model.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Luria-Bertani medium was used for growth of *Escherichia coli* strains, and brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) was used for growth of *L. monocytogenes* and *L. innocua*. For *E. coli* strains (MC1061 or DH5αF′) containing pUC, pKSV7, or pHV1248ΔTn*10* vector derivatives, ampicillin was added to a final concentration of 25 mg/liter in liquid media and 100 mg/liter in solid media. Antibiotics were added to liquid or solid media for the growth and maintenance of *Listeria* strains containing pKSV7 vector derivatives (chloramphenicol, 10 mg/liter) or pHV1248 and pGhost5 derivatives (erythromycin, 5 mg/liter).

Transfer of plasmids. Plasmids were introduced into *E. coli* strains by transformation after $CaCl_2$ treatment (39) and into *Listeria* strains by electroporation. Electroporation was performed as follows. An overnight culture of *L. monocytogenes* diluted 1/100 in BHI was incubated at 37°C until the optical density at 600 nm (OD_{600}) reached 0.2 (final volume, 100 ml). Then penicillin G was added (final concentration, 0.12 mg/liter), and growth at 37°C was allowed to proceed until the OD_{600} of the culture reached 0.8 to 0.9. The culture was then centrifuged at 4°C for 20 min at 4,000 × *g*, washed three times in cold electroporation buffer (816 mM sucrose, 1 mM $MgCl_2$ [pH 6.5 to 7]), and resuspended in 1 ml of the same buffer. A 100-μl volume of bacteria was then mixed with 1 μg of DNA in an electroporation cuvette, incubated for 1 min at 0°C, and subjected to an electric discharge of 2.5 kV at 25 μF and 200 Ω. The sample was immediately transferred to 900 μl of prewarmed BHI broth and incubated at 30°C for 2 to 3 h with agitation. Aliquots of 100 μl were plated on antibiotic-containing BHI agar plates and incubated at 30°C. Electroporants appeared after 2 days.

Molecular cloning and DNA analysis. Unless otherwise stated, all cloning and DNA techniques were carried out by standard methods (39) or as specified by the manufacturers. Chromosomal DNA from *L. monocytogenes* was prepared as described previously (32). Probes for Southern blots were prepared by the PCR or enzymatic digestion, purified from agarose gels with the Geneclean kit (BIO 101 Inc., La Jolla, Calif.), and labeled with the Multiprime system (Amersham). Southern blot hybridizations were performed under conditions of high and low stringency. At high stringency, prehybridization and hybridization were carried out with a rapid hybridization system (Amersham) in a Hybaid hybridization oven at 65°C. At low stringency, prehybridization and hybridization were carried out at 37°C in a solution containing 30% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), and 10× Denhardt's solution. The filters were washed twice for 30 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 37°C and once for 30 min in 1× SSC–0.1% sodium dodecyl sulfate at 42°C. Modifying or restriction enzymes were purchased from Boehringer, Amersham, New England BioLabs, or United States Biochemical Corp. *Taq* polymerase was obtained from Amersham, and HK phosphatase was obtained from Tebu. DNA sequencing was performed with a T7 polymerase kit (Pharmacia).

Culture of cell lines. The human colon carcinoma cell line Caco-2 (ATCC HTB 37) was cultured in Dulbecco's modified Eagle's medium containing 4.5 g of glucose per liter (DMEM; TechGen Laboratories), supplemented with 2mM L-glutamine (Gibco), 10 μg of human transferrin (Sigma) per liter, 1% nonessential amino acids (Gibco), and 10% fetal calf serum (Boehringer). The human hepatocellular carcinoma cell line HepG-2 (ATCC HB 8065) and the murine embryonic hepatocyte cell line ATCC TIB73 were propagated in DMEM supplemented with 2mM L-glutamine and 10% fetal calf serum. S180, S180E-cad, and S180N-cad were grown in DMEM with 15% fetal calf serum. The Caco-2 and ATCC TIB73 cell lines were maintained without antibiotics, while a penicillin-streptomycin solution (Sigma) was added to the medium for HepG-2 cells. All these cells were cultivated at 37°C in 10% CO_2.

Computer sequence analysis. Sequences were analyzed either with the Macintosh software programs Gene Jockey (Biosoft) and DNA Strider (31) or with the University of Wisconsin Genetics Computer Group package (version 8.0) (10). The Blast algorithm (1) was used to search the nonredundant protein and nucleic acid database compilations of The National Center for Biotechnology Information (National Institutes of Health, Bethesda, Md.). The locations of signal peptide cleavage sites were determined at the Signalp World Wide Web server of the Center for Biological Sequence Analysis at The Technical University of Denmark, Lyngby, Denmark (http://www.cbs.dtu.dk/signalp/cbssignalp.html). Multiple alignments of proteins were performed with the Clustal W program, using the BLOSUM62 matrix (44).

Assays for entry of bacteria into cells. Invasivity assays were performed in 24-well tissue culture plates (Costar). Bacteria were grown at 37°C to an OD_{600} of 0.8 to 1, washed twice in DMEM, and diluted in DMEM to 10^7 bacteria per ml. Bacterial suspension (1 ml per well) was added to mammalian cells (~10^5 cells per well). After 1 h of incubation at 37°C to allow bacterial entry, the cells were washed twice with DMEM and overlaid with DMEM containing gentamicin (5 mg/liter) to kill extracellular bacteria. We noticed that two mutants, Δ*inlA-E* and Δ*inlA-F*, were resistant to the concentration of gentamicin we used (5 mg/liter); therefore, a 10-fold-higher concentration of gentamicin (50 mg/liter) was used for these mutants. After 2 h of incubation at 37°C, the cells were washed twice with DMEM and lysed by the addition of Triton X-100 (0.2% final concentration) and the number of viable bacteria was determined on agar plates.

Construction of *L. monocytogenes* mutant strains. (i) In-frame *inlC2* deletion strain (BUG 948). A 4-kb *Bam*HI fragment isolated from a pUC derivative carrying the *inlC2D* region was cloned into pGhost5 (pVE7080). pVE7080 was digested with *Ava*I-*Hin*dIII, filled in with T4 DNA polymerase, and ligated, resulting in plasmid pVE7081 (Table 2) containing an in-frame deletion of 971

TABLE 2. Plasmids used in this work

Plasmid	Host	Marker(s)	Relevant properties	Collection	Source or reference
pHV1248ΔTn*10*	*E. coli*/*Listeria*	Amp/Ery	*ori* pE194ts		21
pGhost5	*E. coli*/*Listeria*	Ery	*ori* pE194ts derivative		30
pKSV7	*E. coli*/*Listeria*	Amp/Cm	*ori* pE194ts	BUG 1004	43
pPE-4	*E. coli*	Amp	pUC18 derivative carrying the 5′ part of *inlC2*	BUG 776	This work
pPE-5	*E. coli*	Amp	pUC18 derivative carrying part of *inlC2* and *inlD*	BUG 771	This work
pPE-7	*E. coli*	Amp	pUC18 derivative carrying the *inlC2DE* locus	BUG 958	This work
p26	*E. coli*	Amp	pUC18 derivative carrying *inlE*	BUG 825	This work
p84	*E. coli*	Amp	pUC18 derivative carrying *inlF*	BUG 946	This work
p7895	*E. coli*	Kana	pUC18 derivative carrying *inlF* and its promoter region	BUG 1322	This work
pVE7081	*E. coli*/*Listeria*	Ery	pGhost5 carrying the fragment with the deletion in *inlC2*		This work
pKSV7-3	*E. coli*/*Listeria*	Amp/Cm	pKSV7 derivative carrying the Δ*inlD-Eco*RI fragment	BUG 1076	This work
pHVΔinlE	*E. coli*/*Listeria*	Amp/Ery	pHV1248ΔTn*10* carrying the Δ*inlE-Hin*dIII fragment	BUG 1003	This work
pHVΔinlF	*E. coli*/*Listeria*	Amp/Ery	pHV1248ΔTn*10* carrying the Δ*inlF-Hin*dIII fragment	BUG 1074	This work
pKSV7-4	*E. coli*/*Listeria*	Amp/Cm	pKSV7 derivative carrying the Δ*inlC2DE-Eco*RI fragment	BUG 1044	This work

bp in *inlC2* (positions 293 to 1265). This in-frame deletion was confirmed by sequencing the junctional region in plasmid pVE7081. pVE7081 was then electroporated into *L. monocytogenes* EGD, and the gene replacement protocol described by Biswas et al. was used (4). Chromosomal DNAs of 12 EryS colonies were analyzed by Southern blot hybridization with an internal *inlC2*-specific probe. One mutant (Δ*inlC2*) was found which contained the expected deletion of the peptide signal, the LRR region, and a large part of the interrepeat region of InlC2.

(ii) *inlD* deletion strain (BUG 1077). Plasmid pPE7 harboring the 5.4-kb *Eco*RI fragment carrying the *inlC2DE* locus was digested with *Sna*BI-*Hpa*I, creating, after blunt-end ligation, a deletion of 1,771 bp (positions 2117 to 3888). The resulting 3.7-kb *Eco*RI fragment was cloned into pKSV7, yielding plasmid pKSV7-3 (Table 2). pKSV7-3 was electroporated into *L. monocytogenes* EGD, and gene replacement was performed by the method described by Camilli et al. (8), resulting in a gene containing only the first eight codons of *inlD*. The deletion ends in the intergenic region.

(iii) In-frame *inlE* deletion strain (BUG 950). The 3.1-kb *Hin*dIII fragment of plasmid p26 was cloned into pUC9. The resulting plasmid was digested with *Sac*I-*Eco*RV, blunt ended with T4 DNA polymerase, and ligated, creating an in-frame deletion of codons 28 to 170 of InlE (positions 4085 to 4513). The deleted *Hin*dIII fragment was cloned into pHV1248ΔTn*10*, yielding plasmid pHVΔinlE. This shuttle plasmid was then electroporated into *L. monocytogenes* EGD, and gene replacement was performed by the method described by Camilli et al. (8).

(iv) *inlF* deletion strain (BUG 1078). The 3.5-kb *Hin*dIII fragment containing *inlF* (p84) was cloned into pHV1248ΔTn*10* and digested with *Xba*I-*Spe*I (compatible ends), and the internal 1,162-bp fragment (positions 1507 to 2669) was deleted after intramolecular ligation of the resulting plasmid backbone. The resulting plasmid, pHVΔinlF, was then electroporated into *L. monocytogenes* EGD, and gene replacement was performed by the method described by Camilli et al. (8). The deletion results in a gene containing only the first 378 codons of *inlF*.

(v) *inlC2DE* deletion strain (BUG 1472). pPE-7 harboring the 5.4-kb *Eco*RI fragment carrying the *inlC2DE* locus was digested with *Pac*I, filled in with T4 DNA polymerase to avoid the creation of an InlC2-E hybrid protein (consisting of the N-terminal part of InlC2 and the C-terminal part of InlE), and ligated, creating a deletion of 3,760 bp (positions 1332 to 5092 as indicated in Fig. 3). This large deletion encompasses the genes *inlC2*, *inlD*, and *inlE*. Note that a truncated InlC2 protein of 362 amino acids is produced by the mutant strain Δ*inlC2DE* (data not shown). The deleted 1.6-kb *Eco*RI fragment was cloned into pKSV7 (pKSV7-4) and electroporated into *L. monocytogenes* EGD. Gene replacement was performed by the method described by Camilli et al. (8).

(vi) *inlABC2DE* deletion strain (BUG 1065). Plasmid pKSV7-4 was electroporated into *L. monocytogenes* EGD:Δ*inlAB* (11), and gene replacement was performed by the method described by Camilli et al. (8).

(vii) *inlABC2DEF* deletion strain (BUG 1080). Plasmid pHVΔinlF was electroporated into *L. monocytogenes* EGD:Δ*inlABC2DE* (BUG 1065), and gene replacement was performed by the method described by Camilli et al. (8).

Virulence assays. The 50% lethal doses (LD50s) were determined by intravenous (i.v.) injection of groups of four or five susceptible BALB/c mice (8-week-old females from IFFA-CREDO) with various dilutions of bacteria. Bacteria were first grown overnight in BHI and then inoculated 1:20 in fresh BHI and grown for approximately 3 h. Glycerol was added to 15% (vol/vol), and culture aliquots were frozen at −80°C. When required, aliquots were thawed, diluted in 0.15 M NaCl, and injected into a tail vein of the mice. The LD50 was estimated by the probit method. To quantitate viable bacteria present in the spleen and liver, these organs were aseptically removed and homogenized in 0.15 M NaCl, and serial dilutions were plated on BHI agar plates as previously described (11).

Nucleotide sequence accession numbers. The sequences of the *inlC2DE* and *inlF* loci have been deposited in the GenBank database under accession numbers U77368 and U77367, respectively.

RESULTS

Internalin multigene family. Evidence that *inlA* was part of a gene family originally came from Southern blot hybridization under low-stringency conditions (15). Hybridization of *Hin*dIII-digested chromosomal DNA from *L. monocytogenes* EGD, with the internal 1.2-kb *Hin*dIII fragment of *inlA* as a probe (probe A), revealed the presence of four bands in addition to the previously known *inlA* and *inlB* genes (Fig. 1). The *inlB* gene corresponds to the 4.2-kb fragment in strain EGD, as shown by reprobing the Southern blot with an *inlB*-specific probe at high stringency (data not shown). Probe A covers most of the *inlA* gene. Therefore, to test whether the other bands corresponded to a particular domain of internalin, Southern blot hybridization at low stringency was repeated with shorter probes corresponding to the different regions of *inlA* (leucine-rich repeat region, interrepeat, and region B). Our results suggested the existence of four new *inl* genes, each containing the three different domains of internalin in *L. monocytogenes* EGD (data not shown). As shown in Fig. 1A, the internalin multigene family is also present in two other wild-type strains of *L. monocytogenes*, strain LO28 (45) and the type strain CIP 82110. Furthermore, as previously described by Gaillard et al. (15), "*inl*-like" genes are also present in other members of the genus *Listeria*, including the invasive species *L. ivanovii* and the noninvasive species *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* (Fig. 1B).

Molecular cloning of the *inl* genes. The *inl* gene-containing fragments were isolated from libraries of chromosomal *Hin*dIII or *Eco*RI DNA fragments from the parental strain EGD. Positive clones were identified by colony hybridization at low stringency with the internal 1.2-kb *Hin*dIII fragment of *inlA* as a probe. Four plasmids, pPE-4, p84, p26, and pPE-5, containing *Hin*dIII fragments of 3.9, 3.5, 3.1, and 2.3 kb, respectively, were isolated. A fifth plasmid, named pPE-7, was obtained by screening the *Eco*RI library. A partial restriction map of these plasmids showed that the 5.5-kb *Eco*RI fragment of pPE-7 overlapped three *inl*-containing *Hin*dIII fragments, which were therefore found to be contiguous on the chromosome of *L. monocytogenes* (Fig. 2A). The *inl*-containing fragments were subjected to DNA sequence determination. Four open reading frames (ORFs) homologous to *inlA* were identified and named *inlC2*, *inlD*, *inlE*, and *inlF*. The *inlC2*, *inlD*, and *inlE* genes are contiguous on the chromosome and may be part of an operon, while *inlF* is present in a different locus. The genetic organization of these regions is shown in Fig. 2. We do not know if the *inl* loci are closely linked, although preliminary evidence based on pulsed-field gel electrophoresis indicates that the six *inl* genes identified in our laboratory are located on a 1,100-kb NotA fragment which also contains the virulence gene cluster (34).

DNA sequence analysis of *inl* genes. Our previous data (15) and the cloning by low-stringency hybridization reported above demonstrate that in *L. monocytogenes* EGD, the internalin multigene family is composed of at least six members contained in three loci: *inlAB*, *inlC2DE*, and *inlF*. Recently, a seventh member has been identified by others and named *inlC* (14), and we have confirmed that this gene was present in our strain EGD (data not shown). The nucleotide sequence of the *inlC2DE* locus showed the existence of three open reading frames homologous to *inlA*, named *inlC2*, *inlD*, and *inlE*. Intergenic sequences were of 200 and 201 bp between *inlC2* and *inlD* and between *inlD* and *inlE*, respectively. Each gene is preceded by a potential ribosome binding site (AAGGAG) located 7 or 8 bp upstream from the start codon, ATG. Putative transcription terminators were detected 12, 11, and 9 bp downstream from the stop codons of *inlC2*, *inlD*, and *inlE*, respectively. Several virulence genes including *inlA*, *inlB*, and *inlC* are regulated by PrfA. A search for the 14-bp dyad-symmetric sequence recognized by the transcriptional activator *prfA* (TTAACANNTGTTAA, where N represents one of the four bases) revealed the presence of such a sequence upstream of *inlE*, but this PrfA box has three mismatches compared to the perfect palindrome. This sequence is located at position −40 compared to a putative Pribnow box located at position −10, suggesting that *inlE* might be transcribed in a *prfA*-dependent manner independently from *inlC2* and *inlD*. An imperfect PrfA box was also detected in the promoter region of *inlD*. It contains four mismatches with respect to the ideal *prfA* target sequence. Partial sequencing of the region upstream of *inlC2* showed the presence of a putative gene encoding a protein similar to a 6-phospho-β-glucosidase. Sequence down-

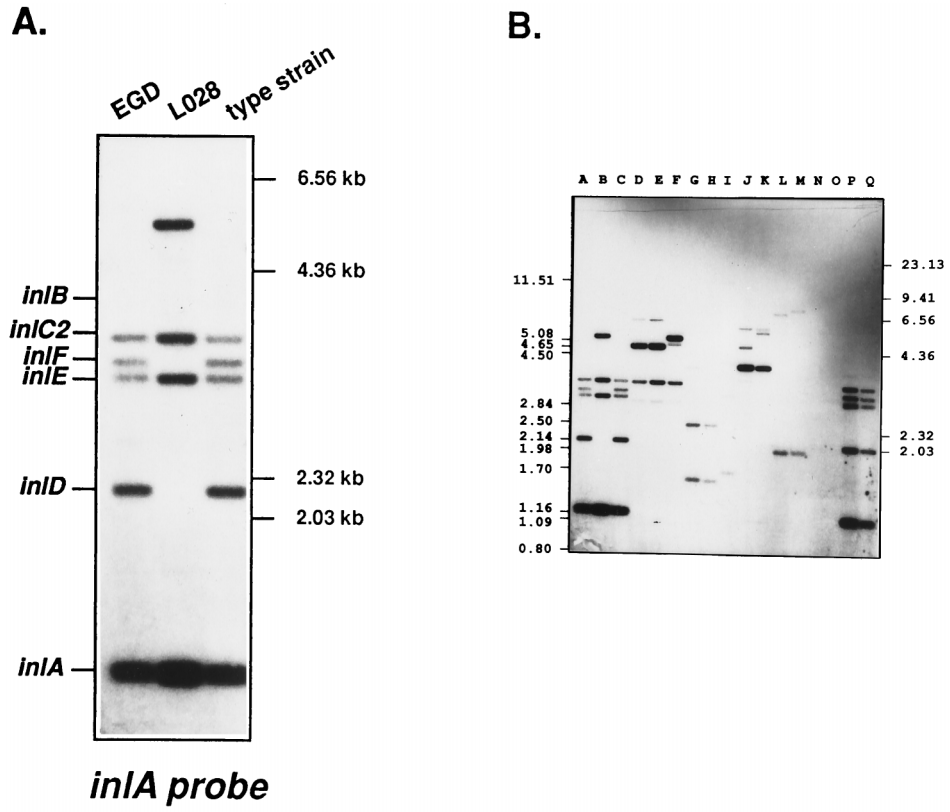

FIG. 1. Detection of sequences hybridizing to *inlA* in different species of *Listeria* by Southern blotting under low-stringency conditions. (A) Hybridization of *Hin*dIII-digested chromosomal DNA from wild-type *L. monocytogenes* EGD (serovar 1/2a), LO28 (serovar 1/2c), and CIP82110^T with the internal 1.2-kb *Hin*dIII fragment of *inlA*. The *Hin*dIII fragment corresponding to *inlB* can be seen in longer exposures of the same blot (not shown). (B) Southern blots of DNAs from six *Listeria* species hybridized with the *inlA* probe as in panel A. Lanes: A to C, *L. monocytogenes* EGD (BUG 600), LO28 (Bof 344), and CIP82110T (BUG 31), respectively; D to F, *L. ivanovii* CIP 7842T (BUG 496), SLCC 4121 (BUG 598), and CLIP 257 (BUG 497), respectively; G to I, *L. seeligeri* SLCC 3954T (BUG 494), SLCC 3503 (BUG 599), and CLIP 9529 (BUG 495), respectively; J and K, *L. innocua* CIP 8011T (BUG 498) and CLIP 11262T (BUG 499), respectively; L and M, *L. welshimeri* SLCC 5334T (BUG 502) and SLCC 5328 (BUG 501), respectively; N, *L. grayi* type strain CIP 76124T (BUG 503); O, DNA from lambda digested with *Hin*dIII (marker); P and Q, *L. monocytogenes* EGDSmR (Bof 297). Molecular weight markers are indicated (in thousands) on the left and right. The *inlA* probe is a *Hin*dIII restriction fragment spanning positions 2616 to 3822 as described previously (15).

stream of *inlE* revealed the presence of a putative protein with homology to a succinyl-diaminopimelate desuccinylase. All of these genes are oriented in the same direction.

We identified a fourth ORF homologous to *inlA*, named *inlF*, by sequencing the *Hin*dIII fragment of plasmid p84. Two possible start codons located at position 355 and 374 were detected. Only the second ATG is preceded by a potential ribosome binding site (AAGGA) located 9 bp upstream from the start codon. Therefore, we predict that this second ATG is the start codon for *inlF*. The nucleotide sequence of the region upstream of the putative start site for *inlF* was obtained by sequencing plasmid p7895 (Fig. 2B). Two putative transcription terminators located 197 bp upstream from the start codon and 4 bp downstream from the stop codon of *inlF* were detected. Partial sequencing of the region surrounding *inlF* revealed the presence of a potential ORF ending 222 bp upstream of *inlF* that does not have similarities to sequences in the databases. We also identified a putative ORF located downstream from *inlF* that encodes a protein similar to *E. coli* phosphoenolpyruvate synthase (Fig. 2B).

Optimal alignment among all the *inl* genes was performed with the FrameAlign program of the Genetics Computer Group software package. The percent identity between any pair of genes of the *inl* family was 55 to 80%. Interestingly, the genes *inlC2*, *inlD*, and *inlE*, which are clustered in a single locus, display the most striking conservation (70 to 80% identity) while the other *inl* genes, including *inlA*, *inlB*, *inlC* and *inlF*, share only 55 to 65% identity (Table 3). A search of the nucleotide databases revealed the existence of a partial sequence of 1,010 bp that shares 91% identity with *inlC2*. This sequence, called lisM51, was isolated and characterized as a *L. monocytogenes*-specific nucleotide sequence from the clinical strain JBL1231 serotype 4b by using a strategy of genomic subtraction between *L. monocytogenes* and *L. innocua* (9).

Sequence of Inl proteins. Figure 3 shows the sequences of all internalins and highlights the principal features of this multigene family. InlA (internalin), InlB, and InlC are 800, 630, and 297 amino acids long, respectively. *inlC2*, *inlD*, *inlE*, and *inlF* are predicted to encode proteins of 548, 567, 499, and 821 amino acids, respectively. The isoelectric points (pI) of InlC2, InlD, InlE, and InlF are 4.8, 4.6, 4.4, and 4.5, respectively, and are very similar to that of internalin (pI 4.9). The pI of InlC is 6.4. The only basic pI is that predicted for InlB (9.8). Only InlB and InlC possess cysteine residues in their amino acid sequence.

The primary sequence of InlC2, InlD, InlE, and InlF displays characteristic features of internalin, including a signal sequence, two regions of repeats, and a hydrophobic C-terminal end preceded by the pentapeptide LPXTG (Fig. 3). All of the Inl proteins contain an N-terminal sequence of 30 to 35 amino

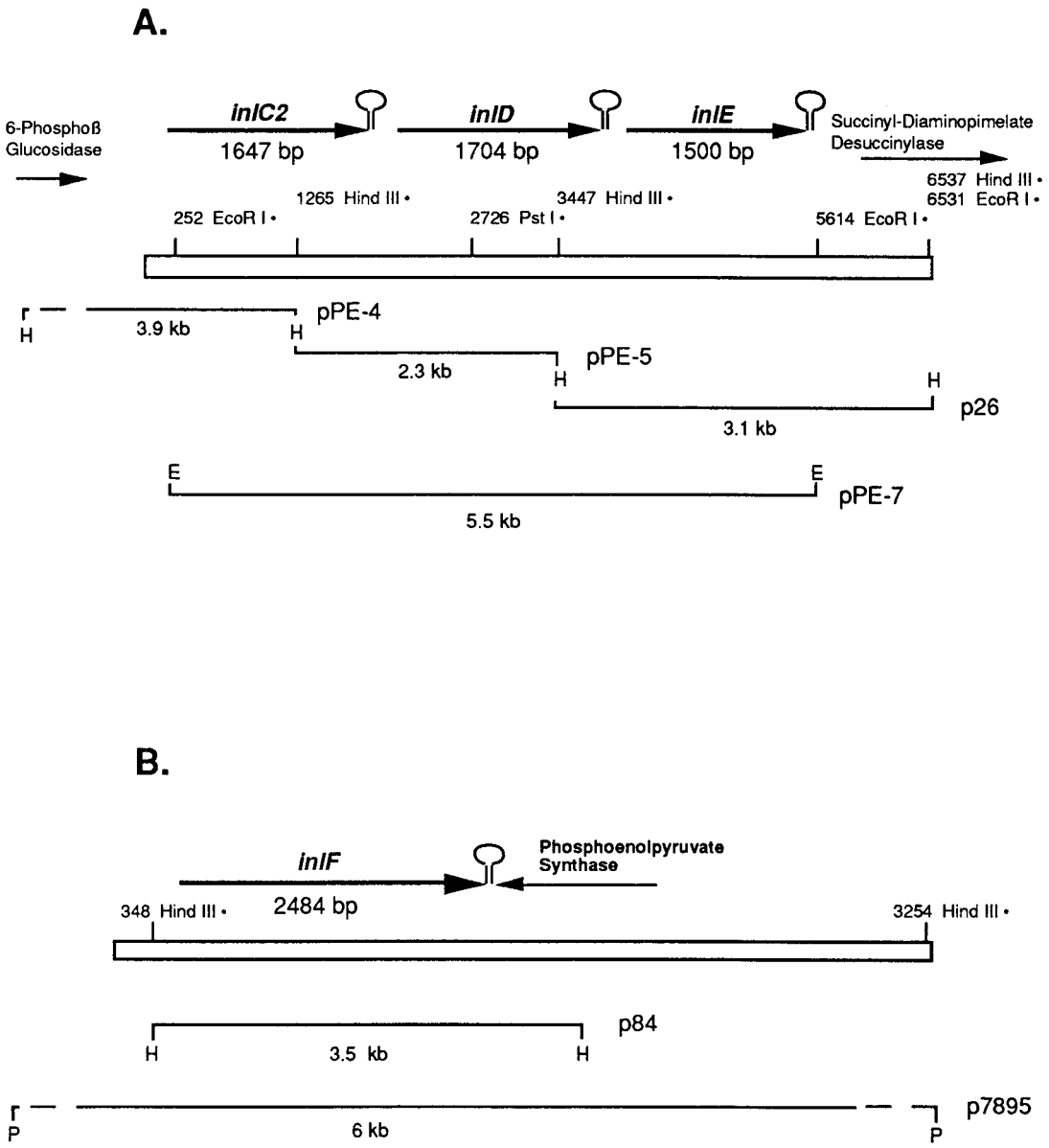

FIG. 2. Genetic organization of the *inlC2DE* and *inlF* loci. A partial restriction enzyme map of the *inlC2DE* and *inlF* regions is presented. The different inserts cloned into pUC vectors are indicated under the map, i.e., pPE-4, pPE-5, p26, pPE-7, and p84. The ORFs *inlC2*, *inlD*, *inlE*, and *inlF* are indicated above the map. Putative transcription terminators are indicated by a ♀ symbol.

acids with characteristics ascribed to bacterial signal peptides (37). Among Inl proteins, the number of repeats varies between 8 and 15 (for region LRR) and between 1 and 4 (for region B), while the length (about 90 amino acids) and amino acid sequence of the interrepeat region are highly conserved (Fig. 4). At the C terminus, except for InlB and InlC, the other Inl proteins have a 21-amino-acid hydrophobic segment preceded by the pentapeptide LPXTG and followed by a short tail of charged amino acids. This C-terminal region serves as a signal for sorting of surface proteins, like protein A of *Staphylococcus aureus*, to the cell wall of gram-positive bacteria (41). Protein A is covalently linked to the peptidoglycan via a peptide bond between the carboxylic end of the threonine residue of the LPXTG motif and a free amino group of the peptidoglycan (40). Interestingly, in InlC2 and InlD, an alanine is found instead of threonine at position 4 of the LPXTG motif, suggesting that these proteins may use a slightly different mechanism of cell wall anchoring.

Like the percent identities observed at the DNA level, the percent similarities at the amino acid level presented in Table 3 show that (i) InlC2, InlD, and InlE are highly related; (ii) InlB is the most divergent member of the internalin family; and (iii) InlC is more closely related to InlB than to InlA.

Inl proteins do not show striking overall sequence similarities to other proteins in the databases. However if each structural domain of internalin is considered separately, the LRR repeats of internalin are closely related to those found in the *sds22* gene product, a regulator of protein phosphatase 1 in *Saccharomyces pombe* (36). Alignment of the LRR domain of internalin with the human homolog of *sds22* (38) reveals 28%

TABLE 3. Pairwise comparison of the amino acid sequences of Inl proteins[a]

Inl protein (size)	% Similarity to[a]:						
	InlA (800 aa)	InlB (630 aa)	InlC (297 aa)	InlC2 (548 aa)	InlD (567 aa)	InlE (499 aa)	InlF (821 aa)
InlA (800 aa)							
InlB (630 aa)	60 (835/8)						
InlC (297 aa)	64 (493/3)	64 (318/1)					
InlC2 (548 aa)	71 (800/6)	53 (611/6)	59 (343/2)				
InlD (567 aa)	69 (799/9)	55 (637/10)	61 (369/3)	75 (572/1)			
InlE (499 aa)	63 (802/5)	57 (569/5)	58 (342/2)	75 (550/2)	65 (574/3)		
InlF (821 aa)	62 (863/4)	52 (826/7)	57 (458/4)	65 (820/9)	65 (823/8)	60 (631/5)	

[a] Overall percentages of similarity are indicated. The amino acid (aa) length of the sequence aligned and the number of gaps (≥5 amino acids) that were considered are given in parentheses. Alignment was performed with the BESTFIT program provided in the Genetics Computer Group package.

identity over a 360-amino-acid sequence with the BESTFIT program. In both internalin and Sds22, the LRR unit consists of 22 amino acids with a conserved asparagine residue that plays a critical role in the formation of a predicted β-helix motif (19a). In addition, the highly conserved interrepeat region of Inl proteins is similar to the p60 protein of *L. seeligeri* (6). Sequence comparison shows 30% identity between InlA (amino acids 422 to 492) and p60 of *L. seeligeri* (amino acids 271 to 341) over a 70-amino-acid overlap. The stretch of amino acids from 311 to 341 is present in the p60 of *L. seeligeri* and *L. ivanovii* but not in the p60 of *L. monocytogenes*. p60, which was first described in *L. monocytogenes* as an invasion-associated protein (*iap*), is a protein of 484 amino acids which has a murein hydrolase activity (22, 23, 46), suggesting that its role in invasion is probably indirect. The functional significance of these similarities is unknown.

Entry of the Δ*inlAB*, Δ*inlCDE*, and Δ*inlF* deletion mutants into various cell lines. The role of the *inlAB* operon was previously studied in our laboratory (for a review, see reference 13), and we found that both genes encode surface proteins that play critical roles in *L. monocytogenes* invasion. InlA is necessary for entry into Caco-2 cells (15) or other cell lines expressing the InlA receptor E-cadherin (33). In contrast, InlB is required for entry into cultured hepatocytes (11) and into the epithelial cell lines HeLa, HEp-2, and Vero (19). It was thus tempting to hypothesize that the other *inl* genes could also be involved in the entry process. To address this point, we constructed isogenic deletion mutants containing deletions in *inlC2* (Δ*inlC2*), *inlD* (Δ*inlD*), *inlE* (Δ*inlE*), *inlF* (Δ*inlF*), and *inlC2DE* (Δ*inlC2DE*). These chromosomal mutants were obtained by allelic exchange in *L. monocytogenes* EGD. We also introduced the *inlC2DE* deletion into the Δ*inlAB* strain (11), creating a Δ*inlA–E* strain. Finally, a strain deleted for all of the *inl* genes except *inlC* (Δ*inlA–F*) was constructed by introducing the *inlF* deletion into the Δ*inlA–E* strain. All the constructs were verified by Southern blotting and PCR sequencing of chromosomal DNA from mutants (data not shown).

These seven mutants, together with the three previously described mutants Δ*inlA*, Δ*inlB*, and Δ*inlAB* (11), were tested for entry into four cell lines: the human enterocyte-like cell line (Caco-2), a murine hepatocyte cell line (ATCC TIB73), a human hepatocyte cell line (HepG-2) and a mouse fibroblast cell line (S180). The percentages of entry of Δ*inlC2*, Δ*inlD*, and Δ*inlE* into these four cell lines were identical to those of Δ*inlC2DE*; therefore, in Fig. 5, we present only the results obtained with Δ*inlC2DE*. Δ*inlC2DE* and Δ*inlF* entered in the same way as the parental wild-type strain into all the cell lines tested, indicating that *inlC2*, *inlD*, *inlE*, and *inlF* are not required for the entry of *L. monocytogenes* into these cells.

Interestingly, we found that InlB is required for entry into mouse fibroblasts. In the S180 fibroblasts, the Δ*inlB* and Δ*inlAB* mutants were approximately fivefold less invasive than the wild-type strain or the Δ*inlA* strain, indicating that *inlB* plays a significant role in fibroblast cell invasion. This result was strengthened by using another murine fibroblast cell line named L2. In these cells, Δ*inlB* and Δ*inlAB* mutants were 20- to 100-fold less invasive than the wild-type and Δ*inlA* strains were. Complementation of the mutant Δ*inlAB* with a plasmid carrying *inlB* restored full invasiveness into L2 fibroblasts (data not shown).

Since E-cadherin, the cellular receptor for InlA (33), and internalin are both members of large protein families, it was tempting to hypothesize that different members of the internalin family might interact with different members of the cadherin family or that different Inl proteins might interact with one type of cadherin with more or less affinity. To address this point, we tested the different *inl* deletion mutants for entry into stably transfected S180 cells expressing either the chicken E-cadherin (S180L-CAM2) or the chicken N-cadherin (S180N-cad). No difference was found between the Δ*inlC2*, Δ*inlD*, Δ*inlE*, Δ*inlF*, and Δ*inlC2DE* mutants and the wild-type strain for entry into these two cadherin-transfected cell lines (data not shown). As expected from the results obtained with the nontransfected S180 cells (Fig. 5), the Δ*inlB* and Δ*inlAB* mutants were fivefold less invasive than was the wild-type strain in S180N-cad whereas in S180L-CAM2, where the *inlA*-dependent pathway plays a major role, the role of *inlB* was apparently masked (data not shown).

Expression of Inl proteins. The absence of a phenotype for mutations in *inlC2*, *inlD*, *inlE*, and *inlF* raised the possibility that these genes were not expressed under our standard growth conditions. RNA dot blot analysis indicated that these genes were transcribed during growth in BHI broth although less efficiently than the *inlAB* operon was (Fig. 6). As previously published (12), the *inlAB* operon is maximally expressed during the exponential phase of growth in BHI medium at 37°C. In contrast, expression of *inlC2*, *inlD*, *inlE*, and *inlF* was maximal during the early exponential phase of growth in BHI medium at 37°C. In summary, *inlC2*, *inlD*, *inlE*, and *inlF* are expressed at the transcriptional level early and transiently during bacterial growth in BHI medium.

Role in virulence. The virulence of the *inl* mutants was evaluated in the mouse model. We decided to determine the LD_{50}s of the parental strain EGD and two isogenic mutants, Δ*inlAB* and Δ*inlA–F*, in susceptible BALB/c mice after i. v. inoculation. Our reasoning was that if a significant difference was found between *inlAB* and *inlA–F* deletion mutants, we could then determine the LD_{50} of each single isogenic mutant individually. The LD_{50}s for EGD, Δ*inlAB*, and Δ*inlA–F* were $10^{4.1}$, 10^5, and 10^5, respectively suggesting that *inlC2*, *inlD*,

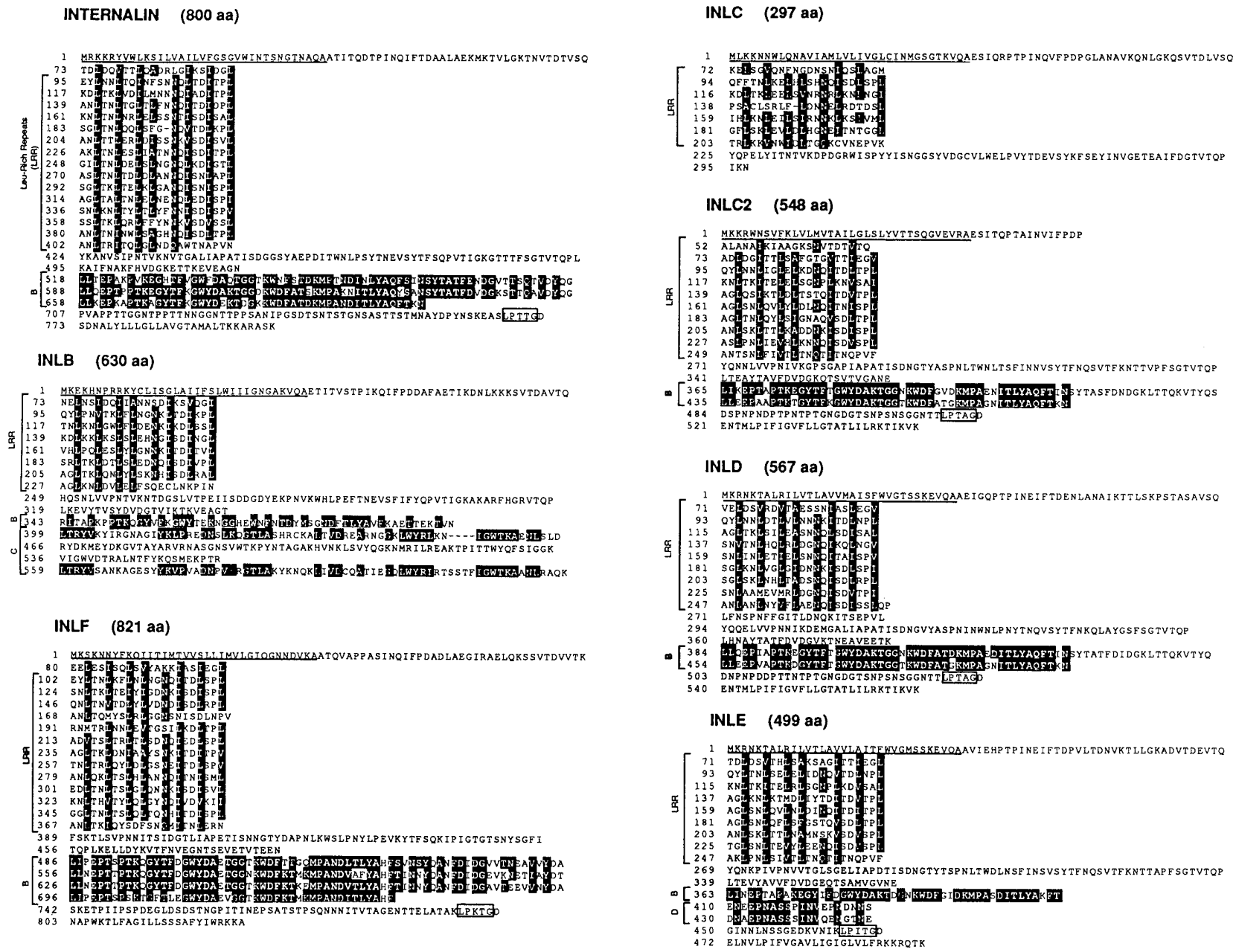

FIG. 3. Principal features of the internalin family. The amino acid (aa) sequences of all presently identified Inl proteins in *L. monocytogenes* EGD are presented. The signal sequences are underlined, and the different regions of repeats are outlined. The consensus pentapeptide LPXTG at the C-terminal end is boxed.

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InlA    424 YKANVSIPNTVKNVTGALIAPATISDGGSYAEPDITWNLPSYTNEVSYTF
InlB    249 .QSNLVVPNTVKNTDGSLVTPEIISDDGDYEKPNVKWHLPEFTNEVSFIF
InlC    225 YQPEY.ITNTVKDPDGRWISPYYISNGGSYVDGCVLWELPVYTDEVSYKF
InlC2   271 YQNNLVVPNIVKGPSGAPIAPATISDNGTYASPNLTWNLTSFINNVSYTF
InlD    294 YQQELVVPNNIKDEMGALIAPATISDNGVYASPNINWNLPNYTNQVSYTF
InlE    269 .QNKPIVPNVVTGLSGELIAPDTISDNGTYTSPNLTWDLNSFINSVSYTF
InlF    383 FSKTLSVPNNITSIDGTLIAPETISNNGTYDAPNLKWSLPNYLPEVKYTF

InlA    474 SQPVTIGKGTTTFSGTVTQPLKAT..FNAKFHVDGKETTKEVEAG..
InlB    298 YQPVTIGKAKARFHGRVTQPLKEV..YTVSYDVDGTVIKTKVEAG..
InlC    274 SEYINVGETEAIFDGTVTQPIKN.....................
InlC2   321 NQSVTFKNTTVPFSGTVTQPLTEA..YTAVFDVDGKQTSVTVGANE.
InlD    344 NKQLAY....GSFSGTVTQPLHNA..YTATFDVDGVKTNEAVEETK.
InlE    318 NQSVTFKNTTAPFSGTVTQPLTEV..YAVVFDVDGEQTSAMVGVNE.
InlF    433 SQKIPIGTGTSNYSGFITQPLKELLDYKVTFNVEGNTSEVETVTEEN
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FIG. 4. Multiple alignment of the interrepeat sequences of Inl proteins given in the single-letter amino acid code. Numbers on the left indicate positions in the amino acid sequence. Black boxes indicate amino acid identities, and grey boxes show similarities (Clustal W multiple-alignment program). Gaps are represented by dots.

inlE, and *inlF* are not essential for virulence. To evaluate more precisely the contribution of *inl* genes to listerial pathogenicity, bacterial counts were measured in liver and spleen 3 days after i.v. injection of approximately 10^4 bacteria. In a previous study, we observed a 10-fold reduction in bacterial counts in the liver after oral inoculation of Δ*inlAB* compared to the parental strain EGD. Results with the new *inl* mutants are shown in Fig. 7, exp 1. We did not detect any differences between the *inl* mutants and the parental strain EGD. Curiously, the Δ*inlA–E* and Δ*inlA–F* mutants did not show a decrease in bacterial counts in the liver that had been observed with the Δ*inlAB* mutant. In another independent experiment (Fig. 7, exp 2), bacterial counts were measured in the liver 3 days after i.v. injection of EGD, Δ*inlAB*, and Δ*inlA–F*. As previously described (11, 16), the Δ*inlAB* mutant was affected (0.5 log unit) whereas the Δ*inlA–F* mutant behaved essentially like the parental strain EGD. We propose two hypotheses to explain this unexpected result. One is that Δ*inlA–E* and Δ*inlA–F* grow more rapidly than EGD or Δ*inlAB*, which is apparently the case in liquid bacterial broth as well as in solid media. The second hypothesis is that Δ*inlA–E* and Δ*inlA–F* are more resistant to killing by Kupffer cells than is Δ*inlAB*. However, some differences were found between the Δ*inlA–E* and Δ*inlA–F* strains with respect to other isogenic strains, such as absence of mannosidase activity and a lower sensitivity to gentamicin. Therefore, we cannot exclude the possibility of secondary compensatory mutations in these strains.

DISCUSSION

In this paper, we report the identification of four genes belonging to the internalin gene family. InlA and InlB were the

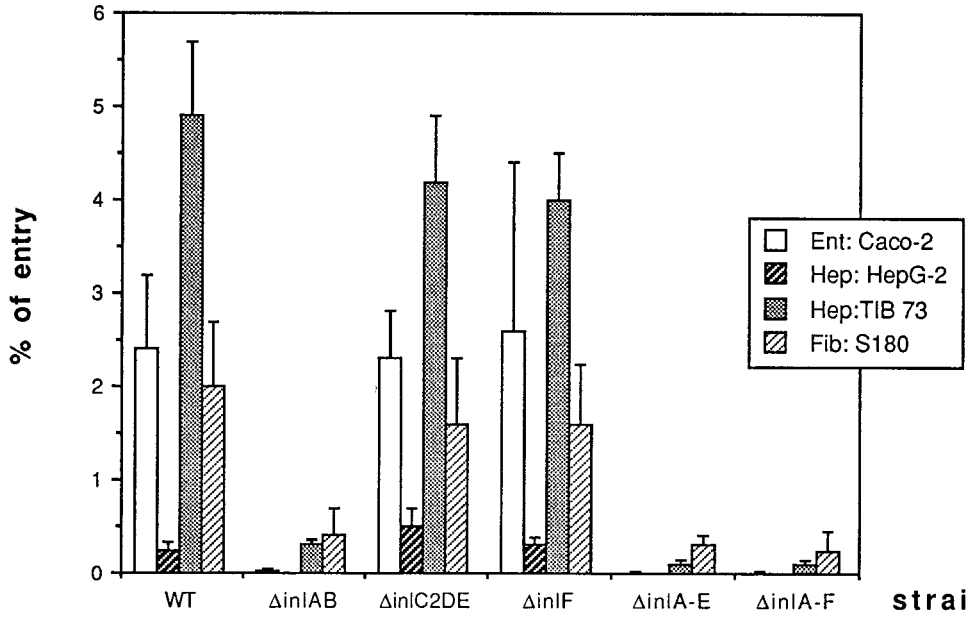

FIG. 5. Entry of *L. monocytogenes* isogenic strains carrying deletions in the *inl* loci into various cell lines. This graph summarizes three to five independent experiments (gentamicin survival assay) performed by the standard method with a multiplicity of infection of 50 to 100 bacteria per cell, a 1-h infection period, and 2 h of further incubation with gentamicin. Values along the vertical axis represent the percentages of entry (the number of bacteria that survived incubation in the presence of gentamicin as a percentage of the number of inoculated bacteria) in the different cell lines. The cell lines used were Caco-2, a human enterocyte-like cell line; HepG-2, a human hepatocytic cell line; TIB 73, a murine hepatocytic cell line; and S180, a murine fibroblastic cell line.

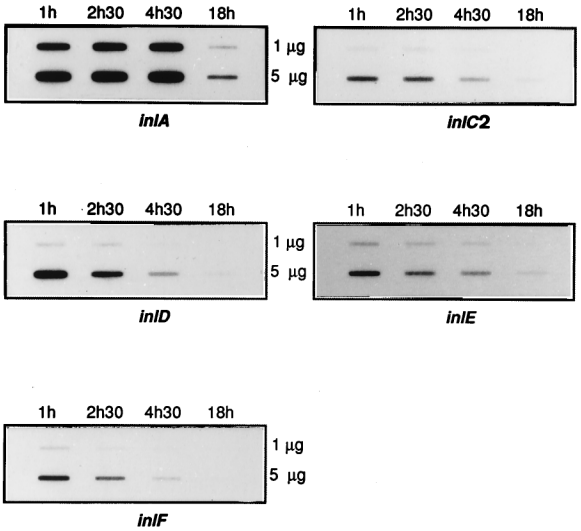

FIG. 6. Transcription of the *inl* genes during growth in bacterial medium. For RNA slot-blot analysis, 1 or 5 µg of RNA isolated during various time points along the growth curve (1h, early-exponential phase; 2h30, exponential phase; 4h30, late-exponential phase; 18h, stationary phase) at 37°C from strain EGD was hybridized with an *inlA* probe (a 1,252-bp fragment produced by PCR with oligonucleotides ppe2-16 [5′ CAAGTAATAAGGTGTCAG 3′] and ppe2-24 [5′ TGGGCATATAAGGTGATG 3′]), an *inlC2* probe (a 1,013-bp *Eco*RI-*Hin*dIII fragment isolated from pPE-7 [Fig. 2]), an *inlD* probe (a 721-bp *Pst*I- *Hin*dIII fragment isolated from pPE-7 [Fig. 2]), an *inlE* probe (a 1,224-bp fragment produced by PCR with sense [5′ CGAAATCCTACTACGCTA 3′] and antisense [5′ GTTCTCAGTGAACTTAGC 3′] oligonucleotides), or an *inlF* probe (a 1.2-kb *Xba*I fragment isolated from plasmid p84).

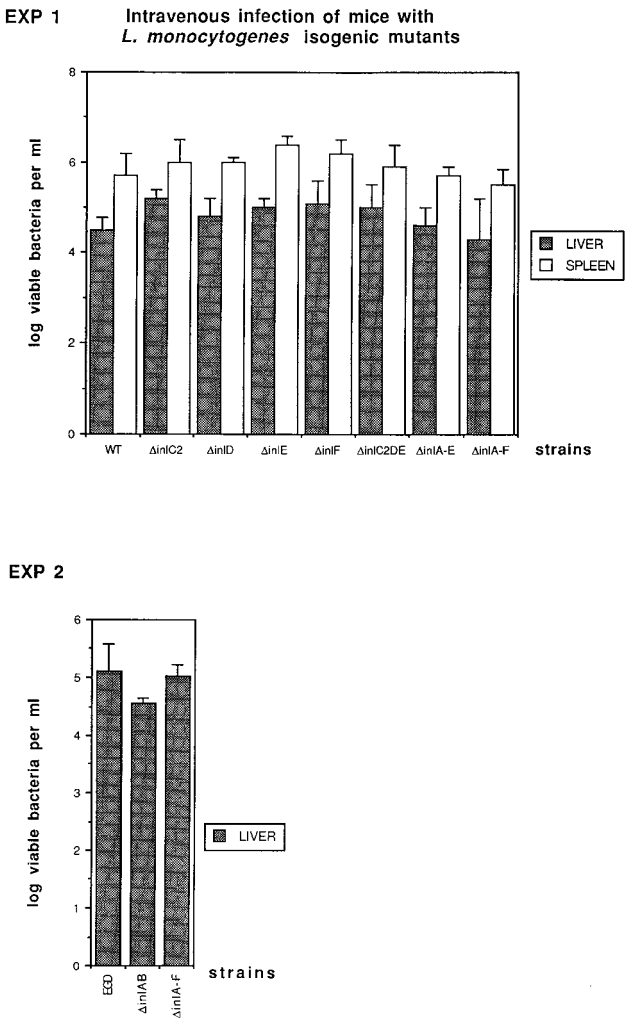

FIG. 7. Viable bacterial counts in organ homogenates of mice infected i.v. with *L. monocytogenes* isogenic mutants. In one experiment (EXP 1), groups of four mice were infected by i.v. injection of ca. 7×10^3 bacteria. Bacterial counts in the liver and spleen of BALB/c mice 3 days after the i.v. challenges are presented, with each point representing the mean ± standard deviation. In another independent experiment (EXP 2), groups of four mice were infected by i.v. injection of ca. 2.5×10^3 bacteria, and the bacterial counts in the liver of mice was determined 3 days after infection.

first members of this family to be described (15). Both are surface proteins that play a crucial roles in the invasion of mammalian cells (for a review, see reference 13). InlA is needed for entry into the enterocyte-like epithelial cell line Caco-2, while InlB is required for entry into other cell lines including hepatocytes (TIB73 and HepG-2), and some epithelial cells (CHO, HeLa, and Vero). Recently, a third member named IrpA (27) or InlC (14) was identified during a search for additional PrfA-regulated genes. InlC is an extracellular 30-kDa protein of unknown function (14). The *inl* genes reported in this work, namely, *inlC2*, *inlD*, *inlE*, and *inlF*, encode proteins of 548, 567, 499, and 821 amino acids, respectively, which share the principal features of internalin (InlA), i.e., a signal sequence, two regions of repeats (one of which is rich in leucine residues), and a putative cell wall anchor at the C terminus. The only surface protein of this family that does not possess this cell wall-anchoring motif is InlB. Interestingly, we have recently identified a new surface protein of *L. monocytogenes* of about 98 kDa named Ami which contains a region of repeats homologous to the C repeats of InlB (Fig. 3), and we have evidence that the C-terminal region of InlB and Ami represents a novel motif necessary for surface attachment of these proteins (5a).

Comparison of *inl* proteins with proteins from various databases did not reveal any striking similarity that might suggest possible functions. Inl proteins belong to the superfamily of leucine-rich repeat (LRR) proteins. LRRs are short sequence motifs (20 to 29 amino acids) present in over 60 different proteins, mostly eukaryotic, most of which appear to be involved in protein-protein interactions (20). Interestingly, proteins from two other pathogenic bacteria contain the LRR motif, YopM of *Yersinia pestis* and IpaH of *Shigella flexneri*. YopM inhibits platelet aggregation by binding thrombin, which normally binds to the GPIb receptor, a LRR protein (25, 26). The relevance of this observation, however, has recently been questioned, since YopM can be delivered to the interior of eukaryotic cells (5). The function of IpaH is unknown, but it is an immunodominant antigen during shigellosis. Multiple copies of *ipaH* are present on the chromosome and virulence plasmid of *S. flexneri* (7, 18), suggesting the existence of another family of LRR proteins in a bacterial pathogen whose intracellular lifestyle is closely related to that of *L. monocytogenes.*

Examples of multigene families flourish in the world of eukaryotes. However, few examples are documented in bacteria. The pathogenic *Neisseria* species (*Neisseria gonorrhoeae* and *N. meningitidis*) provide a paradigm of microorganisms containing gene families. In *N. gonorrhoeae* MS11, a total of 11 variant *opa* genes have been characterized, all of which can be turned on and off independently (2, 3). These variable proteins are involved in a number of adherence functions such as interactions

with human leukocytes and epithelial cells and intergonococcal adhesion. Depending on the *opa* expressed, gonococci are capable of invading cultured epithelial cells (Opa_{50}) or of interacting with human polymorphonuclear neutrophils (other Opa) (24).

While it is clear that *inlA* and *inlB* are needed for host cell invasion, the separate role of each gene is the subject of some controversy. We have found that *inlA* is required for entry into Caco-2 cells while *inlB* is required for entry into other cell lines including some epithelial cells (HeLa, CHO, and Vero) and some cultured hepatocytes (HepG-2 and ATCC TIB73). *inlA* acts synergistically with *inlB* for entry into HepG-2 cells, whereas it is not required for entry into the other *inlB*-permissive cell lines, TIB73, HeLa, Vero, and CHO (11, 19). Our results are not in agreement, however, with those obtained by Lingnau et al., who showed that both *inlA* and *inlB* are required for entry into various epithelial cell lines (Caco-2, HeLa, Ptk2, and HEp-2) (28). The discrepancy between the results of our experiments and those of Lingnau et al. may be due to strain differences, a hypothesis that is currently being tested. Concerning the role of *inlB* in hepatocyte invasion, our results have been independently confirmed by Gaillard et al. for the ATCC TIB73 cell line (16) and by Gregory et al. for a primary culture of murine hepatocytes where expression of *inlB*, but not *inlA*, is required for maximal uptake of *L. monocytogenes* (17). In the present study, we have shown that *inlB* is also involved in the invasion of murine L2 and S180 fibroblasts. These results are also not in agreement with those of Lingnau et al., who showed that entry into WI-38 and HEL 299 fibroblasts was independent of the expression of either *inlA* or *inlB* (28). However, it should be noted that in WI-38 and HEL 299 cells, the *L. monocytogenes* wild-type strain enters with a very low efficiency while in the S180 and L2 cells that we used, the level of invasion of wild-type *L. monocytogenes* was comparable to the level observed in epithelial cells.

To assess the role of *inlC2*, *inlD*, *inlE*, and *inlF*, isogenic chromosomal mutants of *L. monocytogenes* EGD carrying a deletion in each gene (Δ*inlC2*, Δ*inlD*, Δ*inlE*, and Δ*inlF*) or in several genes (Δ*inlC2DE*, Δ*inlA–E*, and Δ*inlA–F*) were constructed and tested for entry into various nonphagocytic cell types, including epithelial, fibroblastic, and hepatocytic cell lines. Our results indicate that the hypothesis that the internalin family repertoire contributes to the tropism exhibited by *L. monocytogenes* in cultured cells—in particular, that it could account for the *inlAB*-independent entry—is too restrictive. Indeed, *inlA* and *inlB* do play roles in entry in different cell types, but as of now, we have been unable to detect a role for *inlC2*, *inlD*, *inlE*, or *inlF*.

In addition, in phagocytic cells, in particular in the murine macrophage cell line J774, uptake and intracellular multiplication of *inl* mutants were not affected (data not shown). The capability of these mutants to spread from cell to cell was also unimpaired as assayed by plaque formation on L2 monolayers (data not shown). Nevertheless, we have shown that *inlC2*, *inlD*, *inlE*, and *inlF* are not pseudogenes, since they can be transcribed during growth in bacterial medium at 37°C. Whether these genes are differentially transcribed within infected cells is under investigation. Preliminary evidence based on Western blot analysis with affinity-purified antibodies against the InlC2-histidine tag and InlE-histidine tag fusion proteins shows that InlC2 but not InlE is detectably expressed during growth in bacterial medium (our unpublished data). Finally, the expression of Inl proteins was also approached by an indirect technique. We reasoned that if internalins are major surface components expressed in infected hosts, antibodies against internalins would be detected during human listeriosis. It is interesting that in the sera of five listeriosis patients, antibodies against InlA, InlB, InlC2, and InlE were not detected whereas antibodies against listeriolysin O were detected in all cases (1a). These results suggested either that these Inl proteins are not expressed in vivo or that they are not immunogenic. We favor the second hypothesis for two reasons: (i) *inlA* can be detected on the bacterial surface in infected cells by immunofluorescence, and (ii) *inlC*, a member of this family, is transcribed in phagocytic J774 cells in a late stage of infection when all bacteria have reached the cytoplasm of host cells and are actively spreading (14).

The *inlAB* and *inlC* deletion mutants exhibited a significantly reduced virulence when injected i.v. in mice (14, 16). The LD_{50}s of these mutants were increased by 1 and 1.5 log units, respectively, by the i.v. route. The other deletion mutants, namely, *inlC2*, *inlD*, *inlE*, and *inlF*, did not show a decrease in virulence in the murine model. Therefore, the role of *inlC2*, *inlD*, *inlE*, and *inlF* in listeriosis, if any, remains to be established. It is still possible that these genes are involved in the passage of *L. monocytogenes* to the brain or the placenta, a situation which could not be tested in the mouse model system and with the type of infections that we performed.

These results lead to the conclusion that there are still other invasion genes in *L. monocytogenes* which remain to be discovered.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

There recently appeared a paper by Domann et al. (E. Domann, S. Zechel, A. Lingnau, T. Hain, A. Darji, T. Nichterlein, J. Wehland, and T. Chakraborty, Infect. Immun. **65:** 101–109, 1997) which reported on the complete sequencing and characterization of the *irpA* gene in *L. monocytogenes* EGD. Amino acid sequence alignment of IrpA with InlC indicates the identity of the two proteins except for two amino acids.

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