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Identification, Cloning, and Characterization of the *lma* Operon, Whose Gene Products Are Unique to *Listeria monocytogenes*

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The *lmaA* gene of *Listeria monocytogenes* encodes a protein capable of inducing delayed-type hypersensitivity reactions in *L. monocytogenes*-immune mice (S. Göhmann, M. Leimeister-Wächter, E. Schiltz, W. Goebel, and T. Chakraborty, M. Microbiol. 4:1091–1099, 1990). Here we show that it is the last gene of the *lma* operon, which now comprises four genes, *lmaDCBA*. Maxicell analysis of peptides encoded by the *lma* operon identified four polypeptides of 16.7, 16.4, 14.9, and 21 kDa which correspond to the gene products encoded by the *lmaD*, *-C*, *-B*, and *-A* genes, respectively. Northern blot analysis of the *lma* operon showed that *lmaA* is expressed by two transcripts: the longer *lmaDCBA* transcript of 2,100 nucleotides, which was observed at growth temperatures of 37 and 20°C, and a shorter transcript consisting of *lmaBA*, which is detected only at low temperatures (20°C). Two promoters, one preceding the *lmaD* gene and another located upstream of the *lmaB* gene, were detected. An extended stem-loop structure resembling box elements found in other gram-positive pathogens was also present in the *lmaC-lmaB* intergenic region. By immunoblot analysis, we found that although LmaA was produced at both temperatures (20 and 37°C), it was secreted into culture supernatants only at 20°C. However, LmaA lacks a bona fide signal peptide sequence and could, like flagellin, be secreted by a type III transport system. DNA hybridization studies indicate that the *lma* operon is species specific and restricted to pathogenic strains of *L. monocytogenes*.

The gram-positive, facultative intracellular bacterium *Listeria monocytogenes* is a foodborne pathogen responsible for infections in immunocompromised persons and pregnant women. Listerial infections can result in meningitis, meningoencephalitis, septicemia, and death (10, 12, 16). The consumption of contaminated food has been shown to be the major route of infection. In addition to causing sporadic listeriosis, *L. monocytogenes* has been implicated in several epidemics in Europe and North America (2, 37). The ability of *L. monocytogenes* to grow at 4°C, a temperature at which many off-the-shelf foodstuffs are stored, opens up a unique environment for listerial enrichment (16, 43). Listerial products that are expressed at low growth temperatures are therefore a relevant area of study.

Virulence factors necessary for entry, intracellular survival, multiplication, and motility have been previously characterized. The genes encoding these factors are organized in two clusters on the listerial chromosome. The main cluster contains the genes coding for hemolysin (*hly*), phospholipases (*plcA*, *plcB*), metalloprotease (*mpl*), and actin nucleation factor (*actA*) and includes the virulence regulator protein PrfA (6, 18–21, 27, 28). The internalin genes *inlA* and *inlB*, which are required for uptake of the bacteria into the eukaryotic cell, are arranged as an operon close to but separate from the other virulence locus (29). The expression of the *inlA* and *inlB* genes is regulated by both PrfA-dependent and -independent mechanisms (8, 9, 23, 24). By restriction fragment analysis with the endonuclease *Not*I and subsequent hybridization studies with specific gene probes for the virulence genes, Michel and Cossart (29) were able to show that the genes required for virulence are clustered in one region of the listerial chromosome. A marker that also localized to this chromosomal region was the previously published *lmaB* and *lmaA* genes (13, 29).

The *lmaA* gene was previously shown to encode a listerial antigen capable of eliciting a delayed-type hypersensitivity reaction in *Listeria*-immune mice (13). These studies also indicated that the *lmaA* gene is a highly specific marker for pathogenic *Listeria* spp. Nucleotide sequencing of the *lmaA* gene revealed that it was probably transcribed as part of an operon because (i) there were no bona fide promoter sequences preceding this gene and (ii) the *lmaA* gene was separated by a sequence of 12 nucleotides (nt), which included a putative ribosome binding site from a second open reading frame located immediately upstream.

In this communication, we report the cloning, sequencing, and characterization of the entire *lma* operon and demonstrate by hybridization studies that the *lma* genes are unique to pathogenic *Listeria*. In maxicell experiments and transcription studies, we analyzed the expression of the *lma* genes. Our studies show that the *lma* operon is differentially regulated depending on growth temperature. Interestingly, despite the lack of secretion signals, the *lmaA* protein is secreted to supernatants of bacteria in a temperature-dependent fashion.

MATERIALS AND METHODS

Bacterial strains and cultivations. The wild-type *L. monocytogenes* serotype 1/2a strain EGD and the *Listeria* spp. used in this study have been described previously (18). The *Escherichia coli* strain DH10β [F′ Δ*mcrA* (*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX174 deoR recA1* ϕ*araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL endA1 nupG*] was used for construction of the isogenic mutants as detailed below. *Listeria* spp. were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at the temperature indicated. For plasmid-containing *Listeria* strains, the medium was supplemented with 5 μg of erythromycin/ml. The recombinant *E. coli* strains were cultured in Luria-Bertani broth (Difco Laboratories, Detroit, Mich.) supplemented with 100 μg of ampicillin or 300 μg of erythromycin/ml depending on the plasmid.

Plasmids. The plasmid cloning vectors pUC19 (46) and pAULA (3) were previously described. The plasmid pLMB1, for expression of *lmaB*, was constructed by amplifying a 964-bp PCR product with oligonucleotides M1 and F1 (Table 1). After digestion of the purified fragment with the restriction endonuclease *Ssp*I, the resulting 490-bp DNA fragment was cloned into the cloning

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TABLE 1. Oligonucleotides used in these studies

Oligonucleotide	Sequence (5′→3′)	Application
B1	GGCGCTTTACCTGCTTCGAC	Construction of pLMC1
D1	GCGGTAATGATGGATTTACCG	Construction of *lmaA* deletion mutant
D2	CGACAAATACTTACTTCAAA	*lmaB* hybridization probe (Northern)
E2	TTCTGCTGGTGCTACAGGTG	*lmaA* hybridization probe (Southern)
F1	CGGTTTACAGTTAGACCTTGTTGGCC	Construction of *lmaA* deletion mutant; *lmaB* hybridization probe (Southern); construction of pLMB1
F2	GGCCAACAAGGTCTAACTGTAAACCG	*lmaA* hybridization probe (Southern); PCR to verify Δ*lmaA*
G1	GGCAATTGTAGCTGACTATAG	Construction of *lmaA* deletion mutant
G2	GCTGCAACTAAACTAATCCCTGATGTTAATTCCACCG	Construction of *lmaA* deletion mutant
H1	GGAAGTGGGAATGGATGGATAG	*lmaDC* hybridization probe (Northern); *lmaD* hybridization probe (Southern); amplification of *lmaDC* in RT-PCR
H2	CGGTAAATCCATCATTACCGC	*lmaDC* hybridization probe (Northern); *lmaC* hybridization probe (Southern)
J1	GGCGGACTAGATATTTCTAT	*lmaB* hybridization probe (Northern)
K1	CCGGGAGCTGCTAAAGCGGT	*lmaA* hybridization probe (Northern)
K2	GCCAAACCACCGAAAAGACC	*lmaA* hybridization probe (Northern); amplification of *lmaCBA* in RT-PCR
L1	TGTCATCCGCTTACTGGACA	Amplification of *lmaDC* in RT-PCR; construction of pLMC1
M1	GATGGAATGTTACGTGGAACG	Amplification of *lmaCBA* in RT-PCR; construction of pLMB1
N1	CTCTTCTATCAAACGCTTTCG	Primer extension of *lmaD*
P1	CTGGAGCATTGTTCTCACTGCTTCC	Primer extension of *lmaB*
P2	CTCGATCATCGTTGTAGGC	Primer extension of *lmaB*
Q1	CTCATACTGACATTCAATCGCCG	*lmaD* hybridization probe (Southern)
R1	GAGCGGTTTCGGATGATTACG	*lmaC* hybridization probe (Southern)
R2	GGAAGCAGTGAGAACAATGCTCCAG	*lmaB* hybridization probe (Southern)
S1	CCCTTTATTCGCAGTCAAAGCTG	PCR to verify Δ*lmaA*

vector pUC19. The *lmaC* gene was cloned by amplifying a 575-bp PCR fragment with the primer set B1/L1 to give the plasmid named pLMC1. For generating a recombinant plasmid encoding LmaD, we digested the plasmid pLM68 (35) with *Eco*RV and cloned the DNA fragment blunt end into the cloning vector pUC19, which was digested with *Sma*I. The resulting recombinant plasmid was named pLM68.1, and it was used for further subcloning. To do this, pLM68.1 was digested by restriction endonuclease *Hin*dIII to give two fragments, and the 750-bp fragment was inserted into pUC19. The resulting plasmid was named pLM68.1.1 and encodes the LmaD polypeptide. For expression of *lmaA*, we chose the recombinant plasmid pLM10, which was described previously (13). The inserts of all constructs used in this study were verified by DNA sequencing.

DNA sequence analysis and homology searches. The nucleotide sequence of the *lmaD* gene was determined from double-stranded plasmid templates by dideoxy sequencing reactions (34). 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-^{32}P]dATP (1,000 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). The labeled reaction products were separated by electrophoresis on urea–6% polyacrylamide gels and dried down, and the sequences were read from X-ray autoradiograms.

To search for homologies, we used the BLASTP (1) and FASTA (32) programs within the Husar-GCG environment at the Deutsche Krebsforschung Zentrum, Heidelberg, Germany.

RNA isolation, Northern (RNA) blot analysis and RT-PCR. Total RNA was isolated from *L. monocytogenes* (optical density at 600 nm of ~0.8) growing at 37 or 20°C by the hot-phenol extraction method (21). RNA analysis was performed by formaldehyde gel electrophoresis. RNA samples (15 μg each) were electrophoretically separated onto a 1.3% agarose gel, transferred onto nylon filters (Hybond N; Amersham), and hybridized with the appropriate radiolabeled DNA fragments under conditions specified by the vendor (11). The probes were generated by PCR amplification with the following oligonucleotides: H1 and H2 for the detection of *lmaDC* (787 bp), J1 and D2 for the amplification of the *lmaB* probe (308 bp), and the primer set K1 and K2 for the generation of the *lmaA* probe (326 bp). Reverse transcription-PCR (RT-PCR) studies were performed with the SuperScript Preamplification System for first-strand cDNA (Gibco, Life Technologies, Eggenstein, Germany) as described by the manufacturer. As specific oligonucleotides for amplification of *lmaDC*, the primer set L1/H1 was used, and for amplification of *lmaCBA* cDNA, the oligonucleotides M1 and K2 were used.

Primer extension studies. For primer extension studies, the oligonucleotides N1, P1, and P2 located 66 bp downstream of *lmaD* and 29 or 104 bp downstream of *lmaB*, respectively, were used to determine the transcriptional end points. The oligonucleotides were labeled at their 5′ ends, and primer extension analyses were performed with the recombinant virus reverse transcriptase (Superscript; Life Technologies) as described previously (20) with a total of 40 μg of RNA (*lmaD*) or 80 μg of RNA (*lmaB*). Dideoxy sequencing reactions (34) with the same primer and an appropriate plasmid DNA template were run in parallel.

Electrophoretic analysis of plasmid-encoded polypeptides. Analysis of plasmid-encoded translational products in an *E. coli* maxicell system was described previously (40).

Southern hybridization. Listerial chromosomal DNA was isolated after lysis of bacteria (14). A total of 5 μg of the DNA was digested with the restriction endonuclease *Hin*dIII (New England Biolabs), separated on an 0.7% agarose gel overnight (30 V), and transferred to positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany) as described by Southern (39). Digoxigenin (Boehringer Mannheim) was used for labeling DNA probes during PCR amplification. Primer pairs E2/F2 (*lmaA* probe), F1/R2 (*lmaB* probe), R1/H2 (*lmaC* probe), and H1/Q1 (*lmaD* probe) were used for PCR amplification. Hybridization was carried out under conditions of high stringency as described by the manufacturer.

PCR amplification of DNA. DNA fragments of *L. monocytogenes* EGD and the *lmaA* deletion mutant were amplified by PCR with chromosomal DNA or single colonies as template in a final volume of 100 μl containing deoxyribonucleoside triphosphates (each at 100 mM), oligonucleotides (20 pmol each; for sequences and applications, see Table 1), and 2.5 U of *Taq* DNA polymerase (Eurobio, France). Reactions were performed using the GeneAmp PCR System (Norwalk, Conn.) as follows: 25 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s.

Construction of the *lmaA* in-frame deletion mutant. The *lmaA* deletion was generated by cloning PCR fragments into the cloning vector pAULA. For generating the *lmaA* deletion, the oligonucleotide pairs D1/F1 and G1/G2 were used for the amplification of two fragments of 930 and 296 bp. The 3′ ends of the PCR products were modified by using the SureClone Ligation Kit (Pharmacia Biotech, Freiburg, Germany), and the two fragments were ligated in a 6-h ligation reaction. The products of this reaction were selectively amplified with the oligonucleotides D1/G2 and cloned into the temperature-sensitive shuttle vector pAULA. Then plasmid transformation into *L. monocytogenes* EGD was done via electroporation (30), and incubation was done on brain heart infusion on agar plates supplemented with 5 μg of erythromycin per ml at 28°C. Recombinant clones were used for integration and excision events as previously described (23). The *lmaA* deletion mutant was verified using PCR amplification with oligonucleotides S1/F2 (*lmaA*) and sequencing (data not shown).

Production of antisera to flagellin and LmaA. Recombinant LmaA was purified from *E. coli* recombinants harboring the plasmid pLM10 by an as yet

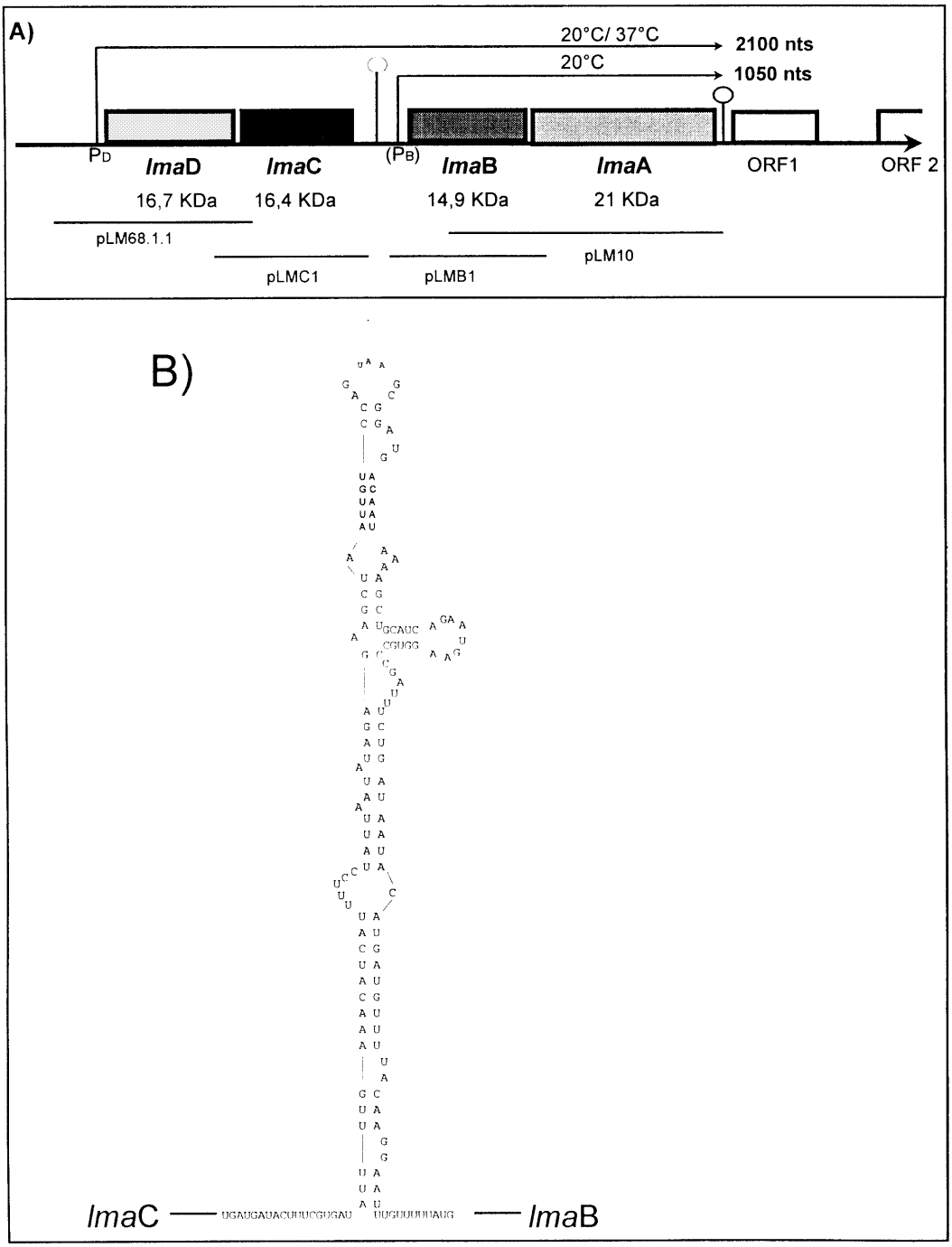

FIG. 1. (A) Genetic organization of the *lma* operon of *L. monocytogenes* EGD. The *lma* genes are represented as shaded boxes, and the molecular masses of the encoded polypeptides are given below the boxes. The Rho-dependent terminator between *lmaA* and open reading frame 1 (ORF1) is depicted, as is the intergenic stem-loop structure between *lmaC* and *lmaB*. Arrows indicate the two transcripts and the temperature at which they were detected together with the transcriptional start sites of *lmaD* (P_D) and *lmaB* (P_B). The different recombinant plasmids used in maxicell experiments are also indicated. (B) The mRNA structure of the intergenic *lmaC*/*lmaB* region following simulated folding shows a characteristic stem-loop structure. The change in free energy upon formation reveales a ΔG of -37 kcal/mol.

unpublished protocol (5). Briefly, cultures were grown for 3 h at 37°C in Luria-Bertani medium, and LmaA expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Growth continued for another 3 h. Under these conditions, about 30% of the total LmaA produced by this strain is present in its culture supernatant. This fraction was used for further purification. Following precipitation with 60% ammonium sulfate, the sample was dialyzed extensively against 20 mM Tris HCl (pH 7.4). The supernatant was loaded onto a Mono Q column equilibrated with the same buffer, and LmaA was isolated using a linear gradient of 0 to 250 mM NaCl. The protein eluted at around 100 mM NaCl and was dialyzed against phosphate-buffered saline and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To confirm that the protein isolated was indeed LmaA, N-terminal sequencing of the purified protein was performed. The N-terminal 14 amino acids of the purified protein were determined to be NH_2-MAFEENLYCDYTPG and corresponded exactly to the N-terminal primary sequence for this protein (see Fig. 3).

Flagellin from *L. monocytogenes* EGD was purified by the method of Peel et al. (32). Rabbits were immunized intradermally with approximately 25 μg of

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LmaA:  MAFEENLYCDYTPGAAKAVAGKDVILAVFNAAGDKLLAVAGQQGLTVNRSKDSIEITSKDTV
       GGWKSKIGGMKEWSIENDGLYVADAESHKELAKYFESDSPVCVKIINQASKKGLFGGLAIVA
       DYSFEAPFDEAMTYSVKLDGMGALVDLTITEGGDQMPGETPVAPAE - 170 aa

LmaB:  VKSLSFMRVLEAVRTMLQEKGGLDISIVMRDQVEMPTTMIEMIDQEEEESQTAWKEKYRFAI
       HHYTNETDLAGVEKIDTLIQTGFTLPEGYKLIAVRHYGKQNLVKENTLIHAKTSFEVSICRE
       LKVKI - 129 aa

LmaC:  MQVLVLPENKDINYIKTVQEVKRFFADFERFRMITGLSKKPHLLRNGFLEEPQFEPVAFSAR
       HNKEVILEARWLVEKYTEMLNQMDDLYRTILMECYVERKQDVAVMMDLPYEIAQFKRIKKRA
       VLELATLMGILVRK - 138 aa

LmaD:  MDRKLLKEKQIQLIFQLEQEENRFIRKRLIEELEFFEALGDREKGLLTAEQKLLILTPSEYR
       EYKRTKSDVQISRIIGVSRSSLAEWKRKKGLNRKKSQPVQQEMIDVLAFHLDKTKEEIGALP
       ASAIECQYEAFVINEANN - 142 aa
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FIG. 2. Peptide sequences of LmaA, -B, -C, and -D from *L. monocytogenes*. The entire nucleotide sequence of the *lma* operon has been deposited in the EMBL database. aa, amino acids.

purified LmaA or flagellin emulsified with complete Freund's adjuvant. At 3 and 6 weeks postinjection, animals were boosted with further doses (25 μg) of antigen in incomplete Freund's adjuvant. Sera collected at various times following immunization were collected and tested for their reactivities to purified protein by Western blot analysis or enzyme-linked immunosorbent assay.

Protein preparation. Supernatants (10 ml) from an overnight culture of *Listeria* spp. were precipitated with trichloroacetic acid, washed twice with acetone, and suspended in 100 μl of sample buffer containing 5% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, and 11% (vol/vol) glycerin. For preparation of the cytoplasmic proteins, protoplasts were first generated as described elsewhere (45), centrifuged, and lysed by suspending the pellet with 1 volume of sterile water.

PAGE and immunoblots. Protein samples (10 μl) were boiled for 3 min and loaded onto a 10 to 20% gradient polyacrylamide gel, and electrophoresis was run in the presence of SDS. For immunoblot reactions, proteins were transformed to a nitrocellulose membrane and reacted with either flagellin (1:2,000) or LmaA antibodies (1:1,000). Then a secondary antibody was coupled with alkaline phosphatase. The enzymatic reaction was performed in the presence of 1 mg of BCIP (5-chloro-4-bromo-3-indolylphosphate)/ml.

Nucleotide sequence accession number. The nucleotide sequence data reported in this study have been deposited in the EMBL and GenBank databases under the accession number Y09161.

RESULTS

DNA sequence analysis of the *lma* genes of *L. monocytogenes*. In a previous study, we demonstrated the cloning of the entire *lma* region of *L. monocytogenes* (35). Restriction endonuclease analysis and sequencing of a 3,027-bp region around the *lmaA* gene revealed the existence of three further open reading frames. The *lmaA* gene is the most distal and is followed by a terminator sequence immediately 3′ of its TAA termination codon. The entire operon is depicted in Fig. 1. The open reading frames preceding *lmaA* encode polypeptides with predicted molecular masses of 16.7, 16.4, and 14.9 kDa. We have designated these genes *lmaD*, *lmaC*, and *lmaB*, respectively (for peptide sequences, see Fig. 2). The intergenic regions between *lmaD* and *lmaC* and between *lmaB* and *lmaA* are relatively short, consisting of 10 and 12 nucleotides, respectively. However, the *lmaC* and *lmaB* genes are separated by an intergenic region of 277 bp. A sequence with features of the −35 and −10 regions of bacterial promoters was detected only upstream of the most proximal open reading frame, suggesting that these genes were transcribed in an operon. Homology searches with the predicted primary sequences of the various open reading frames were performed but revealed no significant sequence similarities to other known proteins in current databases.

Electrophoretic analysis of plasmid-encoded polypeptides. To verify expression of the cloned *lmaDCBA* genes, we performed in vivo transcription/translation analysis using the *E. coli* maxicell strain CSH26ΔF6 (40). To analyze the plasmid-encoded polypeptides, recombinant plasmids harboring individual *lma* genes were constructed. This was important because it allowed us to distinguish between the various Lma polypeptides, which are small polypeptides with highly similar molecular weights. The plasmids constructed for this study and the sizes of their inserts are shown in Fig. 1.

All plasmids, including the vector plasmid pUC19, were transformed into *E. coli* CSH26ΔF6, and the plasmid-encoded polypeptides were labeled with [^{35}S]methionine following UV treatment of the bacterial host cell. The corresponding autoradiogram of the electrophoretically separated polypeptides is shown in Fig. 3. The estimated molecular masses of 15 kDa for *lmaB* and 16 kDa for *lmaC* and *lmaD* were in close agreement with their calculated molecular masses, which were based on their primary sequences. Only the polypeptide corresponding to *lmaA* showed aberrant migration in SDS-PAGE, giving an apparent molecular mass of 21 kDa compared to its predicted molecular mass of 18 kDa.

Transcriptional analysis of the *lma* region of *L. monocytogenes*. To examine for RNA transcripts deriving from the *lma* region, total RNA was isolated from *L. monocytogenes* EGD grown at 20 and 37°C and subjected to Northern blot analysis. The RNA preparations were probed using different PCR-generated fragments that comprised the *lmaDC, lmaB*, or *lmaA* gene. Two results were obtained in this study. First, although relatively

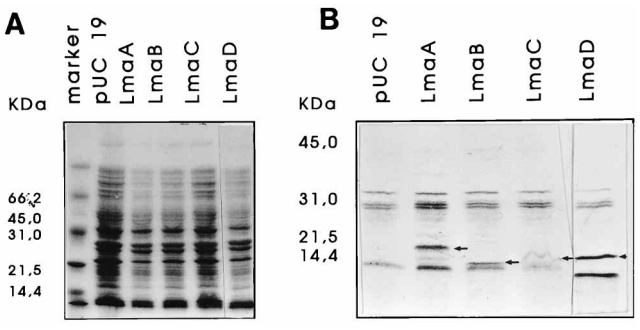


FIG. 3. Maxicell experiment for transcription and translation analysis of the plasmid-encoded Lma polypeptides. Results of SDS-PAGE (A) and the corresponding autoradiogram (B) after labeling of polypeptides with [^{35}S]methionine are shown. The positions of the Lma proteins indicated above the lanes are marked by arrows. Plasmids used were pUC19 (for vector-encoded sequences), pLM10 (for LmaA), pLMB1 (for LmaB), pLMC1 (for LmaC), and pLM68.1.1 (for LmaD). Molecular mass markers are as given above.

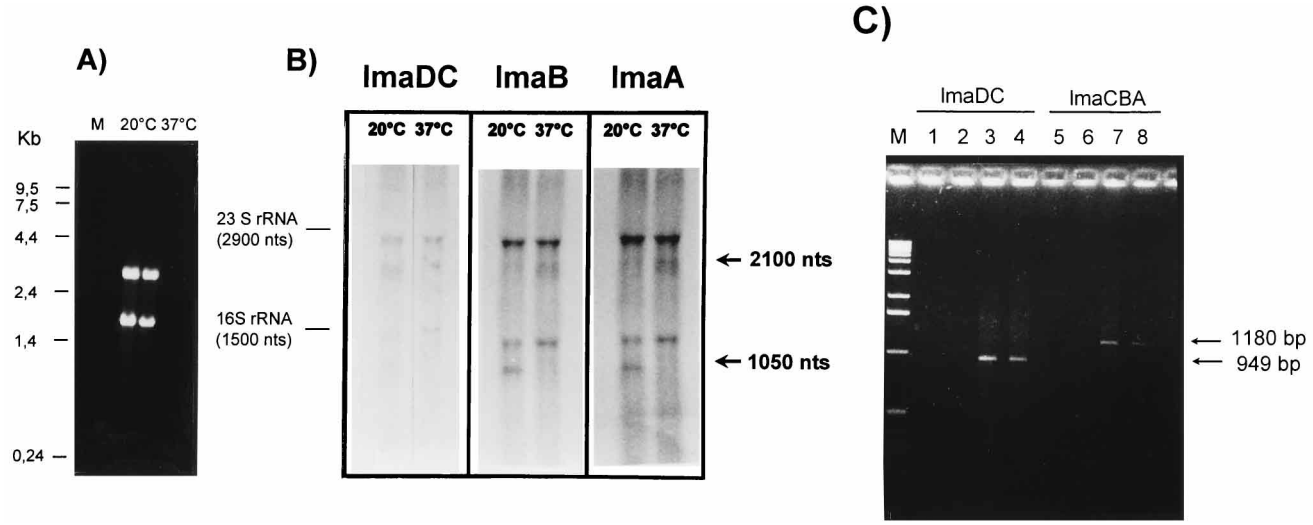


FIG. 4. Northern blot analysis of the *lma* transcripts in *L. monocytogenes*. Total RNA was isolated at 20 or 37°C. (A) Agarose gel electrophoresis with total RNA. Molecular size markers are as indicated in the figure. (B) The corresponding autoradiogram after hybridization with radiolabeled *lmaDC, lmaB,* and *lmaA* probes. (C) Amplification products and *lmaDC* and *lmaCBA* fragments following RT-PCR with specific oligonucleotides. Total RNA was isolated at 20°C (lanes 1, 3, 5, and 7) or 37°C (lanes 2, 4, 6, and 8) and digested with RNase-free DNase. One half of the RNA was incubated without reverse transcriptase (lanes 1, 2, 5, and 6), and the other half was incubated with reverse transcriptase (lanes 3, 4, 7, and 8). Molecular size markers (lane M, from bottom to top): 0.5, 1, 1.6, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 kb.

weak, a 2,100-nt transcript encompassing the *lmaDCBA* genes was detected at both growth temperatures. Second, a shorter transcript of 1,050 nt that harbored the *lmaBA* genes was specifically detected at the lower growth temperature (Fig. 4B). These results indicated that *lmaBA* is transcribed both independently and in combination with *lmaDC*. The detection of polycistronic mRNA provides strong evidence that *lmaDCBA* is transcribed as an operon.

We also performed RT-PCR with specific oligonucleotides for further confirmation of the results of the Northern blot analysis. The cDNA was obtained by reverse transcription of total RNA (isolated from cultures grown at 20 and 37°C) and was used as DNA template to amplify the overlapping *lmaDC* and *lmaCBA* fragments. To exclude DNA contamination, each reaction mixture was prepared in duplicate, but only one reaction mixture was supplemented with reverse transcriptase. This quantitative analysis confirms the presence of a polycistronic *lmaDCBA* transcript (Fig. 4C).

To analyze for promoters upstream of *lmaD* and *lmaB*, we used specific oligonucleotides derived from the 5′ end of each gene. Total RNA from *L. monocytogenes* EGD grown at 20 and 37°C, respectively, was isolated for mapping of the transcriptional start sites. For *lmaD*, the 5′ end of the transcribed RNA lay 13 bp upstream of its initiation codon ATG. A putative promoter with highly conserved −10 and −35 sites was found. The corresponding sequences are depicted in Fig. 5C. In the case of the *lmaB* promoter, we were able to map the 5′ end of this transcript only with RNA isolated from cultures grown at 20°C. In contrast to the promoter of *lmaD*, the promoter of *lmaB* shows weakly conserved consensus sequences when compared with bacterial promoters (Fig. 5C). We noticed a sequence with strong homology to the consensus sequences for prokaryotic promoters further downstream of the mapped *lmaB* promoter. Nevertheless, primer extension experiments using the oligonucleotide P2 with different amounts of total RNA failed to confirm that this sequence is a promoter, suggesting that the promoter with the weaker consensus sequence is indeed required for *lmaBA* transcription at lower growth temperatures.

Expression and localization of LmaA. Since the LmaA protein is an important antigenic component of *L. monocytogenes* in manifestation of the delayed-type hypersensitivity response in *Listeria*-immune mice (13), we raised specific rabbit polyclonal antibody to purified recombinant LmaA to examine its localization in *L. monocytogenes*. Additionally, antibodies were used to further corroborate the data on temperature-dependent expression of the LmaA protein obtained from transcriptional analysis. Supernatant proteins obtained from strains *L. monocytogenes* EGD and NCTC 7973 and *Listeria innocua* 6b grown at 4, 20, and 37°C, respectively, were isolated by trichloroacetic acid precipitation, subjected to SDS-PAGE, transferred to nitrocellulose filters, and treated with specific rabbit anti-LmaA antibodies. The 21-kDa polypeptide specifically detected by the antisera was shown to be LmaA by two findings: first, no cross-reacting polypeptide was observed in an *L. innocua* strain which we had previously shown to lack the *lma* region (see also Fig. 8), and second, supernatants from an isogenic Δ*lmaA* mutant showed no cross-reaction with the anti-LmaA antisera. We observed (Fig. 6 and 7) that although LmaA was detectable at both growth temperatures in cytoplasmic fractions, it was secreted only in cultures grown at 20°C. This effect was specific and was observed for two different strains of *L. monocytogenes* (Fig. 6). The presence of LmaA in bacterial supernatants at lowered temperatures correlated with the presence of flagellin, whose expression has been shown to be temperature regulated (7) (Fig. 6). However, in contrast to LmaA, which could be detected both in the cytoplasm and in the supernatants of listerial cultures, flagellin is not detectable in the cytoplasmic fraction (data not shown).

Detection of sequences homologous to the *lma* operon in *Listeria* spp. The DNA probes used in this analysis were generated by using specific oligonucleotide primer pairs to amplify individual *lma* genes and are depicted in Fig. 8B. A total of 19 strains comprising all known species of *Listeria* were used in this study. In the analysis described, genomic DNAs from all strains were isolated and digested with the restriction endonuclease *Hin*dIII. Using individual *lma* probes, we were able to

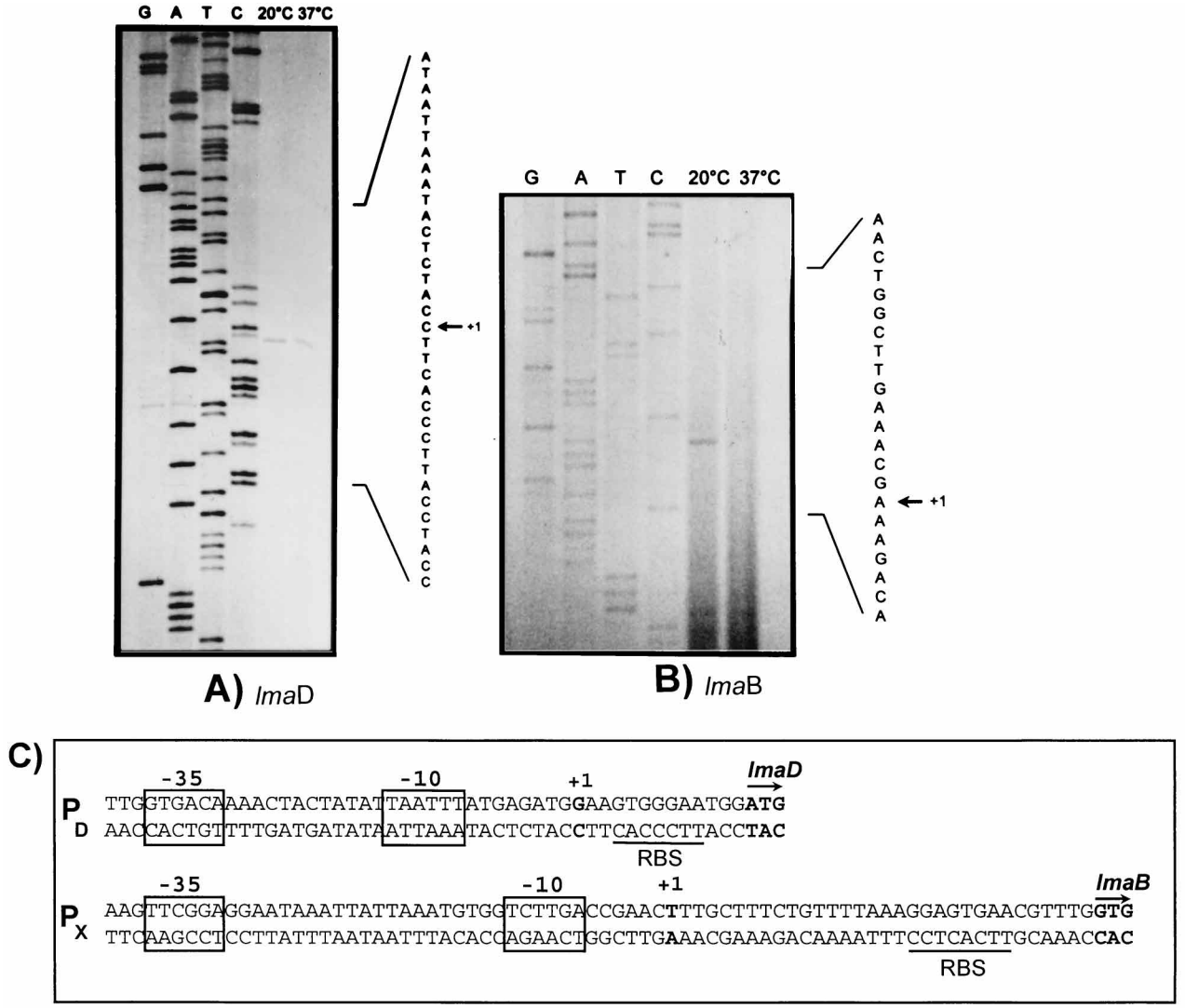


FIG. 5. Mapping and detection of *lmaDCBA* (A) and *lmaBA* (B) transcripts (isolated at 20 and 37°C, respectively) in *L. monocytogenes* by primer extension analysis. γ-^{32}P-end-labeled oligonucleotide primers (see Materials and Methods) were used for reverse transcription of the transcripts. The DNA products were separated on a 6% polyacrylamide gel simultaneously with a dideoxy sequencing reaction ladder, with the same DNA primer and plasmid pLM68 to allow determination of the extension product. The letter above each lane indicates the dideoxynucleotide used to terminate the reaction. Parts of the sequence are indicated at the right side of each panel, and the transcriptional start point is indicated with an arrow as +1. (C) Nucleotide sequences of the regions preceding the *lmaD* and *lmaB* genes. The positions of the transcriptional start sites for P_D and P_B promoters preceding the *lma* genes are indicated by +1. The −10 and −35 regions are boxed.

show that all determinants of this operon were highly specific for *L. monocytogenes.*

An example of this analysis that uses an *lmaB* probe is shown in Fig. 8A. A 3.5-kb *Hin*dIII fragment from *L. monocytogenes* EGD was detected with the *lmaB* probe, while a 2-kb fragment was detected in serotype strains 1/2a, 1/2c, and 3c. In serotype strains 1/2b, 3b, 4b, 4d, 4e, and 7, a *Hin*dIII fragment of only 900 bp was observed to hybridize to the probe. These results suggest considerable restriction fragment length polymorphism in this chromosomal region. Interestingly, *lmaB* and *lmaA* were detectable neither in nonpathogenic species nor in attenuated *L. monocytogenes* strains such as L99 and 4c.

DISCUSSION

Data obtained previously from cloning the *lmaA* gene had suggested that this gene is part of an operon of genes (17, 35). Conventional strategies used to screen several genomic libraries, however, did not yield additional DNA segments upstream of the fragment already present in the plasmid pLM10. Using a strategy that combined both insertional mutagenesis and directional cloning, we succeeded in obtaining recombinants that allowed characterization of the entire *lma* operon (35). The open reading frames predicted a cluster of four genes, which we designated *lmaDCBA* and which were shown to encode proteins of 16.7 kDa (LmaD), 16.4 kDa (LmaC), 14.9 kDa (LmaB), and 21.0 kDa (LmaA). Database searches performed with all Lma proteins revealed no significant sequence homologies to known proteins in the present databases. Also, no significant structural entities could be discerned by using the secondary-structure prediction programs within the Genetics Computer Group software package.

Northern blot analysis confirmed that *lmaDCBA* were transcribed as a polycistronic message. Because of the weak signals obtained in these studies, these results were further corroborated by using the more sensitive RT-PCR technique. Surpris-

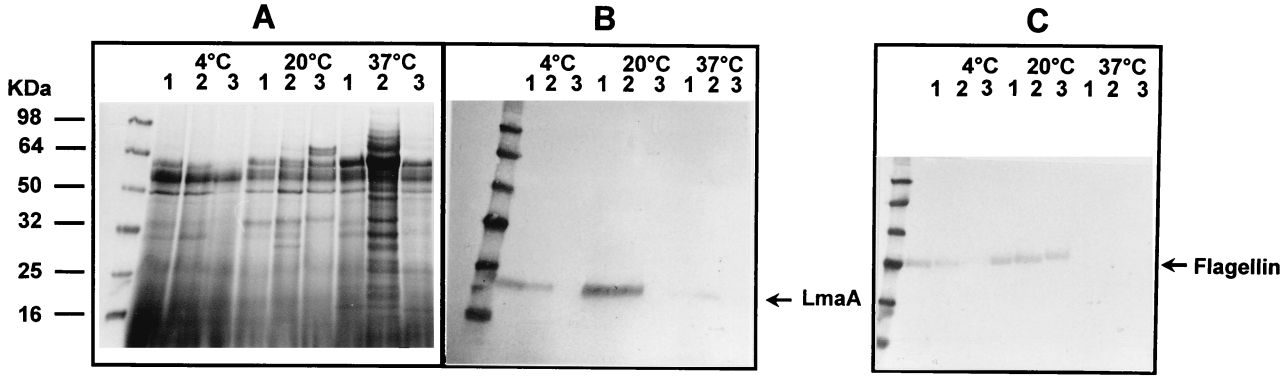


FIG. 6. Analysis of the proteins from supernatants of *L. monocytogenes* EGD (lanes 1), *L. monocytogenes* NCTC 7973 (lanes 2), and *L. innocua* 6b (lanes 3) grown at 4, 20, and 37°C, respectively. The Coomassie-stained SDS gel (A) and corresponding immunoblots after detection with LmaA (B) and flagellin-specific antibodies (C) are shown.

ingly, in bacteria grown at 20°C, we noticed an additional transcript of 1,050 nt that encompassed only the *lmaBA* genes. The 5′ ends of both transcripts were mapped by primer extension analysis. The RNA start site of the *lmaDCBA* transcript was only 13 nt upstream of the ATG initiation codon, with highly conserved −10 and −35 promoter consensus sequences. The point of initiation of the shorter 1,050-nt-long *lmaBA* transcript was mapped to 26 bp upstream of the translational start site of *lmaB*. Examination of the sequences −10 and −35 upstream of the *lmaBA* transcript showed no significant homologies to similar regions of known promoters, suggesting that transcription from this promoter might require additional factors or is mediated by changes in DNA structure. Such explanations would be in keeping with the observation that this transcript is only conditionally expressed, viz., at low growth temperatures.

An interesting feature of the *lma* operon is the 277-bp-long intergenic region between *lmaC* and *lmaB*, which contrasts with the 10 bp separating *lmaD* and *lmaC* and the 12 bp separating *lmaB* and *lmaA*. Simulated folding of the mRNA structure of this region indicated the presence of an extended stem-loop structure comprising 128 bp with a ΔG of −37 kcal/mol (17). This structure is reminiscent of the so-called BOX sequences detected in *Streptococcus pneumoniae* (25). In that species, 25 copies of the BOX sequence have been detected, and all of them are located in the intergenic regions of genes associated with the expression of virulence or competence. For genes involved in colony opacity of *S. pneumoniae*, the BOX element has been shown to be required for expression of phase variation at high frequency (33). Although there are no obvious sequence homologies between the structure described here and the streptococcal BOX elements, these sequences appear to be species specific and may be conserved only in terms of function. Seen this way, it is tempting to speculate that the temperature-dependent expression of the *lmaBA* transcript could result from stable interactions within this structure. Hence, local changes in DNA structure could lead to exposure of binding sites for regulatory factors proximal to the P_B promoter. Mutational analysis of the putative structure will be required to shed light on its role in promoting expression of P_B.

To obtain further evidence for the temperature-dependent expression of transcripts produced within the *lma* operon, we used immunoblotting with specific LmaA antisera to examine its expression. LmaA was produced at both temperatures and could be detected in cellular extracts of bacteria grown at 20 or 37°C. Surprisingly, LmaA was found to be secreted in cultures growing at both 20 and 4°C but not at 37°C. This was documented for two strains used in this study and has been observed for all other LmaA-producing *L. monocytogenes* strains. These data are reminiscent of observations made with the secreted Ipa proteins of *Shigella flexneri*, which suggest that the translocation of Ipa proteins is temperature dependent; i.e., that although these proteins are produced at different growth temperatures, they are secreted only at 37°C (26, 44). Since *L. monocytogenes* is capable of growing at low temperatures, it is possible that novel protein transport systems and surface structures that contribute to the survival of *L. monocytogenes* are conditionally expressed under these circumstances. The lack of a signal peptide sequence in the primary structure of *lmaA* suggests that it may be exported by a type III secretion pathway (for reviews, see references 26 and 41).

Recently a listerial antigen that associates with a murine minor histocompatibility class Ib molecule, H2-M3, was identified (22). The gene encoding *lemA*, for *Listeria* epitope with

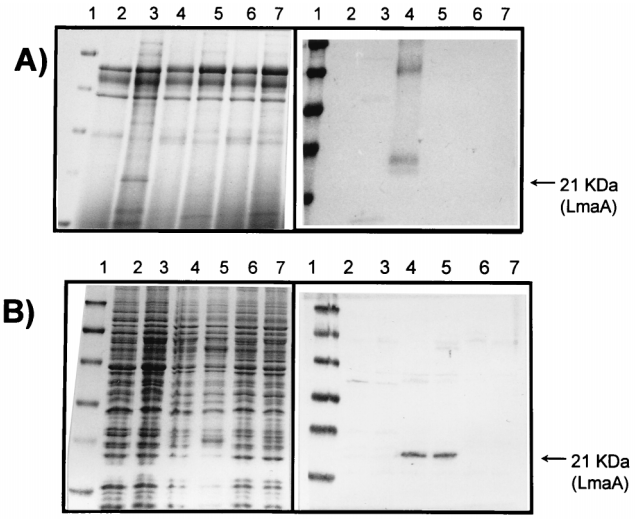
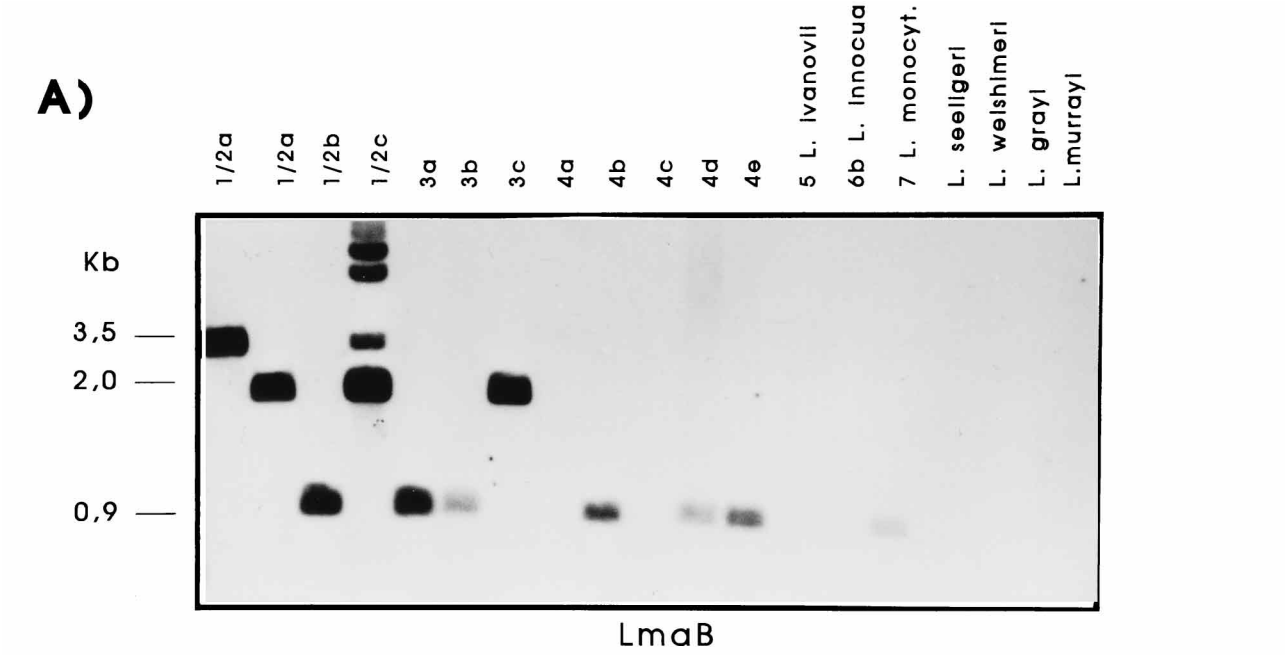
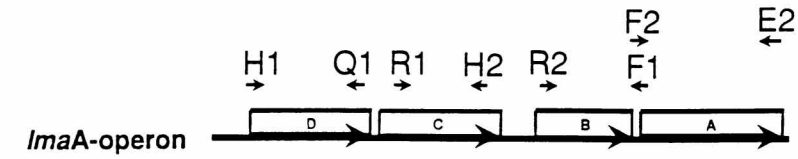


FIG. 7. Analysis of proteins from supernatant and cytoplasm of various *Listeria* strains with specific LmaA antibodies. The localization of LmaA in the supernatant (A) and cytoplasm (B) with overnight cultures grown at 20 and 37°C, respectively, is shown. Lanes 1, molecular mass markers (from top to bottom): 98, 64, 50, 32, 25, and 16 kDa; lanes 2 and 3, *L. innocua* 6b (20 and 37°C, respectively); lanes 4 and 5, *L. monocytogenes* EGD (20 and 37°C, respectively); lanes 6 and 7, *L. monocytogenes* Δ*lmaA1* (20 and 37°C, respectively).



B)



strain		serotype	*lma*D	*lma*C	*lma*B	*lma*A
L. monocytogenes	EGD	1/2a	+	+	+	+
L. monocytogenes	NCTC 7973	1/2a	+	+	+	+
L. monocytogenes	SLCC 2750	1/2b	+	+	+	+
L. monocytogenes	LO28	1/2c	+	+	+	+
L. monocytogenes	NCTC 5105	3a	+	+	+	+
L. monocytogenes	SLCC 2540	3b	+	+	+	+
L. monocytogenes	SLCC 2479	3c	+	+	+	+
L. monocytogenes	L99	4a	+	+	-	-
L. monocytogenes	SLCC 4013	4b	+	+	+	+
L. monocytogenes	ATCC 19116	4c	+	+	-	-
L. monocytogenes	ATCC 19117	4d	+	+	+	+
L. monocytogenes	ATCC 19118	4e	+	+	+	+
L. monocytogenes	SLCC 2482	7	+	+	+	+
L. ivanovii	ATCC 19119	5	-	-	-	-
L. seeligeri	SLCC 3945	1/2b	-	-	-	-
L. welshimeri	SLCC 5334	6a	-	-	-	-
L. innocua	ATCC 33091	6b	-	-	-	-
L. grayi	RIVM 1		-	-	-	-
L. murrayi	RIVM 2		-	-	-	-

FIG. 8. (A) Autoradiogram of the Southern blot of chromosomal DNA from different strains digested with restriction endonuclease *Hin*dIII and hybridized to specific DNA probes, which are described in Materials and Methods. (B) Summary of the data obtained with probes containing the *lmaABCD* genes. Oligonucleotides used for PCR generation of DNA probes are indicated.

M3, predicts a 17-kDa protein that is unique and unrelated to proteins in current databases. The *lemA* gene is part of a cluster of five genes, and the available sequence information suggests that this antigen may also be part of an operon. Interestingly, although this protein lacks a signal peptide sequence, predictions of secondary structure suggest that it may be a membrane-associated protein. We considered it likely that transport of the Lem antigen is also effected by a type III secretion pathway postulated for this bacterial species.

DNA hybridization studies allowed us to extend our previous observations regarding the specificity of the *lmaA* gene for pathogenic *Listeria*. Hence, the entire *lma* operon was detected in pathogenic *L. monocytogenes* strains. In attenuated strains such as the serotype 4a strain L99 and the serotype 4c strain, no *lmaBA*-homologous sequence was detected (4). However, these strains showed hybridization with *lmaD* and *lmaC* probes, suggesting that they probably harbor a deletion of the *lmaBA* genes. Heterogeneity in the expression of virulence traits in strains belonging to serogroup 4 has repeatedly been reported in the literature (4, 38). We also analyzed *L. innocua* 6a, a serotype in which several strains have previously been reported to harbor the *lmaA* gene (15). In an *L. innocua* 6a isolate, we detected a sequence that was 90% homologous to the *lmaBA* genes, but this strain lacks the genes located upstream of *lmaB* (36). In general, however, we observed that *L. innocua* strains lack all genes making up the *lma* operon. Recent data have implicated strains of *L. innocua* as causative agents of meningoencephalitis, suggesting that this species may also harbor a subset of virulence factors present in pathogenic *L. monocytogenes* (42). These data provide hints on the evolution of different *Listeria* species and suggest that the presence of the *lma* operon could predate the divergence of these species.

In conclusion, in this study, we present evidence for a novel cluster of genes in *L. monocytogenes* that constitutes the *lma* operon. Nonpathogenic *Listeria* species and strains attenuated for virulence appear to have lost some or all of these genes. Secretion but not synthesis of the LmaA protein is temperature dependent and is not signal peptide dependent. Further studies will be directed at elucidating the regulatory mechanisms that operate to control expression of individual *lma* genes in survival within the host and in the environment.

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