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Identification and Characterization of a Novel PrfA-Regulated Gene in *Listeria monocytogenes* Whose Product, IrpA, Is Highly Homologous to Internalin Proteins, Which Contain Leucine-Rich Repeats

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The expression of all virulence factors in *Listeria monocytogenes* characterized to date is controlled by the virulence regulator protein, PrfA. To identify further PrfA-regulated proteins, we examined supernatants of *L. monocytogenes* EGD harboring additional copies of the PrfA regulator for the presence of novel proteins. This led to the identification and biochemical purification of a hitherto uncharacterized PrfA-dependent 30-kDa protein (A. Lingnau, T. Chakraborty, K. Niebuhr, E. Domann, and J. Wehland, Infect. Immun. 64:1002–1006, 1996). Oligonucleotide primers derived from internal peptide sequences of this protein allowed the cloning and determination of the entire sequence of the respective gene. The protein comprised 297 amino acids with strong overall homology to the internalins, InlA and InlB, particularly in the region harboring the leucine-rich repeats. The gene has been designated *irpA* for internalin-related protein A gene. Transcriptional studies revealed that the gene was monocistronic and, like the *inlA* and *inlB* genes, was transcribed by PrfA-dependent and PrfA-independent mechanisms. Monoclonal antibodies raised against IrpA indicated that it was produced by *L. monocytogenes* but not by the nonpathogenic species *Listeria innocua*. To examine the role of IrpA in pathogenesis, we constructed an isogenic in-frame deletion mutant that removed all but 116 amino acids of the IrpA protein. This mutant was neither defective for invasion into many tissue culture cell lines nor did it demonstrate reduced intracellular survival. However, in vivo studies using the mouse infection model revealed that the *irpA* mutant showed reduced virulence compared to the parental strain. These results suggest a role for IrpA during disseminated infection by *L. monocytogenes*.

Listeria monocytogenes, a ubiquitously occurring gram-positive pathogen, causes severe food-borne infections in humans and many animals (8, 14, 15). Following ingestion, the intestinal barrier may be breached leading to disseminated infection within the body of the host. Many tissues are infected, and clearly an important virulence trait of pathogenic *Listeria* is its ability to invade a wide variety of nonphagocytic eukaryotic cells including fibroblasts, enterocytes, epithelial cells, and hepatocytes (14). Like other intracellular bacteria such as *Shigella*, *Salmonella*, and *Yersinia* spp., *L. monocytogenes* actively induces its uptake into host cells (27, 33).

Recently, it has been demonstrated that a specific genetic locus is required for invasion of *L. monocytogenes* into various tissue culture cell lines. This locus consists of two highly homologous genes, *inlA* and *inlB*, both of which are located in an operon (13). The generation of isogenic mutants with chromosomal in-frame deletions in either gene has shown that the products of both genes are required for the invasion of *L. monocytogenes* into nonphagocytic tissue culture cell lines. It has been suggested that InlA and InlB are required for differential invasion into epithelial and hepatocytic-tissue culture cell lines, respectively (7, 13, 25). More recently, the receptor engaged by the InlA protein has been identified as E-cadherin, a Ca^{2+}-dependent cell-cell adhesion molecule with essential functions in junctional intercellular adhesion and the maintenance of adult tissue architecture (27).

Both the InlA and InlB polypeptides are cell wall-associated proteins, with apparent molecular weights of 88,000 and 65,000, respectively (25). A striking structural feature of both proteins is the presence of consecutive leucine-rich repeats (LRRs), present 15 times in InlA and 7 times in InlB (13). Homology searches have revealed that InlA and InlB are members of a superfamily of LRR-containing proteins. The members include eukaryotic adhesive proteins such as decorin, fibromodulin, and glycoprotein Ib; signal-transducing receptors such as the *trk* proto-oncogene transmembrane protein, CD14, and the yeast adenylate cyclase; and the plant disease resistance gene proteins RPS2 and N (18, 20). Interestingly, the presence of bacterial proteins containing LRRs has been, to date, only detected in surface-bound proteins of pathogenic bacteria. Hence, members of the *ipaH* gene family of *Shigella flexneri* all encode LRR-containing proteins. Similarly, the YopM protein of *Yersinia pseudotuberculosis* and the filamentous hemagglutinin protein of *Bordetella pertussis* are members of the LRR superfamily (18, 26).

All of the currently known virulence genes of *L. monocytogenes* are found in two genetic clusters present in its chromosome. The majority of these genes are located on a single 10-kb chromosomal locus containing the *hly* gene flanked by the lecithinase (comprising the *mpl*, *actA*, and *plcB* genes) and the *plcA-prfA* operons, while the invasion genes *inlA* and *inlB* are

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TABLE 1. Oligonucleotides and their features

Code letter	Sequence (5′→3′)	Position in *irpA* (5′→3′)	Feature or application
A	CGTCCAACACCAATTAATCAAGTATTTCC	763–791	Created from sequence of peptide 1
B	TTTAATTGGTTGTGTTACTGTTCC	1535–1513	Created from sequence of peptide 6
C	ACTGGTCAGAAATGTGTGAATG	1288–1309	Inverse PCR, downstream region
D	AGTCAGTATCTCTGAGTTCG	1117–1098	Inverse PCR, downstream region
E	CTTTTGTGATACAAGGTCTG	864–845	Inverse PCR, upstream region
F	TCAAATAAGTGACCTTAGTC	969–988	Inverse PCR, upstream region
G	CTCCAGATAGTGCGGCCGCTGATACAAGGTCTGTAACAC	877–839	*Not*I endonuclease restriction site
H	CAGTAATGGTGCGGCCGCTGTAGATGGTTGTGTCCTGT	1392–1429	*Not*I endonuclease restriction site
I	CAACATTCTCCACTCCT	654–637	Promoter mapping
K	TAGCTATGCGTTAGATCTCGGAGCCGGGGACT	1–32	*Bgl*II endonuclease restriction site
L	GCAACAAGCACTAGATCTAGATTAGCCT	2100–2073	*Bgl*II endonuclease restriction site

located in an independent operon (32). The expression of these genes is controlled by both temperature and by a common virulence regulator, PrfA (3, 22, 23). Current evidence suggests that purified PrfA binds to a 14-bp palindromic sequence located in the −35 region preceding the promoters of *hly* and *plcA*. Palindromic sequences with nucleotide substitutions that interrupt double-strand formation have also been found in the same regions of all PrfA-regulated genes, and this has led to the notion of a hierarchy of PrfA-regulated control of genes involved in virulence (10, 25). Hence, in the *inlA-inlB* operon, which has a poor PrfA-binding palindrome, transcription of these genes is also regulated by PrfA-independent promoters.

We have initiated a search for novel PrfA-regulated proteins by examining supernatants of a recombinant *L. monocytogenes* strain harboring multiple copies of the PrfA regulator. In a previous study, we purified to homogeneity and biochemically characterized a 30-kDa protein that is overproduced in the culture supernatants of such a strain. Microsequencing of the peptides obtained from the 30-kDa protein revealed significant homology to the InlA and InlB polypeptides and prompted us to call it internalin-related protein A (IrpA). A highly related 24-kDa protein, isolated from the animal pathogenic species *Listeria ivanovii*, was designated iIrpA (24). Here, we report on the molecular cloning, sequencing, and characterization of the *irpA* gene from *L. monocytogenes* EGD. IrpA is highly homologous to InlA and InlB and is present only in pathogenic *Listeria*. Transcriptional analysis indicated that the mRNA transcript of the gene was monocistronic and transcribed by both PrfA-dependent and -independent promoters. An isogenic mutant harboring an in-frame deletion had no obvious phenotype in in vitro tissue culture assays for invasion and intracellular survival but was nevertheless attenuated for virulence in the mouse bioassay of *Listeria* infection.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The weakly hemolytic *L. monocytogenes* EGD (serotype 1/2a) was originally obtained from G. B. Mackaness and has been described previously (17). The strain served as the parental strain for construction of the isogenic deletion mutant Δ*irpA2*. Listeria strains were grown in brain heart infusion broth (BHI; Difco) at either 28°C, 37°C, or 42°C, and 5 μg of erythromycin per ml was added as appropriate. *Escherichia coli* DH10β [F′ *mcrA*Δ-(*mrr hsdRMS-mcrBC*) Φ*80d lacZ*Δ*M15* Δ*lacX74 deoR recA1* Δ*araD139* (*ara*, *leu*)*7697 galU galK* λ- *rpsL endA1 nupG*] (GIBCO BRL, Eggenstein, Germany) was used for cloning and transformation. It was cultivated in Luria-Bertani broth and supplemented either with 100 μg of ampicillin per ml for multiplication of pCRII plasmids (Invitrogen, Leek, The Netherlands) or with 300 μg of erythromycin per ml for multiplication of plasmid pAUL-A and its derivatives (3, 5). Restriction analysis and plasmid constructions were done by standard techniques as outlined by Sambrook et al. (34). All chemical reagents were purchased from Sigma (Deisenhofen, Germany) unless indicated otherwise.

Cloning of the *irpA* gene. To clone the *irpA* gene, we synthesized oligonucleotides on the basis of the amino acid sequence of short peptides from IrpA (24) using the codon usage of previously cloned genes from *L. monocytogenes*. The largest fragment, of approximately 800 bp, amplified with the oligonucleotide pair A and B (sequences and positions of oligonucleotides A to L are in Table 1) was cloned via the TA cloning system (Invitrogen) into the pCRII vector as outlined by the vendor. A determination of the nucleotide sequence of the cloned DNA fragment revealed that all short peptides of Irp1 through Irp6 were present in the primary sequence (Fig. 1). The 5′ and 3′ flanking regions of the *irpA* gene were cloned by using an inverse PCR strategy (29). First, chromosomal DNA of *L. monocytogenes* was digested with restriction endonucleases *Eco*RI and *Mun*I, both generating compatible adhesive ends. In a subsequent self-ligation reaction circular molecules were obtained. Since restriction endonuclease *Eco*RI digested the *irpA* gene itself (Fig. 1), two different circular molecules, harboring either upstream or downstream regions of the *irpA* gene, were created during the ligation reaction. 5′ and 3′ DNA sequences were specifically amplified by PCR with the oligonucleotide pairs E and F and C and D, respectively (Fig. 1; Table 1). The corresponding amplification products were cloned into the pCRII vector plasmid (see above), and the nucleotide sequence was determined. For further studies, the oligonucleotide pair K and L, both harboring a *Bgl*II restriction site, was employed to amplify and clone a DNA fragment consisting of the *irpA* gene and its 5′ and 3′ flanking regions.

Determination of nucleic acid sequence. The DNA sequence of the cloned *L. monocytogenes* DNA was determined from double-stranded plasmid templates by dideoxy-chain termination (35). Double-stranded templates were denatured, and the sequencing reactions were carried out with T7 DNA polymerase as suggested by the commercial supplier in the product literature. Sequencing reactions were primed from vector- and custom-made oligonucleotide primers. [α-^{35}S]dATPαS (1,000 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). The labeled reaction mixtures were separated by electrophoresis on urea–6% polyacrylamide gels, the gels were dried down, and the sequence was read from X-ray film autoradiograms.

Homology studies. To search for homologies of IrpA polypeptide with polypeptides published in different databases, we used the BLASTP (1) and FASTA (30) programs within the Heidelberg Unix Sequence Analysis Resources-Genetics Computer Group (HUSAR-GCG) environment at the Deutsche Krebsforschung Zentrum, Heidelberg, Germany. Additionally, for comparing peptide sequences with internalin A and B (Fig. 2A), Clustal V (16) was used.

Construction of the chromosomal in-frame deletion mutation *irpA2*. A deletion mutation in the *irpA* gene was generated by PCR by employing specific primers with incorporated restriction sites to introduce an in-frame deletion. To create the *irpA* mutation, the oligonucleotides K and G, which harbored *Bgl*II and *Not*I restriction sites, respectively, were used to amplify an 877-bp DNA fragment from the 5′ region of *irpA*, encoding the first 70 N-terminal amino acid residues. The oligonucleotides H and L, which harbored *Not*I and *Bgl*II restriction sites, respectively, were used to amplify a 709-bp DNA fragment from the 3′ region encoding the last 43 C-terminal amino acid residues of IrpA. Both PCR products were digested with *Not*I and used in a ligation reaction. The ligation product harboring the deletion was selectively amplified with the oligonucleotide pair K and L. The resulting PCR product was digested with the restriction endonuclease *Bgl*II and cloned into the *Bam*HI restriction site of the temperature-sensitive shuttle vector pAUL-A. Plasmid pAUL-Δ*irpA2* was transformed into *L. monocytogenes* EGD by a protoplast transformation method (39). To obtain a chromosomal in-frame deletion of the *irpA* gene, recombinant clones were processed as described previously (2, 25). The gene deletion was confirmed by Southern hybridization (36) and PCR sequencing of chromosomal DNA from mutants (data not shown) and by immunoblotting with the specific monoclonal antibody (MAb) O301 (Fig. 3). DNA manipulations were performed as described by Sambrook et al. (34).

Preparation of culture supernatant fluids. To obtain defined bacterial culture supernatants, BHI medium was prefiltered, with a membrane cutoff of 10 kDa

(Minitan; Millipore, Eschborn, Germany), before inoculation with the bacteria. Overnight cultures of appropriate *L. monocytogenes* EGD strains were diluted 1:50 in 20 ml of fresh BHI broth and incubated under vigorous shaking at 37°C until an optical density at 600 nm (OD_{600}) of ~0.4 to 0.6 was reached. The cultures were centrifuged in 30-ml Corex tubes (Sorvall; 6,000 rpm, 15 min), and the polypeptides in the supernatants were precipitated with 10% trichloroacetic acid on ice overnight. Following a centrifugation step (Sorvall; 10,000 rpm, 30 min) the pellets were carefully washed twice with ice-cold acetone, dried, and resuspended in 100 μl of 1 M Tris-HCl (pH 8.8). The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.

SDS-PAGE and immunoblotting. SDS-PAGE was done by the method of Laemmli with 12% polyacrylamide gels (21). The gels were stained by the use of Coomassie brilliant blue R-250. Immunoblotting was performed by a semidry method, with Immobilon P membranes (Millipore). After incubation with alkaline phosphatase-conjugated secondary antibodies (Dianova, Hamburg, Germany), the blots were reacted with 5-bromo-4-chloro-3-indolylphosphate (BCIP; BIOMOL, Hamburg, Germany) in glycine buffer (0.1 M glycine, 1 mM $ZnCl_2$ [pH 10]), which formed the substrate. Alternatively, a sensitive enhanced chemiluminescence-based immunoblot assay (Amersham Buchler) was used following the instructions provided by the supplier.

Polyclonal antibodies. One rabbit was immunized with incomplete Freund's adjuvant containing approximately 100 μg of purified IrpA. Two boosters were done with incomplete Freund's adjuvant at 2-week intervals, each booster containing 50 μg of purified IrpA. The serum was tested by immunoblotting. To obtain affinity-purified polyclonal IrpA antibodies, purified IrpA (3 mg) was coupled to 1 g of CNBr-activated Sepharose 4B by standard protocols. Ten milliliters of the IrpA antiserum was passed through the column containing immobilized IrpA. Low-affinity antibodies and unspecifically bound material were removed with 0.1 M acetate buffer (pH 4.8) containing 0.5 M NaCl. Specific polyclonal antibodies were then eluted with 0.2 M acetate buffer (pH 2.7) containing 0.5 M NaCl; this was followed by immediate neutralization with 1 M Tris-Cl (pH 8.8).

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated from growing cultures (OD_{600}, ~0.4, ~0.6, ~0.8, and ~1.0) of *L. monocytogenes* by the hot-phenol extraction method. RNA analysis was performed by formaldehyde gel electrophoresis (3). RNA samples (80 μg) were electrophoresed in a 1.3% agarose gel, transferred onto nylon filters (Hybond N; Amersham Buchler), and hybridized with the appropriate radiolabeled fragments under conditions specified by the vendor (9).

Promoter mapping by primer extension studies. Synthetic oligonucleotide I located within the *irpA* coding sequence was used as primer and labeled at its 5′ end. Primer extension analysis with recombinant virus reverse transcriptase (Superscript; Gibco) was performed as described previously with a total of 80 μg of RNA (25). Dideoxy sequencing reactions, with the same primer and an appropriate plasmid DNA template, were run in parallel to allow determination of the endpoints of the extension product (35).

In vitro invasion and plaque formation assay. Caco-2 (ATCC HTB37; the cell line was used between passages 20 and 40 since older passages are less permissive to infection by *L. monocytogenes* [11]), PtK2 (ATCC CCL 56), Henle 407 (ATCC CCL 6), and HeLa (ATCC CCL 2) cells were cultured in minimum essential medium (MEM; Gibco) supplemented with 8% fetal calf serum, glutamine, and nonessential amino acids in the absence of antibiotics. HepG2 (DSM ACC 180) cells were cultivated in Dulbecco's minimum essential medium (Gibco) supplemented with 8% fetal calf serum and glutamine. Bacteria grown as described above were washed twice in sterile phosphate-buffered saline (PBS) before addition to the respective tissue culture cell line in a 24-well microplate, generally 2 days after the formation of a confluent monolayer. One hundred microliters of the bacterial suspension (10^6 bacteria) was 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 MEM or MEM containing gentamicin (25 μg/ml) to kill extracellular bacteria. Samples were incubated for a further hour before being washed twice with PBS, and then 400 μl of cold distilled water was 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.

The plaque formation assay was performed with L929 (ATCC CCL1) cells as described recently for L2 cells (10).

Mouse virulence assay (LD_{50} determination). The 50% lethal doses (LD_{50}) for *Listeria* strains were determined as described by Welkos and O'Brien (38). Outbred female BALB/c mice weighing 20 to 25 g were used at 8 to 16 weeks of age. Animals were bred under specific pathogen-free conditions and were obtained from Harlan (Borchen, Germany) (28). Five mice in each experimental group were infected intravenously in the tail vein with doses of *L. monocytogenes* EGD and the Δ*irpA2* mutant that ranged between 10^3 to 10^7 bacteria and that had been diluted in 70 mM phosphate buffer (pH 7.2) containing 0.25% NaCl. The LD_{50} was recorded on day 7 postinfection.

Nucleotide sequence accession number. The nucleotide sequence reported in this article has been submitted to EMBL (Cambridge, United Kingdom) and assigned the accession number X98458 (*L. monocytogenes irpA* gene).

RESULTS

Cloning and sequencing of the *irpA* gene. Recently, we reported the identification and purification of novel internalin-related proteins which were isolated from supernatants of pathogenic *L. monocytogenes* (IrpA) and *L. ivanovii* (iIrpA) strains. To clone the corresponding gene from *L. monocytogenes*, oligonucleotides were designed on the basis of the determined amino acid sequences of IrpA peptides 1 to 6 by using codon preferences of known listerial proteins (see Materials and Methods). The *irpA* gene comprises 891 bp and encodes a protein of 297 amino acids. An inverted sequence, with strong homology to the canonical PrfA-binding box observed in the promoter regions of all listerial virulence factors, was detected 130 bp upstream of the initiation codon, AUG. Also, a sequence of dyad symmetry resembling a rho-independent terminator (31) was located immediately downstream of the termination codon, suggesting that this gene is transcribed monocistronically. The entire nucleotide sequence of the *irpA* gene and its flanking regions is depicted in Fig. 1.

Features of IrpA and homologies to members of the internalin family. The calculated molecular weight of unprocessed IrpA was 33,104. A signal peptidase cleavage site that was in agreement with the (−3−1) rule was located between amino acid residues 34 and 35, indicating that mature IrpA must be composed of 263 amino acid residues and must have a calculated molecular weight of 29,449. This was confirmed by N-terminal microsequencing of purified IrpA, which showed the first 12 residues to be ESIQRPTINQVF, corresponding to amino acid residues 35 to 47 of mature IrpA. In addition, mature IrpA's predicted molecular weight was in close agreement with that from experimental data obtained with the extremely sensitive mass spectrometric analysis, which revealed a molecular weight of 29,763 for native IrpA (24). The theoretically calculated pI of 5.3 was also highly similar to that obtained previously by isoelectric focusing of purified IrpA at approximately 5.6. These data therefore confirm the identity of the gene that encodes the previously isolated IrpA protein (24).

Protein database comparisons for the entire amino acid sequence of IrpA using the programs BLASTP (1) and FASTA (30) of Heidelberg Unix Sequence Analysis Resources (HUSAR) revealed strong sequence homologies to internalin A and B (13). The primary sequence of IrpA showed 56% overall similarity (35% identity) to InlA and 64% overall similarity (42% identity) to InlB. Alignment of sequence homologies with Clustal V (16) showed the close relationship between the three molecules InlA, InlB, and IrpA (Fig. 2). Despite the different sizes of the polypeptides (InlA, 800; InlB, 630; IrpA, 297 amino acid residues) they shared some features. All three polypeptides had signal peptides with lengths of either 34 or 35 amino acid residues and harbored several copies of a highly conserved LRR. The signal peptides and LRRs in the mature proteins were separated by spacer sequences of precisely 50 amino acids in all three molecules. InlA harbored 15 LRRs, InlB harbored 7 LRRs, and IrpA harbored 6 LRRs. Although the amino acid sequence homologies in the LRRs were not very strong, the relative positions of leucine and isoleucine residues within these repeats were highly conserved. The repeats were generally composed of 22 amino acid residues, and the consensus sequence for these LRRs was NQLX XLXXLXXLXXLXXLXLXX, with either leucines or isoleucines at positions 3, 6, 9, 12, 15, 18, and 20 within the LRRs. Strong homologies between all three proteins were also present in regions outside the LRR. Indeed, IrpA appeared to be a truncated version of either InlA or InlB (Fig. 2).

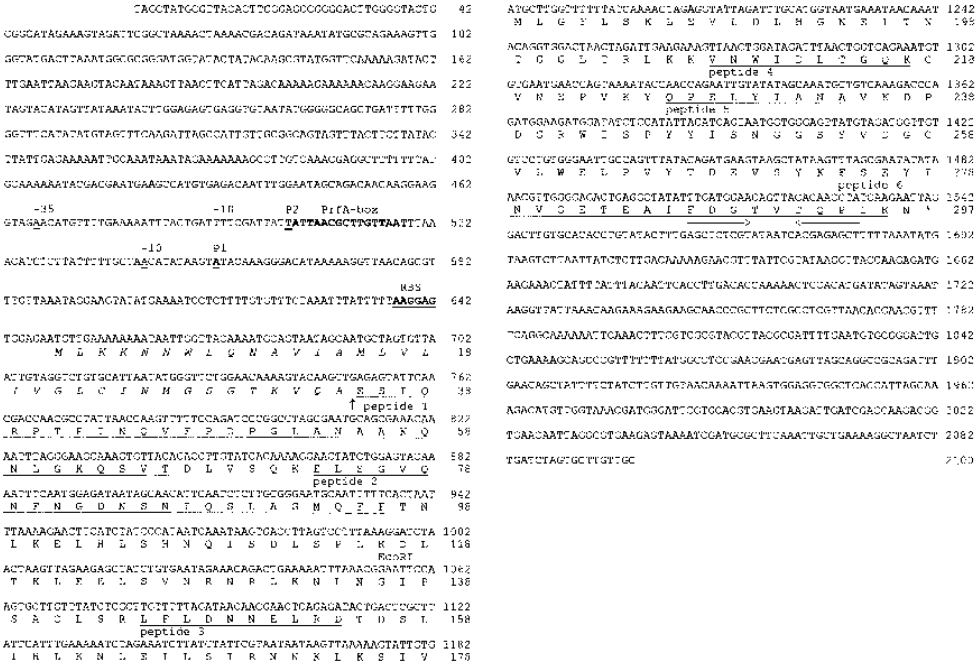

FIG. 1. Nucleotide sequence of the *L. monocytogenes* gene *irpA* and the deduced amino acid sequence of the associated protein, IrpA, including upstream and downstream flanking sequences. The PrfA-independent promoter region and transcriptional start site are indicated by −35, −10, and P2, and the PrfA-dependent promoter region and transcriptional start site are indicated by PrfA-box, −10, and P1. The ribosome binding site (RBS) is in boldface type and underlined, and the 34-amino-acid-long signal peptide of IrpA is in italics. The arrow indicates the signal peptidase cleavage site and the first amino acid residue of mature IrpA, as determined by N-terminal microsequencing. Previously determined short peptides of IrpA, obtained by hydrolysis with endolysin peptidase, are underlined in the deduced primary amino acid sequence and indicated as peptides 1 to 6 (24). The putative rho-independent terminator is indicated by convergent arrows.

Identification of IrpA in recombinant *E. coli* and *L. monocytogenes*. To identify the product encoded by the *irpA* gene, we cloned, by PCR amplification, a 2,100-bp fragment into the TA cloning vector pCRII to generate plasmids pCR-*irpA*-1 and pCR-*irpA*-2. In pCR-*irpA*-2 the *irpA* gene is in the same orientation as the *lac* promoter but is inverted with respect to this promoter in plasmid pCR-*irpA*-1. Cultures of recombinant *E. coli* strains harboring either plasmid were grown for 3 h, induced for expression with IPTG (isopropyl-β-D-thiogalactopyranoside) and allowed to grow for a further 3 h. Bacteria were then harvested, resuspended in lysis buffer, and subjected to SDS-PAGE. IrpA expression was detected by using IrpA-specific MAb O301 with lysates blotted onto nitrocellulose membranes. Expression was only detectable in strains harboring pCR-*irpA*-2, suggesting that expression in *E. coli* is under the control of the *lac* promoter. Two forms of IrpA were detectable (Fig. 3A, lane 2), the 33-kDa form, which is probably the unprocessed form, and the mature 30-kDa form. Signal peptide processing of IrpA in *E. coli* is poor, probably as a result of its N-terminal basic region, which is longer than those of signal peptides normally derived from gram-negative organisms (37).

In order to unambiguously identify the *irpA* gene product in *L. monocytogenes* we constructed a chromosomal in-frame deletion of the *irpA* gene. Using allelic exchange we constructed a mutant, *irpA2*, that harbored an internal deletion of 555 bp. The mutant IrpA2 protein lacks amino acid residues 71 to 254, but as a result of the creation of a *Not*I restriction endonuclease site it harbors three additional alanine residues in this region. Thus, the IrpA2 protein harbors 116 amino acid residues with a calculated molecular weight of 12,554. Analysis of culture supernatants of the wild-type strain and its isogenic mutant by using immunoblotting with IrpA-specific MAb O301 allowed us to demonstrate that mature IrpA has a molecular mass of 30 kDa (24). In the mutant strain a faint band at around 13 kDa was detected, providing further confirmation for the isolation of a mutant strain (Fig. 3B, lanes 1 and 2). Hence, MAb O301 recognizes a portion of the IrpA molecule that flanks its LRR-containing region.

Transcriptional analysis of the *irpA* gene of *L. monocytogenes*. We first examined RNA transcripts produced with the *irpA* gene in the EGD wild-type strain and its isogenic *prfA1* mutant (3) in bacteria grown at 37°C. However, using different growth conditions we were unable to detect transcripts corresponding to the *irpA* gene. We therefore used a second isogenic pair of *L. monocytogenes* strains, the strongly hemolytic NCTC 7973 and its Δ*prfA* variant, SLCC 53. The NCTC 7973 strain has previously been shown to overproduce many PrfA-regulated proteins, possibly as a result of mutations within its PrfA regulator gene (6). We first determined nucleotide sequences upstream of the *irpA* gene of NCTC 7973, including a portion of the structural gene. No differences in nucleotide sequences were observed (4). Total RNA was extracted from growing cultures of either NCTC 7973 or SLCC 53 at OD_{600}s of 0.4, 0.6, 0.8, and 1.0. As a probe, we used a 772-bp DNA fragment amplified by PCR with oligonucleotides A and B and plasmid pCR-*irpA*-2 as template. The results of this analysis are shown in Fig. 4A. Transcripts of approximately 1,150 nt were obtained at all stages of growth in the wild-type strain, but none were observed at any stage of growth in the strain lacking the PrfA regulator. These results confirm that (i) expression of *irpA* is PrfA-dependent and that (ii) *irpA* is transcribed as a single gene.

To determine the transcriptional start site for the *irpA* gene we used a single-stranded oligonucleotide probe derived from the *irpA* gene (see Materials and Methods). The results of

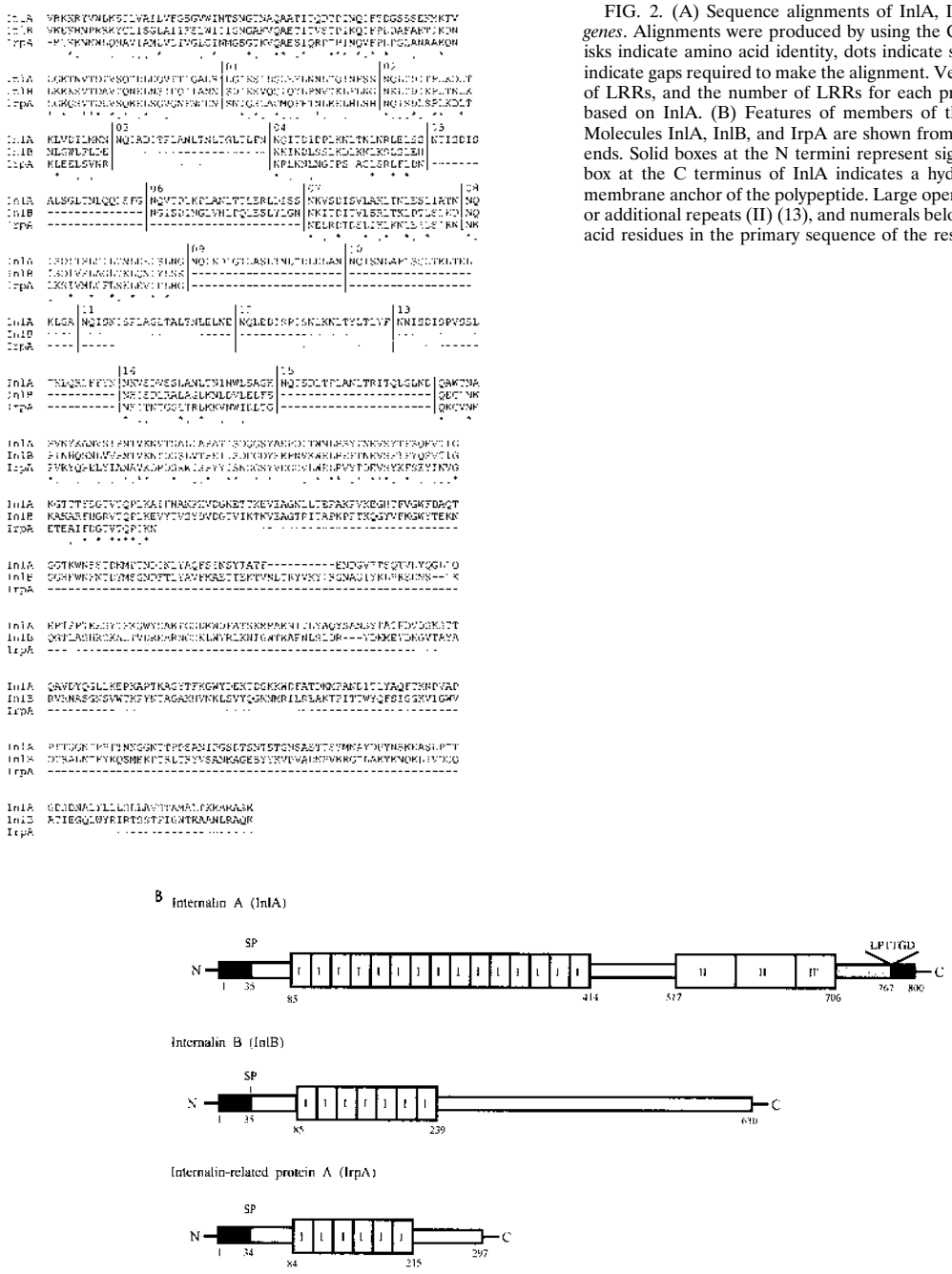

FIG. 2. (A) Sequence alignments of InlA, InlB, and IrpA of *L. monocytogenes*. Alignments were produced by using the Clustal V algorithm (16). Asterisks indicate amino acid identity, dots indicate similar amino acids, and dashes indicate gaps required to make the alignment. Vertical lines mark the boundaries of LRRs, and the number of LRRs for each protein is indicated by numerals, based on InlA. (B) Features of members of the internalin multigene family. Molecules InlA, InlB, and IrpA are shown from their N-terminal to C-terminal ends. Solid boxes at the N termini represent signal peptide, (SP) and the solid box at the C terminus of InlA indicates a hydrophobic domain, the putative membrane anchor of the polypeptide. Large open boxes indicate either LRRs (I) or additional repeats (II) (13), and numerals below the molecules indicate amino acid residues in the primary sequence of the respective polypeptide.

primer extension analysis performed on RNA isolated from strain NCTC 7973 are shown in Fig. 5. We detected two promoters located 97 (P_{irpA1}) and 147 (P_{irpA2}) nt upstream of its initiation codon. The stronger transcript originating from P_{irpA1} harbored a palindromic sequence within its −35 region with strong homology to the PrfA-binding sequence, suggesting that it was PrfA-regulated. The lack of detectable transcripts in the Δ*prfA* strain suggests that P_{irpA2} is a weakly expressed promoter.

Expression of IrpA is dependent on the PrfA regulator and temperature. Apart from dependence on the PrfA regulator, the expression of virulence factors in *L. monocytogenes* is dependent on growth temperature. To examine if this was also the case for IrpA expression, culture supernatants of wild-type strains EGD and NCTC 7973 and their respective isogenic *prfA1* and SLCC 53 mutants were grown at 37°C and 20°C, trichloroacetic acid precipitated, subjected to SDS-PAGE, and probed by immunoblotting with MAb O301 to IrpA (Fig. 6). From these results it is clear that the expression of IrpA is dependent on the presence of a functional PrfA. The low level of expression of IrpA obtained in the *prfA* mutants is probably due to transcription initiated by its PrfA-independent promoter (see above). Furthermore, growth at low temperatures reduced the production of IrpA considerably.

Detection of IrpA in different serotypes of *L. monocytogenes*. To examine the occurrence of the IrpA in pathogenic and

nonpathogenic *Listeria* species, we analyzed culture supernatants of various serotypes of *L. monocytogenes* and *Listeria innocua* strains by immunoblotting using affinity-purified IrpA polyclonal antibodies. IrpA was detectable in all serotypes of *L. monocytogenes* (Fig. 7) but appeared to be produced at different levels. For example, in serotype 4a (SLCC 2374) and 4c (SLCC 2376) strains, the level of expression of IrpA was extremely low (see Fig. 7, lanes 10 and 12). This polypeptide was detected neither in *L. innocua* under any conditions of growth (Fig. 7) nor in other nonpathogenic species of *Listeria* (4; data not shown).

Effect of the *irpA* mutation on invasion, cell-to-cell spread, and virulence. Given the strong homology of IrpA to InlA and InlB, we wished to explore its role in invasion of tissue culture cell lines. To do this, we examined the invasive property of the Δ*irpA2* mutant and compared it to those of the parental strain EGD and isogenic strains lacking either *inlA* or *inlB*. Following incubation of the bacteria for 1 h with tissue culture cell lines Caco-2, HepG2, PtK2, Henle 407, and HeLa, cells were washed and incubated with complete medium containing gentamicin for an additional hour. The results of this assay are depicted in Fig. 8. The Δ*irpA2* mutant strain did not show any defect in its internalization properties for all cell lines tested. The Δ*inlA2* and Δ*inlB2* mutant strains, which served as internal controls in these experiments, showed clear defects in their invasive abilities for the PtK2, Henle 407, and HeLa cell lines. Nevertheless, in agreement with results published previously, bacteria expressing InlA invaded Caco-2 cells (13), while InlB production was required for mediating entry into the hepatocytic cell line HepG2 (7, 12). Therefore, IrpA is not required for in vitro invasion of the tissue culture cell lines Caco-2, HepG2, PtK, Henle 407, and PtK2 by *L. monocytogenes.*

Immunofluorescence studies of intracellular bacterial movement within infected tissue culture cell lines revealed no discernible differences in F-actin assembly. We also compared the ability of the mutant to form plaques on mouse L929 fibroblasts with that of its parental strain. No difference in the sizes of the plaques was observed. A mean diameter of 2.0 ± 0.1 mm was obtained for both strains by measurement of 100 plaques each from three independent experiments, suggesting that in this cell line the Δ*irpA2* strain was defective for neither intracellular growth nor cell-to-cell spread.

The mouse bioassay is an extremely sensitive assay of the virulence potential of *L. monocytogenes* (17). The effect of the *irpA2* mutation on the virulence of *L. monocytogenes* was therefore assessed by LD_{50} determination following intravenous injection of the bacteria into the tail veins of mice. Compared to the wild-type bacteria, the Δ*irpA2* mutant exhibited slightly reduced virulence, since the LD_{50} for the mutant was 8×10^3 compared to 4×10^3 for the wild type. Because of the relatively small difference observed in this assay, these experiments have been repeated with smaller increments in the bacterial dose. As in the previous experiment, the LD_{50} for the mutant was determined to be 8×10^3 compared to 4×10^3 for the parental strain.

DISCUSSION

In this study we report on the cloning of a novel PrfA-regulated gene from a pathogenic strain of *L. monocytogenes* that is highly homologous to internalin A and B proteins. Since it is a new member of the internalin multigene family, we designated it internalin-related protein A (IrpA). We had previously identified and biochemically characterized the IrpA protein as a 30-kDa protein that is specifically overproduced in a strain containing multiple copies of the PrfA regulator (24). The primary sequence of the IrpA protein extends our previous observation, based then on limited peptide sequence homology, that IrpA is a further member of the internalin family of proteins in *L. monocytogenes*. In a broader context, IrpA is an additional member of a superfamily of LRR-containing proteins present in bacteria, plants, and eukaryotes which has been associated with a wide range of cellular activities at diverse cellular locations (18). Transcriptional analysis revealed that *irpA* is transcribed monocistronically by PrfA-dependent and PrfA-independent promoters. An isogenic in-frame deletion *irpA* mutant showed no loss in its invasive properties and behaved like the parental strain in the invasion of various tissue culture cell lines. It was also not compromised in its ability to grow intracellularly and to spread intercellularly, as judged by the plaque-forming assay. However, in the mouse infection model the mutant was slightly attenuated for virulence as determined by LD_{50} analysis.

We determined the sequence of a 2,100-bp region of the listerial chromosome that includes the *irpA* gene. In this region the longest open reading frame corresponded to the product of the previously described IrpA protein. Evidence for this was derived in several ways. First, all six partial peptide sequences previously determined from purified IrpA were found within the coding sequence of this gene. Second, the molecular weight of purified IrpA as determined by mass spectrometric analysis was in very close agreement with that predicted for mature IrpA (29,449 versus 29,763). Third, the sequence preceding the predicted mature protein had all the features predicted for a signal peptide, namely, a charged N-terminal end, a central hydrophobic core, and an alanine residue preceding the cleavage site for signal peptidase, and corresponded exactly to the N-terminal sequence obtained for the purified protein by microsequencing (24). Expression of IrpA in recombinant *E. coli* strains resulted in the detection of both the premature and mature forms of the IrpA protein. Finally, the creation of an isogenic in-frame deletion mutant abrogated production of the 30-kDa IrpA protein. Instead, a 13-kDa protein was detected in supernatants of the mutant strain, exactly as predicted for the deletion generated. An examination of regions flanking the *irpA* gene revealed no further extended open reading frames, and the presence of a putative rho-independent termination sequence suggested that it was transcribed as a single gene. Direct evidence that the *irpA* gene was indeed monocistronically transcribed was obtained by Northern blot analysis of RNA obtained from strain NCTC 7973 grown to different optical densities in BHI. The *irpA* transcript was detected at all stages of growth although it appeared to be produced at higher levels during exponential growth. No transcripts were detectable in the *prfA* mutant strain SLCC 53. Although we were unable to detect any transcript in the EGD strain using Northern blot analysis, reverse transcription analysis for the *irpA* message using *irpA*-specific oligonucleotides indicated that it was present in small, albeit detectable, quantities in this strain (4; data not shown). Primer extension analysis revealed the presence of two promoters, P_{irpA1} and P_{irpA2}, transcribing the *irpA* gene. The sequence of the PrfA-regulated promoter has two base substitutions within its PrfA DNA-binding site, thus resembling the PrfA-dependent promoter preceding the *inlA* and *inlB* genes. Like the *inlA* and *inlB* genes, the *irpA* gene is additionally transcribed by a weak PrfA-independent promoter. Confirmation of the presence of this second promoter comes from the detection of IrpA in supernatants of two isogenic *prfA* mutants. We note that although *irpA* transcripts seem to be of small abundance, the IrpA protein appears to be readily detectable as a Coomassie-stained band in the supernatant of *L. monocytogenes* strains. Hence, this protein is un-

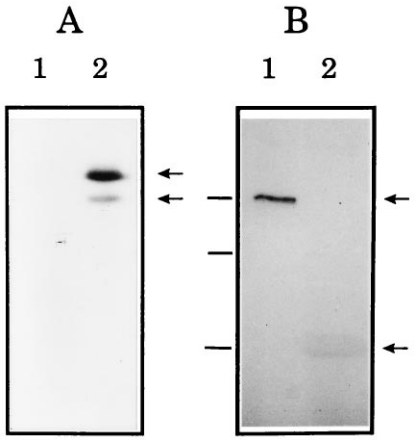

FIG. 3. Identification of the IrpA and ΔIrpA polypeptides analyzed by immunoblotting with MAb O301. (A) Immunoblot of polypeptides in whole-cell extracts of *E. coli* strains harboring either plasmid pCR-*irpA*-1 (lane 1) or pCR-*irpA*-2 (lane 2). The upper arrow indicates the unprocessed 33-kDa form of IrpA, and the lower arrow indicates the processed 30-kDa form of IrpA. (B) Immunoblot of polypeptides in culture supernatants of either *L. monocytogenes* EGD (lane 1) or its isogenic in-frame deletion mutant strain Δ*irpA2* (lane 2). The upper arrow indicates the 30-kDa wild-type form of IrpA, and the lower arrow indicates the 12.5-kDa deletion variant of IrpA. Molecular weight markers, indicated as dashes, are, from top to bottom, 30, 21.5, and 14 kDa.

usually stable, or its secretion to the supernatants of these bacteria, where there is no detectable protease activity, could account for its apparent stability.

The IrpA protein is the third and to date the smallest member of a family of LRR-containing proteins present in *L. monocytogenes*. Although all three proteins contain signal peptides and are exported proteins in *L. monocytogenes*, they are differentially localized with respect to the bacterial surface. Thus, InlA harbors a bona fide cell wall anchor at its C terminus and

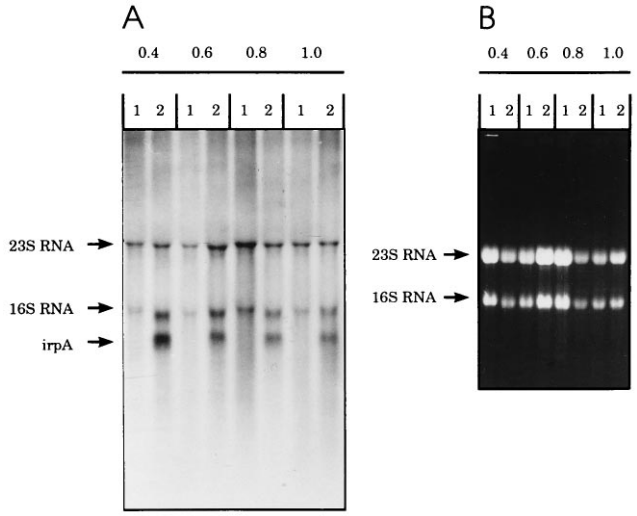

FIG. 4. Northern blot analysis of *irpA* transcripts in the *L. monocytogenes* NCTC 7973 strain (lane 2) and its isogenic SLCC 53 mutant strain (lane 1). Total RNA was isolated from strains growing at 37°C at OD_{600}s of ~0.4, ~0.6, ~0.8, and ~1.0, separated by electrophoresis, blotted onto Hybond N (Amersham), and probed with radiolabeled *irpA*-specific fragment. From the corresponding autoradiogram, a specific transcript for *irpA* of approximately 1,150 nt was detected. (B) Agarose gel electrophoresis of total RNA. Because of the extended exposure time required for detecting the *irpA* transcript, weak unspecific hybridization to the ribosomal 23S and 16S RNA is seen.

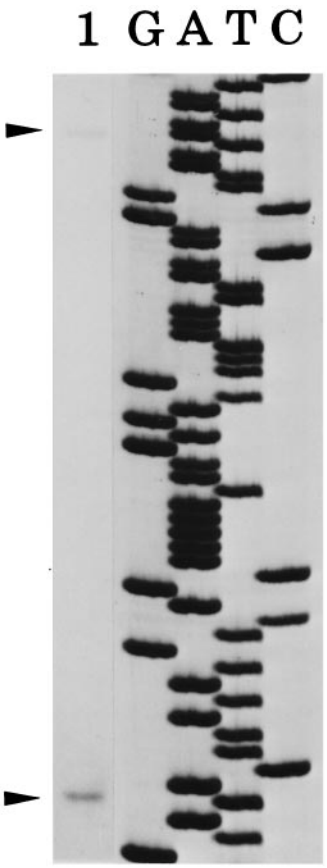

FIG. 5. Mapping and detection of *irpA* transcript in *L. monocytogenes* by primer extension analysis. γ-^{32}P-end-labeled oligonucleotide primers (see Materials and Methods) were used for reverse transcription of the transcript. The DNA products were separated on a 6% polyacrylamide gel simultaneously with a dideoxy sequencing reaction ladder, with the same DNA primer and plasmid pCR-*irpA*-2 to allow determination of the extension products. Lane 1, the primer extension reaction done with strain NCTC 7973; lanes G, A, T, and C, tracts of the sequencing reaction. Arrows indicate the detected extension products.

is located mainly on the surface of invading bacteria but can also be detected in small amounts in supernatant fluids. InlB does not contain features of cell wall-associated proteins of gram-positive bacteria but is predominantly found in this location. On the other hand, IrpA is a secreted protein found only in the supernatants of growing bacteria. Realignment of the LRRs of InlA, InlB, and IrpA show that all members of this family contain a series of 22-amino-acid-long LRRs, present 15 times in InlA, 7 times in InlB, and 6 times in IrpA (Fig. 2B). As discussed previously, LRRs are present in a number of eukaryotic proteins with diverse functions and cellular locations. In bacteria these LRRs are found exclusively in pathogenic bacteria, such as the *ipaH* gene family in *S. flexneri*, the YopM protein from *Yersinia pestis*, and the filamentous hemagglutinin protein of *B. pertussis* (18, 26). Currently, the structure of one LRR-containing protein, the porcine ribonuclease inhibitor, has been determined (19). A LRR unit in this protein consists of 28 or 29 amino acid residues comprising a β sheet followed by an α helix. However, the LRR unit present in all three listerial LRR-containing proteins is only 22 amino acids long and appears to be more representative of such repeats present in other members of this superfamily (18). It has recently been suggested that a 22-amino-acid LRR could fold significantly differently, leading to the creation of a novel right-handed helix

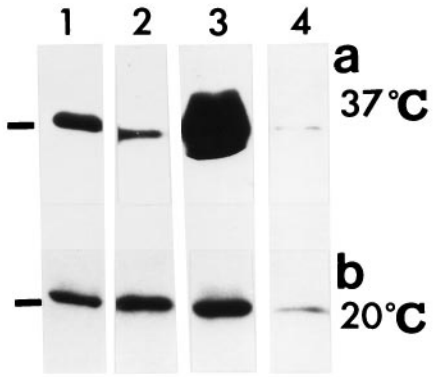

FIG. 6. PrfA- and temperature-dependent expression of IrpA. IrpA polypeptides in culture supernatants derived from *L. monocytogenes* EGD (lane 1), the *prfA1* mutant strain (lane 2), *L. monocytogenes* NCTC 7973 (lane 3), and *L. monocytogenes* SLCC 53 (lane 4) were detected by chemiluminescence with MAb O301. Note that exposure times at 37°C (a) were 2 s and at 20°C (b) were 10 s. The molecular mass markers (left) are both 30 kDa.

termed the β helix, a structure that was first detected in the pectase lyase of *Erwinia chrysanthemi* (40, 41). Since IrpA is the smallest member of this LRR prototype and biochemical purification of this protein has been achieved, crystallization studies using this protein would be of great interest to resolve the question of how these repeats are structured.

The strong similarity of IrpA to InlA and InlB suggested that IrpA might be involved in promoting the uptake of *L. monocytogenes* in tissue culture cell lines. This, however, turned out not to be the case. Indeed, the isogenic deletion mutant did not appear to lack any properties required for invasion, intracellular survival, and intercellular spread in tissue culture cell lines. In the mouse infection model a Δ*irpA* mutant exhibited a slightly reduced virulence compared to the wild type. The LD_{50} for this mutant was found to be 8×10^3 compared to 4×10^3 for the wild type in two independent sets of experiments. A modest increase in LD_{50} values was also obtained for an independently constructed *irpA* deletion mutant (Δ*irpA3*) in which the six LRRs had been precisely removed (4). Virulence phenotypes corresponding to this level of attenuation have previously been reported for the *inlA* and *inlB* mutants, which are deficient for invasion into Caco-2 cells and hepatocytes (12, 13, 25). Hence, IrpA deficiency may be too subtle to be detected in the tissue culture models and could require a complex environment, such as that observed within the host cell tissue, to manifest itself. Our current studies are directed at determining if IrpA is indeed expressed within the eukaryotic cell and at the subsequent biochemical purification of potential host factors that could associate with purified IrpA.

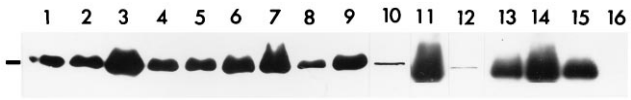

FIG. 7. Detection of IrpA polypeptides in culture supernatants derived from various serotypes and strains of *L. monocytogenes* and from *L. innocua* by immunoblotting. Proteins were separated by SDS-PAGE, and the subsequent immunoblots were processed with affinity-purified IrpA polyclonal antibodies. The following *L. monocytogenes* strains were used: EGD serovar (sv) 1/2a (lane 1), SLCC 2371 sv 1/2a (lane 2), NCTC 7973 sv 1/2a (lane 3), SLCC 2755 sv 1/2b (lane 4), SLCC 2372 sv 1/2c (lane 5), LO 28 sv 1/2c (lane 6), SLCC 7179 sv 3a (lane 7), SLCC 2540 sv 3b (lane 8), SLCC 2479 sv 3c (lane 9), SLCC 2374 sv 4a (lane 10), SLCC 1444 sv 4b (lane 11), SLCC 2376 sv 4c (lane 12), SLCC 2377 4d (lane 13), SLCC 2378 sv 4e (lane 14), SLCC 2482 sv 7 (lane 15), and *L. innocua* NCTC 11288 sv 6a (lane 16). Molecular weight marker, 30 kDa.

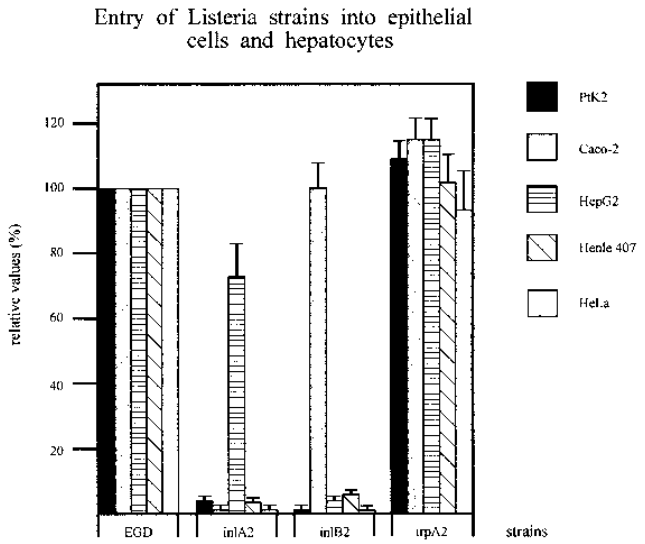

FIG. 8. Entry of *L. monocytogenes* EGD and Δ*inlA2*, Δ*inlB2*, and Δ*irpA2* mutant strains into the rat kangaroo epithelial-like cell line PtK2, the human epithelial-like cell line Caco-2, the human hepatocyte cell line HepG2, the human embryonic intestine cell line Henle 407, and the epitheloid carcinoma cell line HeLa. Gentamicin survival assays were performed as described previously (25). Values along the vertical axis are given relative to the invasion of wild-type strain EGD, which is arbitrarily fixed at 100. Results are means; error bars indicate standard deviations.

In summary, our studies have led to the identification and cloning of a novel PrfA-regulated protein from pathogenic *L. monocytogenes*. IrpA possesses strong homology to the LRR-containing InlA and InlB proteins, but unlike these proteins it is exclusively a secreted protein. Features common to the regulation of other listerial virulence factors, in particular of InlA and InlB, also operate to control production of the IrpA protein. IrpA is also only produced by the pathogenic *Listeria* species, *L. monocytogenes* and *L. ivanovii*. No differences in interaction with tissue culture cell lines in vitro could be discerned for an isogenic deletion *irpA* mutant compared to its parental strain. However, this mutant was slightly attenuated for infection of mice, suggesting that IrpA is a further novel virulence factor of *L. monocytogenes*. Finally, the use of biochemical methods to purify and characterize proteins whose production is regulated by virulence regulators is a powerful tool to identify novel virulence factors. Indeed, as our studies here suggest, none of the well-characterized in vitro assays that were instrumental for the characterization of virulence factors for *L. monocytogenes* (32) would have led to the identification of the *irpA* gene.

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