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Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* Genes, Whose Products Mediate Bacterial Entry into Tissue Culture Cell Lines, by PrfA-Dependent and -Independent Mechanisms

ANDREAS LINGNAU,[1] EUGEN DOMANN,[2] MARTINA HUDEL,[2] MAREN BOCK,[1] THOMAS NICHTERLEIN,[3] JÜRGEN WEHLAND,[1] AND TRINAD CHAKRABORTY[2]*

Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig,[1] Institut für Medizinische Mikrobiologie, Justus-Liebig Universität Giessen, D-35392 Giessen,[2] and Institut für Medizinische Mikrobiologie und Hygiene, Klinikum Mannheim, D-68167 Mannheim,[3] Germany

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Internalization of *Listeria monocytogenes* into nonphagocytic cell lines in vitro requires the products of the *inlAB* locus (J.-L. Gaillard, P. Berche, C. Frehel, E. Gouin, and P. Cossart, Cell 65:1127–1141, 1991). By generating isogenic mutants with a chromosomal in-frame deletion in either *inlA* or *inlB*, we have identified InlA and InlB as surface-bound proteins of *L. monocytogenes* with molecular weights of 88,000 and 65,000, respectively. These results were obtained with monoclonal antibodies raised against either protein and corroborated by N-terminal end sequencing of InlA and InlB. By immunoblot analysis, the production of both polypeptides was found to be strongly dependent on growth temperature and, particularly for InlB, on the presence of the PrfA regulator protein. Expression of InlA was not strictly dependent on the presence of the PrfA regulator protein. Transcription analysis of the *inlAB* locus revealed that the *inlA* gene was transcribed by several promoters, of which only one is PrfA dependent. This PrfA-dependent *inlA* promoter, which contains two base substitutions within its putative PrfA DNA-binding palindrome, is responsible for transcription of both *inlA* and *inlB* genes. A hitherto unrecognized promoter located 51 bp upstream of the GTG start codon of the *inlB* gene was also detected. Hence, *inlA* and *inlB* are transcribed both individually and in an operon by PrfA-dependent and -independent mechanisms. Tissue culture invasion assays employing various epithelial cell lines demonstrated that both InlA and InlB are required for invasion. In vivo studies using the mouse infection model revealed that both internalin mutants were attenuated for virulence.

Listeria monocytogenes is a gram-positive bacterium responsible for severe infections in humans and animals (12, 17). The entry into the host normally occurs in the gut after ingestion of listeria-contaminated food. The precise locus of bacterial invasion is not known, but during an acute infection, many tissues are infected, demonstrating the ability of these bacteria to invade numerous eucaryotic cells in different tissues (16). Elegant electron microscopic studies have demonstrated penetration of *L. monocytogenes* into epithelial cells of both the cornea and the intestine in vivo (32, 33). Tissue culture assays of bacterial invasion revealed that *L. monocytogenes* is capable of penetrating various cell types, including hepatocytes and fibroblasts (6).

Adherence and uptake of *L. monocytogenes* on and into macrophages require involvement of complement components C1q and C3b via their receptors present on the eucaryotic cells (1). Also, macrophage scavenger receptors bind to *L. monocytogenes* by recognizing lipoteichoic acids present on the bacterial surface (11). Uptake of listeriae by nonphagocytic cells is also dependent on rearrangements of the cortical actin cytoskeleton, but both the mechanism of adherence and internalization and the receptors on the host cells participating in this uptake are poorly understood.

By transposon-induced mutagenesis, noninvasive mutants of *L. monocytogenes* have been isolated (15). The point of insertion of the transposon in these noninvasive mutants was localized in a region preceding two highly homologous genes, designated *inlA* and *inlB*, where its presence exerted a polar effect on the transcription of both genes. The *inlAB* operon is organized by two transcripts: a larger 5,000-nucleotide (nt) transcript that spans *inlAB* and a smaller 2,900-nt transcript that covers only *inlA* (10, 15). Evidence that transcription of the *inlAB* locus is both temperature dependent and strictly dependent on the PrfA virulence regulator has been presented (10). Nevertheless, contradictory claims have been made with regard to PrfA-mediated regulation of the *inlAB* locus (37). Transformation of a noninvasive *Listeria innocua* strain with a plasmid harboring the *inlA* gene alone rendered this strain invasive for the enterocyte-like cell line CaCo-2 (15). However, this strain was fourfold less invasive compared with the wild-type *L. monocytogenes* strain, despite increased *inlA* gene dosage due to the high copy number of the plasmid. Finally, a polyclonal antiserum raised against an *Escherichia coli* maltose-binding fusion protein, MalE::InlA, allowed the identification of InlA as an 88-kDa polypeptide that is present in the cell wall and supernatants of *L. monocytogenes* (10, 15). The product of the *inlB* gene remains to be identified.

In this communication, we report on the unequivocal identification of the *inlA* and *inlB* gene products and show that they are present in cell wall and supernatant fractions of growing bacteria. By generating peptide antibodies and monoclonal antibodies (MAbs) specifically recognizing both proteins, we have assessed expression of either protein in cultures grown at different temperatures and in the presence and absence of the PrfA regulator. These data were further extended by analysis of RNA transcripts of either gene and determination of their relative start sites. We show that both *inlA* and *inlB* are essential for entry into many, but not all, nonphagocytic cell types

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie, Frankfurterstr. 107, D-35392 Giessen, Germany. Phone: 0049-641-702-7388. Fax: 0049-641-702-7387.

and that these mutants are attenuated for virulence in a mouse infection model.

MATERIALS AND METHODS

Bacterial strains, cultivation, and reagents. The wild-type *L. monocytogenes* serotype 1/2a strain EGD, its isogenic derivatives EGD*prfA1* and EGD*actA1*, and EGDpERL3 50-1 have been described previously (4, 8, 25). Construction of the *inlA* and *inlB* mutants is detailed below.

Listeria strains were grown in brain heart infusion (BHI) (Difco, Detroit, Mich.) at 37 or 20°C with continuous shaking. Sodium dodecyl sulfate (SDS) extracts and trichloroacetic acid-precipitated supernatants were derived from cultures that had optical growth densities of between 0.9 and 1.1 at 600 nm. Where required, the culture medium was supplemented with 5 μg of erythromycin per ml. All reagents were purchased from Sigma, Deisenhofen, Germany, unless indicated otherwise.

Construction of the chromosomal in-frame deletion mutations *inlA2* and *inlB2*. Deletion mutations in the internalin locus were generated by PCR employing specific primers with incorporated restriction sites to introduce an in-frame deletion in either the *inlA* or *inlB* gene. To create the *inlA* mutation, the oligonucleotide pair A (5′-ACTTCATCTGCTGCAAGGCTTAAAAGCA-3′) and B (5′-AACTTGGTCTGGATCCGTTTGCGAGAC-3′), which harbored a *Bam*HI restriction site, were used to amplify a 1,245-bp DNA fragment from the 5′ region of *inlA* (positions 520 to 1766 of the published sequence), encoding the first 78 N-terminal amino acid residues (10, 15). The oligonucleotide pair C (5′-ATGAACGCTTAGGATCCTTATAATTCA-3′), harboring a *Bam*HI restriction site, and D (5′-TACTTTACCACGCATGCTAAATTGATA-3′) served to amplify an 1,835-bp DNA fragment from the 3′ region encoding the last 47 amino acids of InlA (positions 3792 to 5627). The two PCR products were digested with *Bam*HI and used in a ligation reaction. The ligation product harboring the deletion was selectively amplified with the oligonucleotide pair A and D. The resulting PCR product was digested with the restriction endonucleases *Kpn*I and *Xba*I and cloned into the temperature-sensitive shuttle vector pAUL-A (4). To create the *inlB* mutation, the oligonucleotide pair E (5′-CTA AAAGAACCAAAGGTACCAACGAAAGCCGGA-3′), harboring a *Kpn*I restriction site, and F (5′-CACGGTGATAGGATCCGCTTGTACTTTCGC-3′), incorporating a *Bam*HI site, were used to amplify a 632-bp fragment from the 5′ region of *inlB* (positions 3507 to 4139), encoding the first 40 N-terminal amino acid residues. The oligonucleotide pair G (5′-GCAGCTAATTTAAGGGATC CGAAATAACTGAAAAAGACCT-3′), carrying a *Bam*HI restriction site, and H (5′-GTCATTAAATCTAGACGATTCCATACA-3′), with an *Xba*I restriction site, served to amplify a 733-bp DNA fragment at the 3′ region (positions 5866 to 6618) encoding the last eight C-terminal amino acids of InlB. Following digestion of the two restriction fragments with the restriction endonuclease *Bam*HI, they were ligated and the ligation product harboring the deletion was selectively amplified with the oligonucleotide pair E and H. The resulting PCR product was digested with the restriction endonucleases *Kpn*I and *Xba*I and cloned into the temperature-sensitive shuttle vector pAUL-A (4).

Plasmids pAUL-*inlA2* and pAUL-*inlB2* were transformed into EGD via electroporation, and the recombinants were isolated at a growth temperature of 28°C from BHI agar plates supplemented with 5 μg of erythromycin ml^{-1}. The recombinant clones were screened by plasmid DNA extraction from a single colony and then digested with appropriate restriction enzymes. For integration events, a single colony of the respective *L. monocytogenes* recombinant was incubated on BHI agar plates with 5 μg of erythromycin ml^{-1} at 42°C for 2 days. This procedure was repeated three times, and the presence of the integrated plasmid was confirmed by Southern blot analysis of chromosomal DNA (38). The meroploid intermediates contained both wild-type and deleted alleles. To obtain spontaneous excision of the integrated plasmids through intramolecular homologous recombination, the strains were incubated twice at 28°C and without erythromycin in BHI broth for 24 h each and then incubated at 42°C overnight, before being diluted and plated onto BHI agar plates. The bacterial colonies were transferred by replica plating onto BHI plates containing 5 μg of erythromycin ml^{-1}, and erythromycin-sensitive colonies were screened for the presence of the deletion by PCR with specific oligonucleotides flanking the respective gene. A shorter amplified PCR product was diagnostic for those strains with an allelic exchange of the deleted version for the wild-type allele on the chromosome. The appropriate gene deletions were confirmed by PCR sequencing of chromosomal DNA from mutants (data not shown) and further verified by immunoblotting with the appropriate specific antibody (see Fig. 2). DNA manipulations were performed as described in the work of Sambrook et al. (35).

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was done by the method of Laemmli with 7.5 and 10% polyacrylamide gels (23). The gels were stained by use of either Coomassie brilliant blue R-250 or a silver staining kit (Bio-Rad, Munich, Germany). Immunoblotting was performed by a semidry method, with Immobilon P membranes (Millipore, Eschborn, Germany). After incubation with horseradish peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany), the blots were reacted with chloronaphthol and hydrogen peroxide in phosphate-buffered saline (PBS) as substrates. A sensitive chemiluminescence-based immunoblot assay (ECL; Amersham Buchler, Braunschweig, Germany) was used, according to the instructions provided by the vendor.

Extraction of bacterial cell wall proteins and preparation of culture supernatants. Bacterial cultures were harvested by centrifugation and washed with PBS. Pellets were immediately resuspended in 2% SDS (wt/vol) in PBS and incubated for 15 min at 37°C with gentle shaking. The suspension was then centrifuged, and the supernatants were aliquoted and stored at −70°C.

To obtain defined bacterial culture supernatants, the BHI medium was prefiltered, with a membrane with a cutoff of 10,000 Da (Minitan; Millipore), before inoculation with the bacteria. Following growth of cultures, bacteria were removed by repeated centrifugation. Supernatant proteins were precipitated by addition of 10% trichloroacetic acid and allowed to stand at 4°C overnight. In some instances, supernatant fluids were further concentrated by ultrafiltration.

Additionally, some supernatants were precipitated with ammonium sulfate at 60% saturation (4°C, overnight). Pelleted fractions were dialyzed extensively against PBS and analyzed by SDS-PAGE and immunoblotting.

Amino acid sequence analysis. For sequence analysis, fractions of SDS extracts and ammonium sulfate-precipitated supernatants were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Problot; Applied Biosystems, Weiterstadt, Germany). The regions corresponding to the polypeptides of interest were excised following staining with Ponceau S and subjected to analysis on an Applied Biosystems gas phase Sequenator (model A470) equipped with an on-line phenylthiohydantoin amino acid analyzer.

Antibody production. (i) (MAbs). Female BALB/c mice were immunized subcutaneously with inactivated concentrated culture supernatants or SDS extract of *L. monocytogenes* EGDpERL3 50-1 broth cultures injected into both hind legs on days 17, 14, 10, 7, 4, and 1 before the fusion.

In the case of InlA MAbs [M160(IgG1) and L244(IgG1)], formalin-inactivated concentrated supernatants of EGDpERL3 50-1 were used for immunization. To raise MAbs against InlB [IC100(IgG1)], SDS extract of the same strain was used. The antigen was emulsified in Freund's complete adjuvant for the first injection. The following immunization was done with incomplete adjuvant followed by antigen preparation in PBS. Each time, approximately 5 μg of protein was injected in a volume of 20 μl. Three mice were sacrificed for each antigen preparation, the popliteal lymph nodes were removed, and the fusion was performed with myeloma cells (X63Ag8) as described previously (30, 31). Myeloma and hybridoma cells were grown in OPTIMEM (Gibco, Eggenstein, Germany) supplemented with 5% fetal calf serum, penicillin-streptomycin, and glutamine. Hybridoma selection was performed by adding azaserine-hypoxanthine to the culture medium.

For screening of hybridoma supernatants, concentrated listerial supernatants or SDS extracts were loaded onto slotless gels and tested after blotting with a miniblotting apparatus (Biometra, Göttingen, Germany).

Positive clones were subcloned once or twice by limiting dilution. Immunoglobulin subclasses of the MAbs were determined by using an isotyping kit (Medac, Hamburg, Germany).

(ii) Antipeptide antisera. Synthetic peptides were synthesized according to amino acid residues 127 to 146 (InlB-1), 269 to 285 (InlB-2), and 355 to 343 (InlB-3), predicted to occur within the putative InlB polypeptide (15), by using Millipore peptide synthesizer 9050. An N-terminal cysteine residue was added to allow cross-linking to ovalbumin with *m*-maleimidobenzoyl-*N*-hydroxy-sulfosuccinimidester (Pierce-Chemicals, BA oud Beijerland, The Netherlands). Antisera were then raised in rabbits by standard immunization procedures as described previously (8). The antisera of InlB-1 and InlB-3 were purified by cross-linking of the peptide to EAH Sepharose 4B (Pharmacia, Freiburg, Germany), and specific antibodies were eluted with 0.1 M acetate–0.5 M NaCl, pH 2.7.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated from growing cultures (optical density at 600 nm of ~0.8) of *L. monocytogenes* by the hot phenol extraction method. RNA analysis was performed by formaldehyde gel electrophoresis (4). RNA samples (20 μg) were electrophoresed in a 1.3% agarose gel, transferred onto nylon filters (Hybond N; Amersham), and hybridized with the appropriate radiolabeled fragments, under conditions as specified by the vendor (13). The probe used to detect the listeriolysin transcript has been described previously (4).

Primer extension studies. The synthetic oligonucleotides 5′-AGTAGCTA ATCTGTCTAC-3′ and 5′-GACACGGTGATAGTCTCCGCTTG-3′ located either 133 bp upstream of the *inlA* gene or within the *inlB* coding sequence, respectively, were used as primers and labeled at their 5′ ends. Primer extension analysis with recombinant virus reverse transcriptase (Superscript; Gibco) was performed as described previously with a total of 40 μg of RNA. Dideoxy sequencing reactions (36), with the same primer and an appropriate plasmid DNA template, were run in parallel to allow determination of the endpoints of the extension product.

In vitro invasion assay. PtK2 cells (ATCC CCL 56) were cultured in minimum essential medium (Gibco) supplemented with 10% fetal calf serum, glutamine, sodium phosphate, and nonessential amino acids in the absence of antibiotics. HeLa (ATCC CCL 2), Henle (ATCC CCL 6), CaCo-2 (ATCC HTB 37), HEL 299 (ATCC CCL 137), A549 (ATCC CCL 185). HEp-2 (ATCC CCL 23), and WI-38 (ATCC CCL 75) cells were cultivated in Dulbecco's minimum essential medium (Gibco) supplemented with 10% fetal calf serum and glutamine. Bacteria grown as described above were washed twice in sterile PBS before addition to the respective tissue culture cell line in a 24-well microplate. One hundred

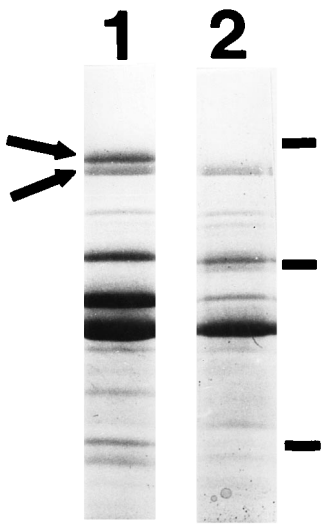

FIG. 1. Analysis of culture supernatants of *L. monocytogenes* EGDpERL3 50-1 (lane 1) and EGD*actA1* (lane 2) by SDS-PAGE. The supernatants were precipitated with ammonium sulfate (60% saturation), and the gel (10%) was stained with Coomassie blue. The upper arrow marks the position of ActA, and the lower marks that of the 88-kDa InlA polypeptide. Molecular mass markers from top to bottom are 97, 66, and 45 kDa.

microliters of the bacterial suspension (10^6 bacteria) were added to approximately 10^5 tissue culture cells per well in a final volume of 500 μl. After 1 h of incubation to allow entry of bacteria, cells were washed once with sterile PBS before the addition of supplemented Dulbecco's minimum essential medium or minimum essential medium containing gentamicin at 25 μg/ml to kill extracellular bacteria. Samples were incubated for a further hour before being washed twice with PBS and then having 400 μl of cold distilled water added. After the addition of 100 μl of fivefold PBS, serial dilutions of cells were plated onto BHI agar plates. Colonies were counted after overnight incubation at 37°C.

In vivo virulence assay. This assay was performed as described previously (21). Mice (Naval Medical Research Institute) were infected intraperitoneally with approximately 10^4 bacteria in 70 mM phosphate buffer (pH 7.2) containing 0.23 M NaCl. Bacterial numbers were determined in spleens and livers at days 1, 3, and 6. Organs were homogenized, and then 0.1-ml portions of appropriate dilutions were plated as described above. Results are expressed as mean values and standard deviations of tests for three mice.

RESULTS

Identification of InlA in culture filtrates. When analyzing the culture supernatant of the EGD strain complemented with additional copies of the *prfA* gene on plasmid pERL3 50-1, we noted that expression was induced for a number of polypeptides, among them two polypeptides, of 90,000 and 88,000 Da, that were particularly enriched when culture supernatants were precipitated with 60% ammonium sulfate (Fig. 1, lane 1). Immunoblotting with specific polyclonal antibodies to ActA (31) showed that the 90-kDa polypeptide was ActA: no cross-reaction was seen with the 88-kDa polypeptide (data not shown). This polypeptide was also present in the ammonium-sulfate precipitate of the EGD*actA1* strain, which produces a truncated 50-kDa ActA polypeptide (Fig. 1, lane 2). In order to identify this novel polypeptide, the proteins of the ammonium-sulfate-precipitated supernatant of the *actA1* mutant were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. The 88-kDa polypeptide was then subjected to N-terminal protein sequencing. A search of protein data banks with the resulting amino acid sequence ATIT QDT showed that it was unique. Surprisingly, the corresponding nucleotide sequence exactly matched a sequence in the noncoding 5′ region of the *inlA* sequence recently published by Gaillard et al. (15). Sequencing of a PCR-amplified fragment of the 5′ region of the *inlA* gene revealed an additional G (TC TAGCGGAAA) between positions 1694 and 1695 of the published sequence (10), resulting in an open reading frame with 56 additional N-terminal amino acid residues for InlA. Our N-terminal protein sequence data for InlA confirmed the position of the previously predicted signal peptide cleavage site (9, 10, 15).

Construction of chromosomal in-frame deletions in *inlA* and *inlB* and identification of InlA and InlB. The region corresponding to the *inlAB* locus was cloned from *L. monocytogenes* EGD with an oligonucleotide probe derived from the N-terminal protein sequence obtained above (pLMH1 [20]). In order to identify the products of the *inlA* and *inlB* genes, we constructed isogenic strains with chromosomal in-frame deletions in either gene (see Materials and Methods).

MAbs raised against a number of major polypeptides detected in the supernatant fluids of *L. monocytogenes* EGDpERL3 50-1 (30) were screened for their ability to recognize the 88-kDa polypeptide in SDS extracts and culture supernatants of (i) the same strain and (ii) the isogenic *actA1* mutant (31). We thus identified several MAbs specific for the InlA polypeptide of *L. monocytogenes* EGD. An immunoblot analysis of the wild-type strain and the *inlA2* mutant performed with the InlA-specific MAb L244(IgG1) is depicted in Fig. 2a and b. This MAb recognizes the 88-kDa InlA polypeptide present in SDS extracts of the EGD wild-type strain but not in extracts of the isogenic *inlA2* mutant (cf. lanes 1 and 2 with 3 and 4). Increased amounts of InlA were detected in the *inlB2* mutant. This is likely to be due to increased stability of *inlA* message, since *inlB* deletion in this strain now fuses the terminator of the *inlAB* operon to the *inlA* gene.

The gene product encoded by the *inlB* gene (15) has not yet been identified. Immunoblotting analysis with polyclonal anti-

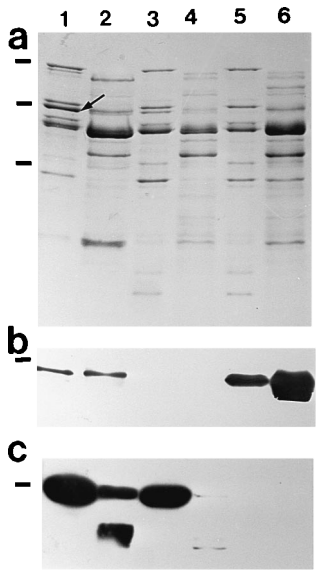

FIG. 2. Identification of the InlA and InlB polypeptides in cell wall extracts and culture supernatants of *L. monocytogenes* EGD and its isogenic *inlA* and *inlB* mutants analyzed by SDS-PAGE and immunoblotting. The figure shows detection of InlA and InlB in SDS extracts (lanes 1, 3, and 5) and supernatants (lanes 2, 4, and 6) of EGD (lanes 1 and 2) and its isogenic *inlA2* (lanes 3 and 4) and *inlB2* (lanes 5 and 6) mutants. (a) The Coomassie blue-stained SDS gel. (b and c) Immunoblots processed with InlA-specific MAb L244 (b) and InlB-specific MAb IC100 (c). The position of InlB in SDS extracts is indicated with an arrow in panel a. Molecular mass markers from top to bottom are 97, 66, and 45 kDa (a); 97 kDa (b); and 66 kDa (c).

bodies against three different peptides that were synthesized according to the predicted primary amino acid sequence of the InlB polypeptide showed that they cross-reacted with a 65-kDa polypeptide (data not shown). This polypeptide was readily detectable by Coomassie blue staining in SDS extracts of the EGD wild-type strain and its *inlA2* mutant but not in the *inlB2* mutant (Fig. 2a, cf. lanes 1, 3, and 5). N-terminal sequencing of the 65-kDa polypeptide gave the amino acid sequence ETIT VPTPIKQIF, which was identical to the predicted InlB sequence starting at amino acid residue 36 (15) and confirmed the presence of a signal peptide.

Since InlB was found to be associated with SDS extracts from whole cells, MAbs were raised against this fraction derived from EGDpERL3 50-1, a strain overproducing PrfA-regulated polypeptides (25, 37). By comparing the hybridoma supernatants on immunoblots with SDS extracts of the EGD strain and its isogenic *inlA2* and *inlB2* mutant strains, we were able to identify several specific InlB MAbs. The reaction obtained with MAb IC100(IgG1) is depicted in Fig. 2a and c (cf. lanes 1 to 4 with 5 and 6). Like the peptide antibodies, this MAb recognizes the same 65-kDa polypeptide in the wild-type strain.

The 88-kDa InlA polypeptide was present in SDS extracts and supernatants of both EGD and the *inlB2* mutant (Fig. 2a and b, lanes 1, 2, 5, and 6). In the wild-type strain, the InlB polypeptide was detected in both SDS extracts and supernatant fluids but in highly reduced amounts in these fractions of the *inlA2* mutant. Unlike InlA, InlB detected in the culture supernatants was proteolytically cleaved to give distinct degradation products (Fig. 2c, lanes 1 and 2).

Production of InlA and InlB in wild-type EGD, its isogenic *prfA* mutant, and EGDpERL3 50-1 harboring multiple copies of the *prfA* gene, at various growth temperatures. Contradictory results have been reported for the regulation of expression of the InlA and InlB polypeptides in pathogenic *L. monocytogenes* (10, 37). The availability of MAbs permitted a careful analysis of the expression of either polypeptide with different growth temperatures and isogenic mutants. The pattern of expression was examined with immunoblotting of SDS extracts and supernatants of strains EGD and EGDpERL3 50-1 and the isogenic *prfA1* mutant grown at 37 or 20°C. These results are depicted in Fig. 3. Production of both InlA and InlB in the wild-type strain was strongly affected by growth temperature (cf. Fig. 3A, panels a′, a″, b′, b″, lanes 1, with the same panels in Fig. 3B). This was also the case for the PrfA-overproducing strain, in which increased *prfA* gene dosage led to the production of higher levels of InlA and InlB (cf. Fig. 3A, panels a′, a″, b′, b″, lanes 2, with the same panels in Fig. 3B). Note that the immunoblots were processed by the sensitive chemiluminescence method. The filters depicted in panels a′ and a″ were exposed for 5 s, while panels b′ and b″ represent 60-s exposures. In the *prfA1* mutant strain, increased amounts of the InlA product were detected regardless of the growth temperature employed (cf. Fig. 3A and B, panels a′ and b′, lanes 3) while a low level of InlB was detected in the *prfA1* mutant strain (cf. Fig. 3A and B, panels a″ and b″, lanes 3). These results suggested that, unlike InlB, the regulation of which appears largely to resemble that of bona fide PrfA-regulated virulence proteins, production of InlA seems to be only partially PrfA dependent. These apparently conflicting data prompted us to examine the transcripts of either gene in cultures grown at different temperatures and to determine the transcriptional start sites for the individual promoters.

Transcriptional analysis of the *inlAB* locus of *L. monocytogenes* EGD. We examined RNA transcripts produced with the *inlAB* locus in the EGD wild-type strain and its isogenic *prfA1* mutant in bacteria grown at 20 and 37°C (Fig. 4). As a control, we also included analysis of the listeriolysin (*hly*) gene where both *prfA* and temperature regulation has been unequivocally demonstrated (24, 25). Since *inlA* and *inlB* are highly homologous genes, care was taken to derive probes from regions exhibiting low homologies (see Materials and Methods and

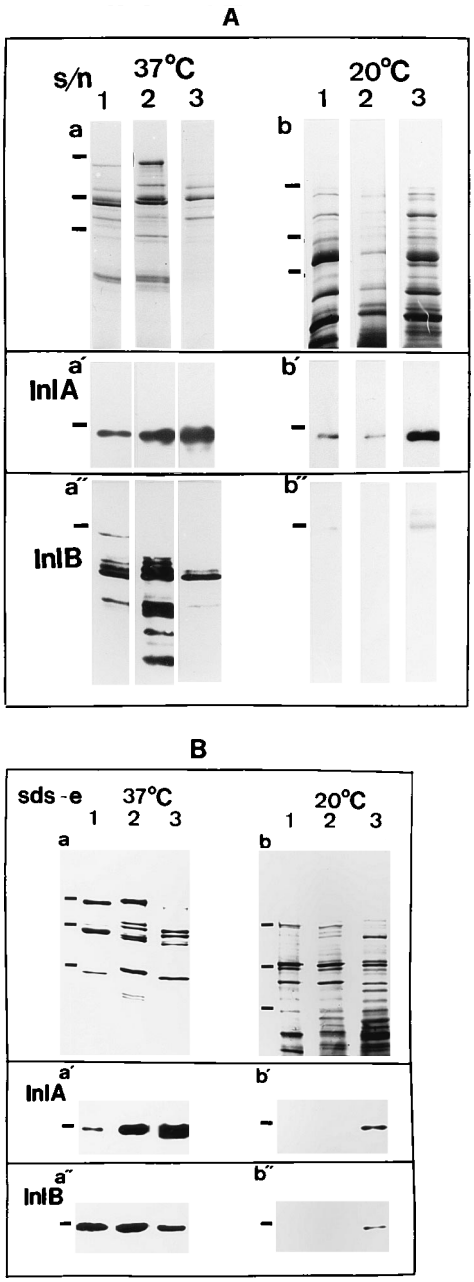

FIG. 3. Detection of the InlA and InlB polypeptides in culture supernatants (A) and SDS extracts (B) of EGD (lanes 1), EGDpERL3 50-1 (lanes 2), and EGD*prfA1* (lanes 3) grown either at 37°C (panels a, a′, and a″) or at 20°C (panels b, b′, and b″). (a and b) Silver nitrate-stained culture supernatants (A) or SDS extracts (B). (a′ and b′) The corresponding immunoblot developed with InlA-specific MAb M160. (a″ and b″) Identical immunoblots processed with the InlB-specific MAb IC100. Note that the blots were developed by chemoluminescence and that exposure times were 5 s for the results shown in panels a′ and a″ and 60 s for panels b′ and b″ in both panels A and B. Molecular mass markers are as follows: (a and b) from top to bottom, 97, 66, and 45 kDa; (a′ b′) 97 kDa; and (a″ b″) 66 kDa.

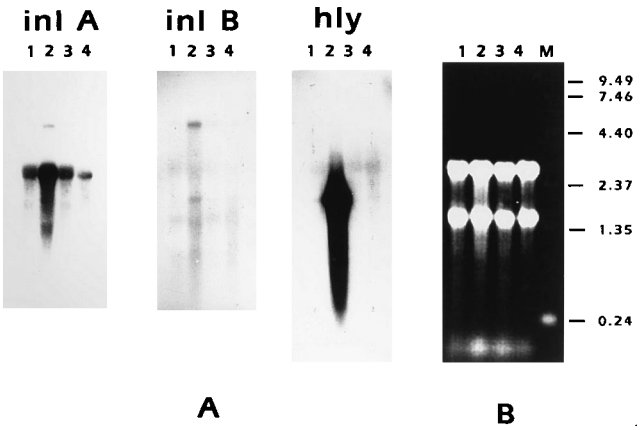

FIG. 4. Northern blot analysis of transcripts within the *inlAB* locus in the *L. monocytogenes* EGD wild-type strain (lanes 1 and 2) and its isogenic *prfA1* mutant (lanes 3 and 4). Total RNA was isolated from strains growing either at 20°C (lanes 1 and 3) or at 37°C (lanes 2 and 4), from exponentially growing cultures, separated by electrophoresis, blotted onto nitrocellulose filters, and probed with radiolabeled *inlA*-, *inlB*-, or *hly*-specific fragments (A). From the corresponding autoradiograms, the following transcripts were detected: 4,800 and 2,900 nt for *inlA*, 4,800 and 1,900 nt for *inlB*, and 1,800 nt for *hly*. Agarose gel electrophoresis of total RNA is shown in panel B. Molecular size markers are in kilobases.

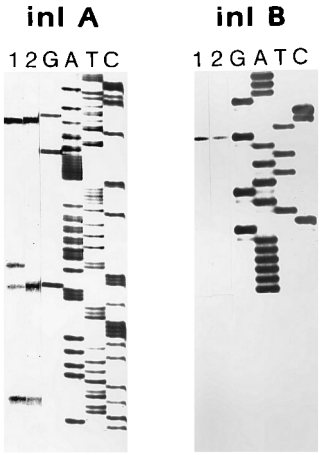

FIG. 5. Mapping and detection of *inlAB* transcripts in *L. monocytogenes* by primer extension analysis. γ-^{32}P-end-labeled oligonucleotide primers (see Materials and Methods) were used for reverse transcription of the transcripts. The DNA products were separated on a 6% polyacrylamide gel simultaneously with a dideoxy sequencing reaction ladder, with the same DNA primer and plasmid pLMH1 to allow determination of the extension product. Lane 1, the primer extension reaction done with strain EGD; lane 2, reaction done with the isogenic EGD*prfA1* mutant; lanes G, A, T, and C, tracts of the sequencing reaction.

Fig. 6A). The results of this analysis are shown in Fig. 4. In the wild-type strain grown at 37°C, abundant transcripts of 2,900 and 4,800 nt in length were detected with the *inlA* probe, indicating that it is transcribed both individually and in combination with the *inlB* gene. The longer transcript was both *prfA* and temperature regulated, while the transcript harboring only the *inlA* gene remained largely unaffected under these conditions. With the *inlB* probe, two transcripts were also detected, one of 4,800 nt encompassing the entire *inlAB* operon and a shorter transcript of 1,900 nt corresponding to the *inlB* gene. The *inlAB* transcript was, as seen above, both *prfA* and temperature regulated. Reduced amounts of *inlB* transcript were also observed in cultures grown at the lower temperature. The amount of *inlB* transcript present in the *prfA1* mutant strain grown at 37°C was variable and difficult to reproduce. When the same RNA preparations were analyzed with the *hly* probe, a strict dependence on the presence of the PrfA regulator and the higher growth temperature was observed. Hence, under identical conditions, transcripts originating from the listeriolysin gene are much more abundant than those deriving from the *inlAB* locus.

To determine promoters for both the *inlA* and *inlB* genes in the wild-type strain and its corresponding *prfA1* mutant, we used single-stranded oligonucleotide probes deriving from the *inlAB* locus (see Materials and Methods) to map the transcription start sites for either gene. Primer extension analysis performed on RNA isolated from these strains is depicted in Fig. 5. For *inlA*, four promoters located 365 (P_{inlA1}), 391 (P_{inlA2}), 396 (P_{inlA3}), and 438 (P_{inlA4}) nt upstream from its initiation codon were detected. Transcription originating from the P_{inlA3} promoter was completely abrogated in the *prfA1* mutant (Fig. 5). A promoter for the *inlB* gene was located 51 nt upstream of its initiation codon in the intergenic region between the *inlA* and *inlB* genes and was expressed independently of the PrfA regulator (Fig. 5).

Both InlA and InlB are required for in vitro invasion. We first examined the ability of the *prfA1* mutant to invade three different epithelial cell lines and compared this with the invasive properties of the *inlA2* and *inlB2* mutants. This was done by incubating bacteria with the various tissue culture cell lines for 1 h at 37°C, after which cells were washed and incubated with complete medium containing gentamicin for an additional hour. All three mutants were clearly defective for invasion (Table 1). Hence, both conditions, gross reduction in the amounts of internalins expressed due to lack of the PrfA regulator and abrogation of either internalin by genetic manipulation, strongly affect the invasive ability of the strain.

The ability of the *inlA2* and *inlB2* strains to penetrate both epithelial-like and fibroblast-like tissue culture cell lines was next examined. As had already been observed, both *inlA* and *inlB* mutants were defective for entry into the epithelial-like HeLa, CaCo-2, PtK2, Henle, A549, and HEp-2 tissue culture cell lines and invaded with an efficiency of between 0.3 and 2.5% of the wild-type strain (Table 2). In contrast, the parental strain invaded fibroblast-like cell lines WI-38 and HEL 299 only poorly, and there was no discernible difference in the invasive ability of strains lacking either the *inlA* or the *inlB* gene (Table 2).

Mouse infection studies. The mouse bioassay is an extremely sensitive assay for examining the pathogenicity of *L. monocytogenes* (21). The effect of the *inlA2* and *inlB2* mutations on the virulence of *L. monocytogenes* was therefore assessed by the mutants' abilities to grow in host tissues when injected intraperitoneally into mice. Both mutants were attenuated for vir-

TABLE 1. Comparison of the invasive abilities of *L. monocytogenes* EGD, EGD*prfA1*, EGDΔ*inlA2*, and EGDΔ*inlB2*

Strain	% Entry into cell line[a]:		
	CaCo-2	PtK2	A549
EGD	2.8	1	0.22
EGD*prfA1*	0.35	0.03	0.005
EGDΔ*inlA2*	0.07	0.02	0.003
EGDΔ*inlB2*	0.07	0.007	0.001

[a] Percent entry was calculated from the number of bacteria that survived incubation in the presence of gentamicin with respect to the total number of inoculated bacteria.

TABLE 2. Invasive properties of the *L. monocytogenes* EGD, EGDΔ*inlA2*, and EGDΔ*inlB2* strains

Strain	% Entry into cell line[a]							
	HeLa	CaCo-2	PtK2	Henle	A549	HEp-2	WI-38	HEL 299
EGD	0.024	4.3	0.9	0.15	0.28	0.52	0.0036	0.002
EGDΔ*inlA2*	0.006	0.012	0.03	0.04	0.03	0.02	0.0027	0.002
EGDΔ*inlB2*	0.004	0.008	0.01	0.022	0.002	0.001	0.0032	0.003

[a] Percent entry was calculated from the number of bacteria that survived incubation in the presence of gentamicin with respect to the total number of inoculated bacteria.

ulence in this animal model. Retarded growth of these strains was observed within the spleens and livers of infected mice, and bacteria were only slowly eliminated from these organs (Table 3).

DISCUSSION

The results of this study have led to the unequivocal identification of InlA and InlB as 88,000- and 65,000-Da polypeptides, respectively, in *L. monocytogenes* EGD. Both InlA and InlB are cell wall-associated proteins of *L. monocytogenes*. However, these proteins are also released to the supernatant fluids of these bacteria. Using specific antibodies raised against InlA and InlB, we showed that the expression of these polypeptides, particularly that of InlA, is not strictly dependent on the presence of the PrfA regulator protein. Transcriptional analysis of the RNA transcripts and their respective start sites revealed that the *inlA* and *inlB* genes are transcribed both as an operon and individually. Of the five RNA start sites mapped within this locus, only one promoter appeared to be dependent on the presence of the PrfA regulator. Tissue culture invasion assays showed that both InlA and InlB are required for efficient invasion of epitheloid cell lines. Nevertheless, both mutants were relatively unimpaired for invasion of fibroblast-like cell lines. Finally, virulence studies with the mouse infection model revealed that both mutants were attenuated for virulence.

Introduction of additional copies of the *prfA* gene into the wild-type *L. monocytogenes* strain results in the overproduction of several PrfA-regulated proteins (2, 25, 37) and led us initially to the identification of the InlA polypeptide. N-terminal microsequencing indicated that InlA is a secreted protein confirming the recently revised sequence for the predicted InlA sequence (10). Although InlA and InlB have been associated with internalization of listeriae by epitheloid cell lines (10, 15), currently available mutants are polar insertions located within the promoter region of the *inlA* gene and reduce expression of both the InlA and InlB polypeptides. To establish the role of each gene in the invasive process, we created mutations in either gene by generating chromosomal in-frame deletions in both *inlA* and *inlB*.

The availability of site-specific *inlA* and *inlB* deletion mutants enabled us to screen a panel of MAbs raised against *Listeria* surface proteins for specific MAbs to either gene product. Hence, InlA was identified as a polypeptide of 88 kDa and was detected in both SDS extracts of whole cells and supernatant fractions. These results are in good agreement with those reported previously (10, 15). InlB was identified as a 65-kDa polypeptide, present also in SDS extracts and supernatant fractions. Unequivocal confirmation that the 65-kDa polypeptide is InlB was obtained by N-terminal microsequencing and revealed that the mature InlB is the result of cleavage following amino acid residue 35 by signal peptidase. Use of InlB-specific antibodies revealed that, unlike InlA, this protein is extensively degraded in the supernatant fluids (Fig. 2).

Previous studies regarding the expression of InlA and InlB and its dependence on the PrfA regulator have yielded conflicting results (10, 37). In an attempt to resolve this issue, we used the MAbs isolated to examine the expression of InlA and InlB under various growth conditions in the *L. monocytogenes* EGD wild-type strain, its isogenic *prfA1* mutant, and EGD-pERL3 50-1 carrying multiple copies of the *prfA* gene. In the wild-type strain, production of InlA was dependent on growth temperature. Unexpectedly, expression of InlA in the *prfA1* mutant strain was greatly increased compared with its parental wild-type strain. Also, a higher dosage of the *prfA* gene increased the production of InlA in the EGDpERL3 50-1 strain (Fig. 3).

Northern blot analysis showed that at 37°C InlA is expressed by at least two transcripts, a 4,800-nt-long transcript encompassing the *inlAB* genes and a shorter 2,900-nt transcript consisting of only the *inlA* gene. Primer extension studies revealed that there are four promoters preceding the *inlA* gene, of which only one is *prfA* regulated (Fig. 5). Since the 4,800-nt transcript is not produced in the *prfA1* mutant (Fig. 4), this *prfA*-regulated promoter is responsible for transcription of both genes, while transcription from the remaining three promoters is responsible for the monocistronic *inlA* transcript. Note that while the amount of *inlA* transcripts detected in the *prfA1* mutant is lower than that in the parental strain, this mutant produces higher amounts of InlA than its wild-type counterpart. Hence, either the protein produced in the mutant strain is more stable or InlA is proteolytically degraded by a protease whose production is regulated by the presence of PrfA.

Unlike results obtained previously with another *L. monocytogenes* strain, EGDSmR (10), we detected, by Northern blot analysis and primer extension analysis, a low-level promoter, particularly evident in strains grown at 37°C, that was respon-

TABLE 3. Growth of *L. monocytogenes* EGD, EGDΔ*inlA2*, and EGDΔ*inlB2* in spleens and livers of infected mice following intraperitoneal injection

Strain	Log_{10} number injected i.p.[a]	Log_{10} number of bacteria/g of tissue (± SD)[b]					
		Day 1		Day 3		Day 6	
		Spleen	Liver	Spleen	Liver	Spleen	Liver
EGD	3.66	3.2 ± 0.4	3.7 ± 0.7	6.1 ± 0.7	5.7 ± 0.9	4.1 ± 0.9	3.6 ± 0.8
EGDΔ*inlA2*	4.02	3.3 ± 0.7	2.7 ± 0.4	3.8 ± 1.3	3.4 ± 1.4	3.1 ± 1.2	1.2 ± 0.6
EGDΔ*inlB2*	4.34	2.9 ± 0.3	2.3 ± 0.4	3.3 ± 0.4	2.7 ± 0.3	2.6 ± 0.4	1.2 ± 0.4

[a] i.p., intraperitoneally.
[b] Log_{10} mean values for three mice.

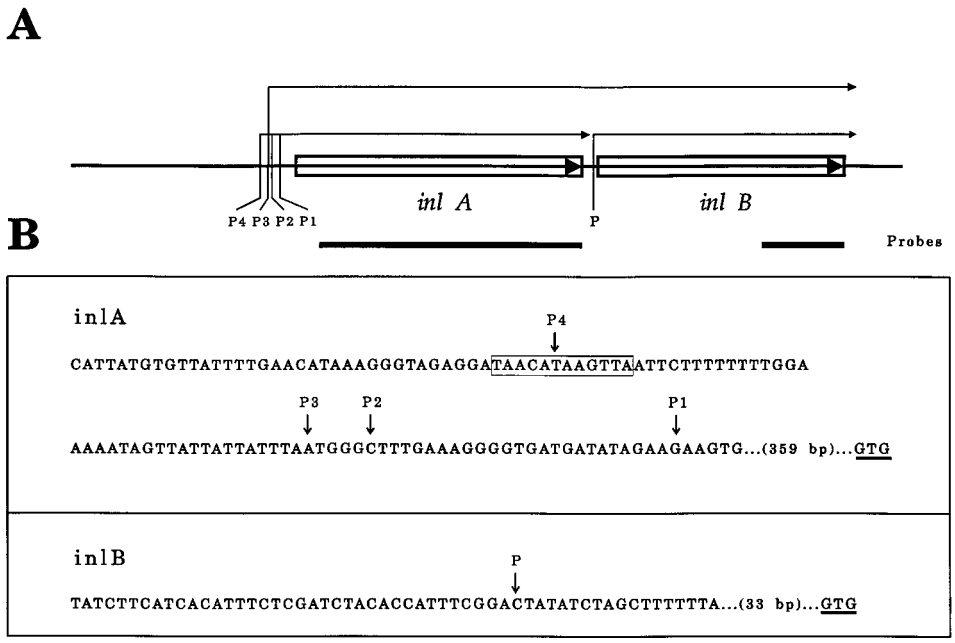

FIG. 6. (A) Schematic representation of the transcriptional organization of the *inlAB* locus. The positions of the *inlA* and *inlB* genes are boxed. The arrowed lines represent transcripts deriving from the various promoters mapped in this study. (B) Nucleotide sequence of the regions preceding the *inlA* and *inlB* genes. The positions of the transcriptional start sites for the P_1, P_2, P_3, and P_4 promoters preceding the *inlA* gene are indicated with vertical arrows. The putative palindromic *prfA* recognition sequence preceding the P_3 promoter is boxed. The position of a promoter preceding the *inlB* gene is also indicated.

sible for *inlB* expression. Note, however, that the *inlB* gene is largely expressed from the longer 4,800-nt transcript originating from the *inlA* promoter. Expression of InlB was, unlike that of InlA, highly dependent on the presence of PrfA and on temperature and reminiscent of that of other PrfA-regulated genes such as listeriolysin (Fig. 5) (25). Hence, PrfA-independent transcripts for the *inlA* gene appear to terminate in the intergenic region preceding the *inlB* gene. Antitermination of the transcript produced by the *prfA*-dependent promoter would result in the expression of both genes, leading to the strong dependence of expression of *inlB* on the PrfA regulator. Antitermination has previously been postulated for the *plcA-prfA* (3, 28) and *mpl-actA-plcB* (29, 39) operons of *L. monocytogenes*, both of which are regulated by the PrfA regulator.

The sequence of the PrfA-regulated promoter located upstream of the *inlA* gene has two base substitutions within its putative PrfA DNA-binding site (10) (Fig. 6). It has been postulated previously that single substitutions within the palindromic sequence preceding the *hly* and *plc* genes, such as those located upstream of *mpl* and *actA*, reduce the affinity of PrfA for these promoters (14). Hence, higher levels of PrfA are required to activate these promoters, a result that is consistent with the observation made with respect to increased expression of InlA and InlB in the EGDpERL3 50-1 strain. The emerging picture of a hierarchy of PrfA-regulated promoters exclusively controlling virulence genes in *L. monocytogenes* now includes the *inlAB* locus, whose expression is clearly controlled by both PrfA-dependent and PrfA-independent promoters.

It has been noted previously that at least one of the promoters preceding the *inlA* gene could be controlled by the sigma 28 factor (10), a secondary sigma factor involved in the control of a regulon, expressing such diverse genes as those required for flagellar morphogenesis, chemotaxis, or nutrient stress responses (19). The role of the other two promoters identified is unclear at this moment but provides an explanation as to why the expression of InlA is virtually constitutive. The multiplicity of promoters required for the expression of InlA is reminiscent of other systems in gram-negative bacteria, such as in the *gal*, *arg*, or *deo* operons, where multiple promoters control gene expression (5). In these cases, the presence of more than one promoter results in greater flexibility in the control of gene expression. Hence, expression of InlA may be required under many growth conditions, ranging from those that exist prior to infection to those that exist within infected host tissue. A summary of the data obtained from our studies is depicted in Fig. 6.

Tissue culture invasion assays with the isogenic *inlA2* and *inlB2* mutants showed that the invasion phenotype was dependent on the expression of both InlA and InlB. The wild-type *L. monocytogenes* strain showed strong variability in its ability to enter different epitheloid cell lines, suggesting that a number of receptors for *Listeria* internalization are present on the surface of these cell lines. We noticed that the fibroblast-like cell lines used in this study were permissive for uptake of *L. monocytogenes*, independent of the expression of either InlA or InlB. This suggests that *L. monocytogenes* may have more than one mechanism of inducing uptake by nonphagocytic cell lines. However, as listeriae appear to invade fibroblast cell lines only poorly these results further suggest a propensity of these bacteria to selectively invade epithelial cells.

A search of the current GenBank library for homologies to the InlA and InlB primary sequences revealed that these proteins are members of a superfamily of leucine-rich proteins (22). These members include regulatory subunits of the enzyme adenylyl cyclase, cell adhesion proteins such as GPIbα (27), and other bacterial virulence factors such as the *ipaH* gene from *Shigella flexneri* (18) or the YopM protein from *Yersinia pestis* (26). Hence, the repeat I-I-L-L-L-L-L found 15 times within the InlA sequence is highly similar to the sequence L-L-L-L-L-L-X repeated 6 and 9 times within the $IpaH_{7.8}$ and $-_{4.5}$ proteins of *S. flexneri*, respectively (40). The

YopM protein, which harbors nine copies of a similar repeat, is thought to promote virulence by interfering with the platelet-mediated inflammatory response mediated by GPIbα, a critical component of hemostasis (34). However, the relative ubiquity of the leucine-rich motif in many proteins that have different functions and cellular locations suggests that they are involved in protein-protein interactions. Whether the InlA and InlB proteins interact with each other as part of a complex to promote internalization or whether they serve as ligands for host receptor proteins is at present a matter of conjecture. With the availability of specific reagents to detect either protein, immunodetection of these proteins during the invasion process is now feasible. Purification protocols for either protein can be established and should eventually lead to the identification of further proteins that interact with InlA and InlB.

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