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Differences in Virulence and in Expression of PrfA and PrfA-Regulated Virulence Genes of *Listeria monocytogenes* Strains Belonging to Serogroup 4

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***Listeria monocytogenes* isolates belonging to serogroup 4 (subtypes 4a, 4ab, 4b, 4c, 4d, and 4e) exhibit different levels of virulence in mice. Molecular studies indicate that in comparison with the control strain EGD (serotype 1/2a), these strains differ in the expression of the PrfA-regulated virulence genes, including *prfA* itself. Strains of serotypes 4c, 4d, 4e, and especially 4a show a low level of invasiveness in Caco-2 cells, which correlates in part with the low level of expression of the *inlA* gene. All serotypes reach the cytoplasm, at the latest, 2 h postinfection and become surrounded by polymerized actin within the next hour, but actin tail formation by serotype 4a, 4c, 4d, and 4e strains is drastically reduced. The *actA* genes in these serogroup 4 strains are expressed in minimum essential medium and within the phagocytic cell line J774. However, the amounts and (in part) the sizes of the ActA proteins in these strains differ under these conditions. The reduced actin tail formation by serotype 4a, 4c, 4d, and 4e strains may be due to the low level of in vivo expression of ActA. In addition, the loss of one repeat unit in the ActA proteins of serotype 4a and 4e strains may also contribute to the less efficient actin tail formation observed with these strains.**

Listeria monocytogenes belongs to the group of facultative intracellular bacteria. This gram-positive pathogen can cause severe infections in certain risk groups, such as pregnant women, newborns, and immunocompromised patients. The microorganism seems to be widespread in nature and is transferred to humans by contaminated milk products, meats, and vegetables (2, 15, 16, 47). Small epidemics, caused mainly by *L. monocytogenes*-contaminated cheeses, have been observed in recent years in several countries around the world (25, 29, 34).

Several types of *L. monocytogenes* may be distinguished by using specific antibodies and bacteriophages (35, 48), but most isolates from *L. monocytogenes* infections in humans and animals fall into the serotypes 1/2a, 1/2b, and 4b (15, 21). Most molecular studies on listerial virulence factors and the regulation of virulence genes performed in recent years were carried out with *L. monocytogenes* strains belonging to serotype 1/2a or 1/2c (for reviews, see references 28, 40, and 41). These investigations have revealed a virulence gene cluster on the chromosomes of these strains which seems to be inherent to all *L. monocytogenes* isolates tested. This gene cluster consists of five genes and is regulated by a positive regulatory factor called PrfA (32). The products encoded by these genes comprise two phospholipases, a cytolysin (listeriolysin), a metalloprotease, and the ActA protein, which is involved in actin polymerization occurring around the listerial cells when they reach the host cell cytoplasm. This event is necessary for the intra- and intercellular movement of the bacteria (10, 11). The roles of the other gene products in the intracellular replication cycle of *L. monocytogenes* have also been extensively studied in recent years. Listeriolysin, together with the phosphatidylinositol-specific phospholipase C (PlcA), has been shown to be required for the disruption of the phagosomal membrane and hence for the release of the bacteria into the cytoplasm (7, 20, 42). The other phospholipase C (PlcB), a lecithinase, is involved in the release of the bacteria from the double membrane which is formed when the bacteria enter a neighboring host cell (51). The metalloprotease Mpl (12, 45) converts the inactive precursor form of PlcB into the active, mature extracellular lecithinase (44). Recent data (49) show that there are still other proteins produced by *L. monocytogenes* under specific culture conditions which are under the control of the regulatory factor PrfA. Synthesis of internalin, a membrane protein (19) which triggers the uptake of *L. monocytogenes* by normally nonphagocytic mammalian cells (19), also seems to be under the control of PrfA (3, 14, 33).

In addition to these PrfA-dependent proteins (PdPs), other proteins, which are not controlled by PrfA, have been shown to be connected with the virulence of *L. monocytogenes*. The secreted protein p60 affects adherence of *L. monocytogenes* to certain mammalian cells, and mutants impaired in the synthesis of this protein are avirulent (24, 43). The roles of the recently characterized catalase and superoxide dismutase in the virulence of *L. monocytogenes* have yet to be determined (5, 22).

Hybridization studies and enzyme activity assays have shown that at least some of the virulence genes identified in *L. monocytogenes* strains of serotypes 1/2a and 1/2c are also present in *L. monocytogenes* isolates of the other known serotypes (37, 52). Nevertheless, there is clear evidence that some of these isolates are considerably less virulent in mice and are only rarely found in infections of humans or animals (15).

In this study we have analyzed the virulence and the expression of the known PrfA-regulated genes in *L. monocytogenes* strains belonging to the six subtypes of serogroup 4, i.e., 4a, 4ab, 4b, 4c, 4d, and 4e. The most frequently occurring clinical

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TABLE 1. Bacterial strains used

Species and strain[a]	Serotype	Source[b]
L. monocytogenes		
EGD	1/2a	S. H. E. Kaufmann
NCTC 7973	1/2a	NCTC
SLCC 2755	1/2b	SLCC
NCTC 5348	1/2c	NCTC
NCTC 5105	3a	NCTC
SLCC 5543	3b	SLCC
SLCC 2479	3c	SLCC
SLCC 2482	7	SLCC
L 99	4a	T. Chakraborty
SLCC 4561	4ab	SLCC
SLCC 4013 ($4b^1$)	4b	SLCC
LL 195 ($4b^2$)	4b	J. Bille (human isolate, 1987 Swiss epidemic)
LL 201 ($4b^3$)	4b	J. Bille (human isolate, 1987 Swiss epidemic)
ATCC 19116	4c	ATCC
ATCC 19117	4d	ATCC
ATCC 19118	4e	ATCC
L. innocua NCTC 11288	6a	NCTC

[a] All strains originated from patients or were isolated from contaminated food.
[b] ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, England; SLCC, Special *Listeria* Culture Collection of the Institute of Hygiene and Microbiology at the University of Würzburg, Würzburg, Germany.

isolates, belonging to serotype 4b, show virulence in mice similar to that of serotype 4ab, while isolates of serotypes 4c, 4d, and particularly 4a and 4e are much less virulent in this animal model.

Our data show further that these *L. monocytogenes* strains also differ in the expression of PrfA-dependent genes (including *prfA* itself). In addition, the amino acid sequences (and possibly the functions) of the ActA proteins of some of these strains are different from the known ActA sequences of *L. monocytogenes* 1/2a and 1/2c strains. The observed differences in the expression of PrfA-regulated genes and in the sequence of ActA seem to affect the efficiency of invasion, actin polymerization, and hence intra- and intercellular movement of these *L. monocytogenes* strains in the host cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *L. monocytogenes* strains used in this study are summarized in Table 1. All strains either originated from patients or were isolated from contaminated food, and some of them were described previously (9, 37). *L. monocytogenes* serotype 1/2a strain EGD was obtained from S. H. E. Kaufmann (University of Ulm, Ulm, Germany), and *L. monocytogenes* serotype 4a strain L99 was kindly provided by T. Chakraborty (Institute for Medical Microbiology, Giessen, Germany). The *L. monocytogenes* serotype 4b strains LL 195 and LL 201 were isolated during the Swiss epidemic (1987) (originating from human patients) and were kindly provided by J. Bille (Institut de Microbiologie, Lausanne, Switzerland). All other strains were purchased from type culture collections. The bacterial strains were cultured in brain heart infusion (BHI) (Difco) at 37°C with aeration. Below, the strains are referred to by their serotype designations (e.g., the serotype 4a strain is called strain 4a; the three serotype 4b strains are called $4b^1$, $4b^2$, and $4b^3$).

Mouse infection model. Female NMRI mice were obtained in a specific-pathogen-free state from Harlan (Borchen, Germany). Mice aged 8 to 12 weeks were infected intraperitoneally with bacteria grown at 37°C for 22 h in tryptose broth (Difco, Augsburg, Germany). The CFU in the spleens and livers of the infected mice were determined at days 1, 3, and 5 postinfection. After homogenization of spleens and livers in 10 ml of distilled water by using a Tenbroeck tissue grinder (Wheaton, Millville, N.J.), bacterial numbers were determined by plating on tryptose agar (Difco). The following initial inocula from overnight cultures were used: strain 4a, 1.2×10^5 bacteria; strain $4b^1$, 1.2×10^5 bacteria; strain $4b^2$, 6×10^4 bacteria; strain $4b^3$, 1×10^5 bacteria; strain 4ab, 5×10^4 bacteria; strain 4c, 1.2×10^5 bacteria; strain 4d, 1.2×10^5 bacteria; strain 4e, 1.2×10^5 bacteria; and strain EGD, 7.5×10^4 bacteria.

Labelling of proteins in minimum essential medium (MEM). Labelling of proteins was carried out as described previously (49) with the modifications that the preincubation time was 20 min and the labelling was carried out for 30 min.

Immunoblotting. After sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of isolated surface proteins, they were transferred from the gel to nitrocellulose filters (Schleicher & Schuell) by the semidry electroblotting method of Kyhse-Andersen (30).

RNA isolation. Bacteria were grown to an optical density at 600 nm of 1.0. The total cellular RNA was isolated prior to and after a 15-min shift in MEM by a previously described method (38) with the modification that the hot phenol-chloroform-isoamylalcohol incubation was prolonged to 20 min.

Northern (RNA) blot analysis. Six micrograms of RNA was used for each lane and denatured in sample buffer (1) at 70°C for 5 min. RNA was separated on a 1% agarose gel with 1.1% formaldehyde, transferred to nylon filters (Hybond N; Amersham), and exposed to UV light or baked for 1.5 h at 80°C to fix the RNA on the membrane. The hybridization step (prehybridization for 2 to 3 h and hybridization) was performed with formamide at a final concentration of 50%. For specific DNA probes we used the following primer pairs: 5′-TCGCAAAG AATTCTAGACCAAG-3′ and 5′-AATCTGTCCCGGGATTACCAAA-3′ for *inlA*, 5′-CAGCTGAGCTATGTGCGAT-3′ and 5′-ACCAATGGGATCCACA AG-3′ for *prfA*, 5′-TTCGGGGAATTCCATGATTAG-3′ and 5′-CACTACTC CCGGGACTGAC-3′ for *plcA*, 5′-CGCGGATGAATTCGATAAG-3′ and 5′-GTCATACCCGGGAAATCAATG-3′ for *hly*, and 5′-ACGGGACCAATATA CGAA-3′ and 5′-TCACCACTATTCAGCGAAC-3′ for *actA*. PCR products were separated on 1% agarose gels, DNA was extracted with a Sephaglas-Band Prep Kit from Pharmacia, and ^{32}P labelling was performed with a Ready To Go DNA-Labelling Kit from Pharmacia. After purification of radiolabelled DNA from unincorporated nucleotides with Nuc Trap push columns (Stratagene), the hybridization was carried out at 42°C overnight. Before being exposed to X-ray films, filters were washed twice with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 58°C for 20 min.

DNA sequencing. The primer pair M 579 (5′-AGTGAAGAGGTAAAGCTT CG-3′) and M 628 (5′-AAGGAACCGGGCTGCTAGCAAA-3′) was used for DNA amplification by PCR. The cloned DNA sequence was determined from double-stranded plasmid templates by dideoxy chain termination (46). The sequencing reactions were performed with T7 DNA polymerase according to the instructions of the supplier.

Invasion of mammalian cells by *Listeria* strains. J774 macrophages were cultured in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (FCS) and L-glutamine. Caco-2 cells were cultured in MEM (GIBCO) supplemented with 10% FCS, L-glutamine, and nonessential amino acids.

Semiconfluent Caco-2 monolayers were washed with phosphate-buffered saline (PBS) before being infected. Infection of cells was performed at a ratio of 1:1 in MEM for 60 min. Contact between bacteria and cells was facilitated by centrifugation ($2,000 \times g$, 10 min, 20°C). After three washes, the medium was supplemented with gentamicin (10 μg ml^{-1}) and incubated for an additional 6 h. Invasion was determined 60 min after gentamicin treatment, and cellular multiplication was determined by lysing the washed monolayers at various times postinfection. The appropriate serial dilutions were plated on BHI plates.

J774 cells were infected with 0.5 bacteria per cell, and gentamicin treatment was performed with 10 μg ml^{-1}. Intracellular multiplication was monitored for 10 h.

Fluorescence staining of F-actin. Staining of actin filaments was performed as described by Karunasagar et al. (26). Briefly, Caco-2 and J774 cells were seeded out on coverslips in tissue culture plates and infected as described above. At different times after gentamicin treatment, the cells were washed with PBS, permeabilized, and fixed in acetone. For staining of F-actin, coverslips were incubated in a 10-U/ml solution of fluorescein isothiocyanate-labelled phalloidin (Sigma).

Electron microscopy. Electron microscopy of ultrathin sections of *Listeria*-infected Caco-2 cells was performed as described by Karunasagar et al. (26).

Plaque formation in L2 cells. The plaque formation assay was performed as described by Sun et al. (50) with the following modifications. The rat epithelial cell line L2 (ATCC CCL-149) was cultured in RPMI 1640 medium supplemented with 10% FCS and L-glutamine. At 24 h before infection, 3×10^5 cells were seeded out into each well of a 12-well tissue culture plate. Bacteria were grown to logarithmic phase in BHI broth, centrifuged, and diluted 1:10 in PBS. L2 monolayers were washed once with PBS containing calcium and magnesium and infected with 0.5, 5, and 50 μl of bacterial suspension. After 2 h, the monolayers were washed three times with PBS containing calcium and magnesium and overlaid with 0.8 ml of RPMI 1640 medium containing 0.5% agarose, 20% FCS, 2 mM L-glutamine, and 10 μg of gentamicin per ml. After 24 h at 37°C, a second overlay containing 0.5% agarose in RPMI 1640, 0.01% neutral red, and 15 μg of gentamicin per ml was added to each well, and plaque formation was observed 24 h later. Plaque size was determined by projecting tissue culture plates on a screen and measuring the diameters of 20 plaques per sample with a ruler. The average plaque size of each serovar was normalized against the plaque size of strain EGD.

Labelling of intracellular synthesized proteins. J774 monolayers were infected with *Listeria* bacteria at a ratio of 10 bacteria per macrophage for 30 min. After the monolayers were washed three times with PBS, the incubation with gentamicin (15 μg ml^{-1}) was continued for 4 h. At 45 min prior to the addition of [^{35}S]methionine at a concentration of 10 μCi ml^{-1}, the eukaryotic protein bio-

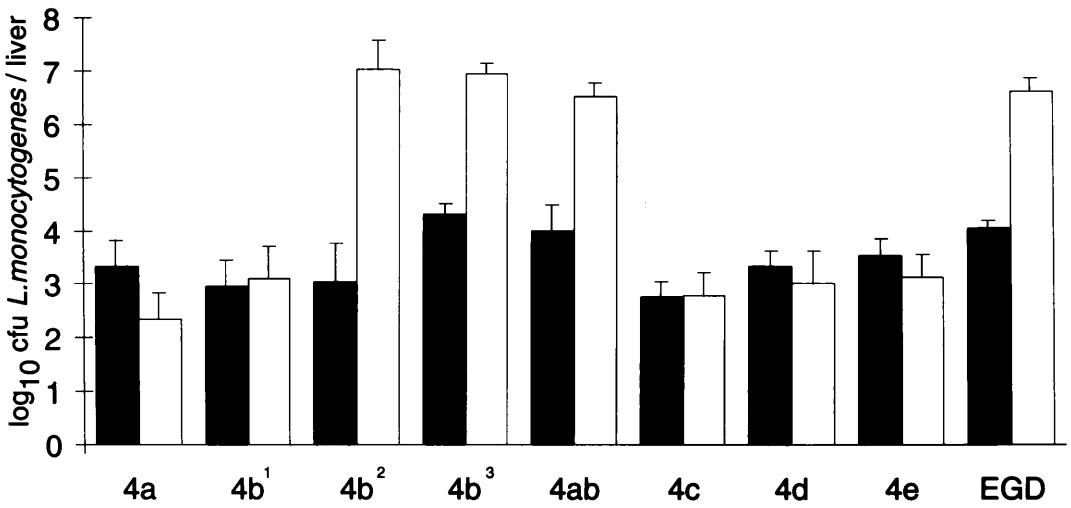

FIG. 1. Bacterial counts in livers of female NMRI mice at day 1 (black bars) and day 3 (white bars) of infection with strains of different *L. monocytogenes* serotypes. Error bars indicate standard deviations of tests for three mice.

synthesis was inhibited with cycloheximide (150 μg ml^{-1}). Labelling was carried out in MEM (without L-glutamine and L-methionine) for 1 h.

Surface protein isolation. After being washed with PBS, monolayers were lysed with cold distilled water and centrifuged (50 × g, 5 min) to remove the cellular fragments. Bacteria were pelleted (5,000 × *g*, 20 min), resuspended in Laemmli sample buffer (31), and heated to 95°C for 15 min. The dissolved surface proteins in the supernatant were loaded onto a 13% polyacrylamide gel. The gels were stained with Coomassie brilliant blue R-250, destained as previously described (31), dried, and exposed to X-ray films for 48 h.

Nucleotide sequence accession number. The nucleotide sequences of the *actA* fragments reported in this paper have been submitted to EMBL and assigned the accession numbers X91146 for *L. monocytogenes* 4a and X91147 for *L. monocytogenes* 4e.

RESULTS

Virulence of *L. monocytogenes* strains belonging to serogroup 4 tested in the mouse model. The *L. monocytogenes* strains 4a, $4b^1$, $4b^2$, $4b^3$, 4ab, 4c, 4d, and 4e were injected intraperitoneally into mice at an initial dose of about 10^5 bacteria per mouse. The inocula were dilutions from overnight cultures that had reached the same stationary phase. The fate of the bacteria in mice was determined by measuring the viable bacterial counts in the liver and the spleen after days 1 and 3 postinfection. This is the time period in which an increase in viable bacteria was previously observed in this mouse model when challenge was with a nonlethal dose of *L. monocytogenes* EGD (23). This strain, which belongs to serotype 1/2a, was used in this experiment as a control. As shown in Fig. 1 for the liver (the values were very similar for the spleen), only the $4b^2$ and $4b^3$ (LL 195 and LL 201, respectively, both of which derived from the Swiss epidemic of 1987) and 4ab strains showed an increase in the number of viable bacterial counts to day 3 similar to that of the EGD control strain. Unexpectedly, the number of viable bacterial counts of strain $4b^1$ (SLCC 4013, obtained from a culture collection) increased only slightly during this time up to day 3 as compared with the other two 4b strains and the control 1/2a strain. The other strains belonging to the subtypes 4c, 4d, 4e, and 4a did not increase in viable counts from day 1 to day 3. There was no further increase from day 3 to day 5 in the more virulent *L. monocytogenes* strains, i.e., strains 1/2a, 4ab, $4b^2$, and $4b^3$, and also in the less virulent $4b^1$ strain, and there was a drop of more than 1.0 $\log_{10}$ unit in the strains of serotypes 4a, 4c, 4d, and 4e (data not shown). The strain of serotype 4a exhibited the lowest virulence in this in vivo test system.

The efficiency of invasion and the capability of intra- and intercellular movement differ significantly among the serogroup 4 strains. We next tested whether the different in vivo virulences of the serogroup 4 isolates correlate with their abilities to invade and replicate in the Caco-2 epithelial cell line and the macrophage cell line J774. The efficiency of intracellular growth shown in the non-professional phagocytic Caco-2 cells was determined by the invasion, cytoplasmic replication, and intra- and intercellular movement due to actin polymerization and tail formation. Invasion is not a limiting step in the phagocytic J774 cells. The intracellular growth of strains 4a, 4ab, $4b^1$, 4c, 4d, and 4e was measured in both host cell types over a period of 6 to 10 h. Infection of Caco-2 cells and J774 macrophages by each of the serogroup 4 strains was performed with equal bacterial counts (one bacterium per cell), and gentamicin was added 1 h postinfection. As shown in Fig. 2A, all *Listeria* serogroup 4 strains were taken up by J774 cells with a similar efficiency, as expected. The rate of intracellular replication was also similar within the observed time period (10 h after gentamicin treatment). In contrast, invasion into Caco-2 cells, determined by measuring the viable bacterial counts 1 h after gentamicin treatment, was different among these listerial strains. As shown in Fig. 2B, strains 4ab and $4b^1$ penetrated into and replicated within Caco-2 cells with an efficiency similar to that of the control strain EGD. Invasion of Caco-2 cells by strains of serotypes 4c, 4d, and 4e was significantly reduced, but the rate of replication of the internalized bacteria was again similar to that of the control strain EGD. The efficiency of invasion of the 4a strain was even lower, but again the rate of replication of the bacteria taken up by the Caco-2 cells was not significantly altered compared with that of strain EGD. The rate of intracellular replication in both host cells shown in Fig. 2 probably reflects more the efficiency of bacterial multiplication within the infected cells than the efficiency of cell-to-cell spread, since microscopy of Giemsa-stained 4a, 4c, and 4d bacteria showed a large number of bacteria in a few host cells and many uninfected cells (data not shown). This observation shows that cell-to-cell spread was reduced in strains of serotypes of 4a, 4c, and 4d. This conclusion was further supported by the smaller plaque size of these latter strains, which was only

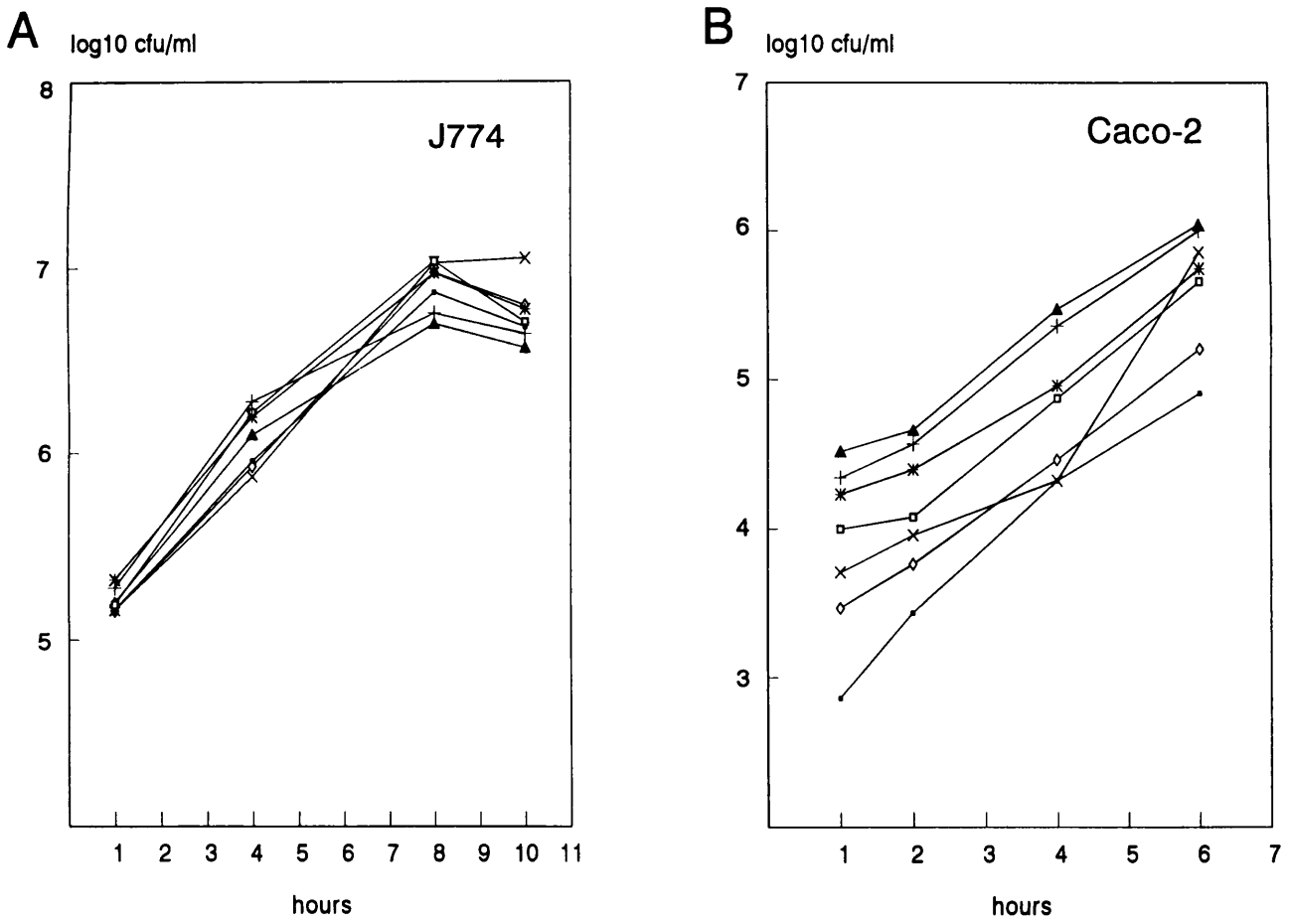

FIG. 2. Intracellular multiplication of *L. monocytogenes* EGD and the serogroup 4 strains in the macrophage-like cell line J774 (A) and the enterocyte-like cell line Caco-2 (B). CFU counts were performed at different times after gentamicin treatment. Values represent means from three experiments. Symbols: ▲, EGD; ■, 4a; +, 4ab; ∗, 4b[1]; □, 4c; ×, 4d; ◇, 4e.

55% for strain 4a and 60% for strains 4c and 4d compared with the plaque size of strains EGD, 4ab, and 4b[1]. The efficiency of plaque formation of the 4e strain was only slightly decreased, resulting in a plaque diameter of 90% compared with strain EGD (Fig. 3).

All serogroup 4 strains polymerized actin in J774 and Caco-2 cells as determined by the staining of the intracellular bacteria with phalloidin. However, only strains 4ab and 4b[1] showed actin tail formation with an efficiency similar to that of the control strain EGD, whereas tail formation in strains 4a, 4c, 4d, and 4e was reduced compared with that in the EGD strain (Table 2). Of 100 internalized listeriae of serotypes 4c, 4d, and 4e, all of which showed an actin coat around the bacterial body, only 1 to 5% possessed actin tails 4 h postinfection, while practically all cells of the control strain EGD carried actin tails at that time. Serotype 4a exhibited retarded actin polymerization, and actin tails were hardly observed in the internalized bacteria.

Actin polymerization around the bacterial cells was analyzed in more detail by electron microscopy of Caco-2 cells infected with the 4a and 4c strains. While the length of the few actin tails observed on these two strains was comparable to that for the control strain EGD, membranous material surrounding the actin-coated bacterial cells was observed at a frequency of about 80% (Fig. 4), suggesting either that the phagosomal membrane was incompletely dissolved or, more likely, that the actin-tailed bacteria were trapped in the double membrane formed during cell-to-cell spread.

Synthesis of PdPs in *L. monocytogenes* strains belonging to serogroup 4 after a shift from BHI culture medium into MEM. As previously shown (49), synthesis of most PdPs in the two *L. monocytogenes* serotype 1/2a strains EGD and NCTC 7973 is induced when the bacteria are shifted from a rich culture medium (BHI) into MEM. There are, however, quantitative differences between these two strains in the PrfA-dependent transcription of the genes encoding these PdPs (4).

In order to analyze whether the observed differences in the virulence of the *L. monocytogenes* strains belonging to the serogroup 4 may be correlated with differences in the expression and/or the size of the known PdPs, we studied the production of these proteins on the transcriptional and translational levels in each of the serogroup 4 strains (4a, 4ab, 4b[1], 4b[2], 4b[3], 4c, 4d, and 4e). Cultures of these strains were shifted from BHI to MEM, and the patterns of the PdPs were compared with that for the serotype 1/2a control strain EGD (Fig. 5A). Differences were indeed observed with respect to the sizes and the amounts of some PdPs. Although the differences in the amounts of the PdPs shown in Fig. 5A are based on the different labelling of the proteins in MEM, these differences indeed reflect quantitative differences in the amount of protein: (i) equal amounts of protein were applied to each lane, which was confirmed by the equal intensities of the major

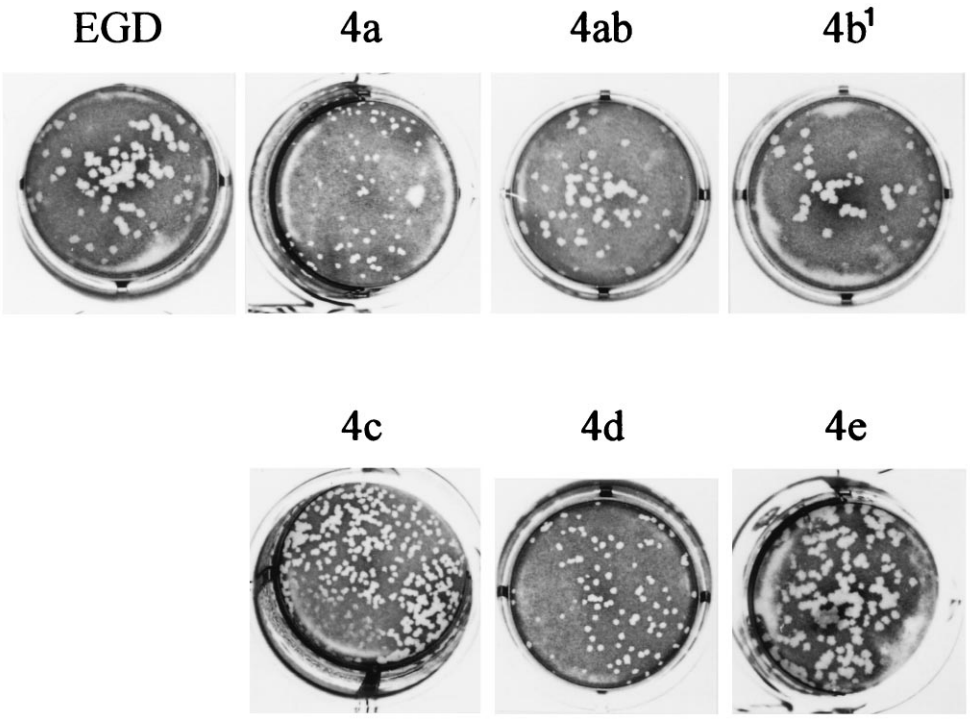

FIG. 3. Plaque formation by different serotypes of *L. monocytogenes* in L2 cells.

protein bands after staining with Coomassie blue, and (ii) the specific radioactivities of the labeled cellular protein (in counts per minute per microgram of protein) in the strains were almost identical (data not shown).

Size differences were particularly apparent for ActA. Three different size classes of ActA proteins, with molecular masses of 92 kDa (strains 4b[1] and 4d), 90 kDa (strains 4ab and 4c), and 88 kDa (strains 4a and 4e), were recognized by immunoblotting, as shown in Fig. 5B. Interestingly, ActA proteins of *L. monocytogenes* strains from other serotypes also fell into one of these three size groups, as shown in Fig. 5B. Extensive proteolytic degradation of ActA caused by Mpl protein occurred in strains EGD and NCTC 7973 (Fig. 5B, lanes 1 and 6). This is shown by the comparison of the wild-type strain EGD with the *actA* and *mpl* insertion mutants (Fig. 5B, lanes 1, 15, and 16). It was less pronounced in all serogroup 4 strains (Fig. 5B). The reason for the reduced ActA degradation in these strains may be the smaller amount of Mpl protein, which did not seem to be induced in MEM in these strains.

Whereas listeriolysin at 58 kDa and phospholipase C (PlcA) at 34 kDa were synthesized in all serogroup 4 strains in amounts comparable to those in the control strain EGD, the amount of PlcB (precursor form of 32 kDa and processed form of 29 kDa) seemed to be small in all of these strains, with the exception of the 4ab strain (Fig. 5A).

To test whether the observed differences in the amounts of the PdPs were caused by different expression of their genes at

TABLE 2. Levels of specific transcripts and corresponding gene products in MEM and actin tail formation of different *L. monocytogenes* strains

Strain	Level of[a]:								Actin tail formation (%)[b]
	prfA	*plcA*	PlcA	*hly*	Listeriolysin	*actA*	ActA	ActA*[c]	
EGD	+(+)	++	++	++++	+++	+++	++	+++	100
4a	+	++	+(+)	+++	++	+	(+)	+(+)	<1
4ab	++	++(+)	++	+++++	+++	++++	++	NT[d]	100
4b[1]	+	++	++	++	++	+	(+)	++	100
4b[2]	+	++	++	NT	NT	++	+(+)	NT	NT
4b[3]	+	++	++	NT	NT	++	+(+)	NT	NT
4c	+	++	++	++++	+++	+	+	+(+)	1–2
4d	+	+(+)	++	++(+)	++(+)	+	+	+(+)	1–2
4e	(+)	+	++	+(+)	++	(+)	+	++	5

[a] Specific transcripts were detected by Northern blot analysis; gene products were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. (+), very weak reaction; +, weak reaction; ++, strong reaction; +++, ++++, and +++++, very strong reactions. PrfA, *mpl*, and Mpl were not detected.
[b] Actin tail formation in Caco-2 cells 4 h postinfection.
[c] ActA*, ActA expressed in J774 cells.
[d] NT, not tested.

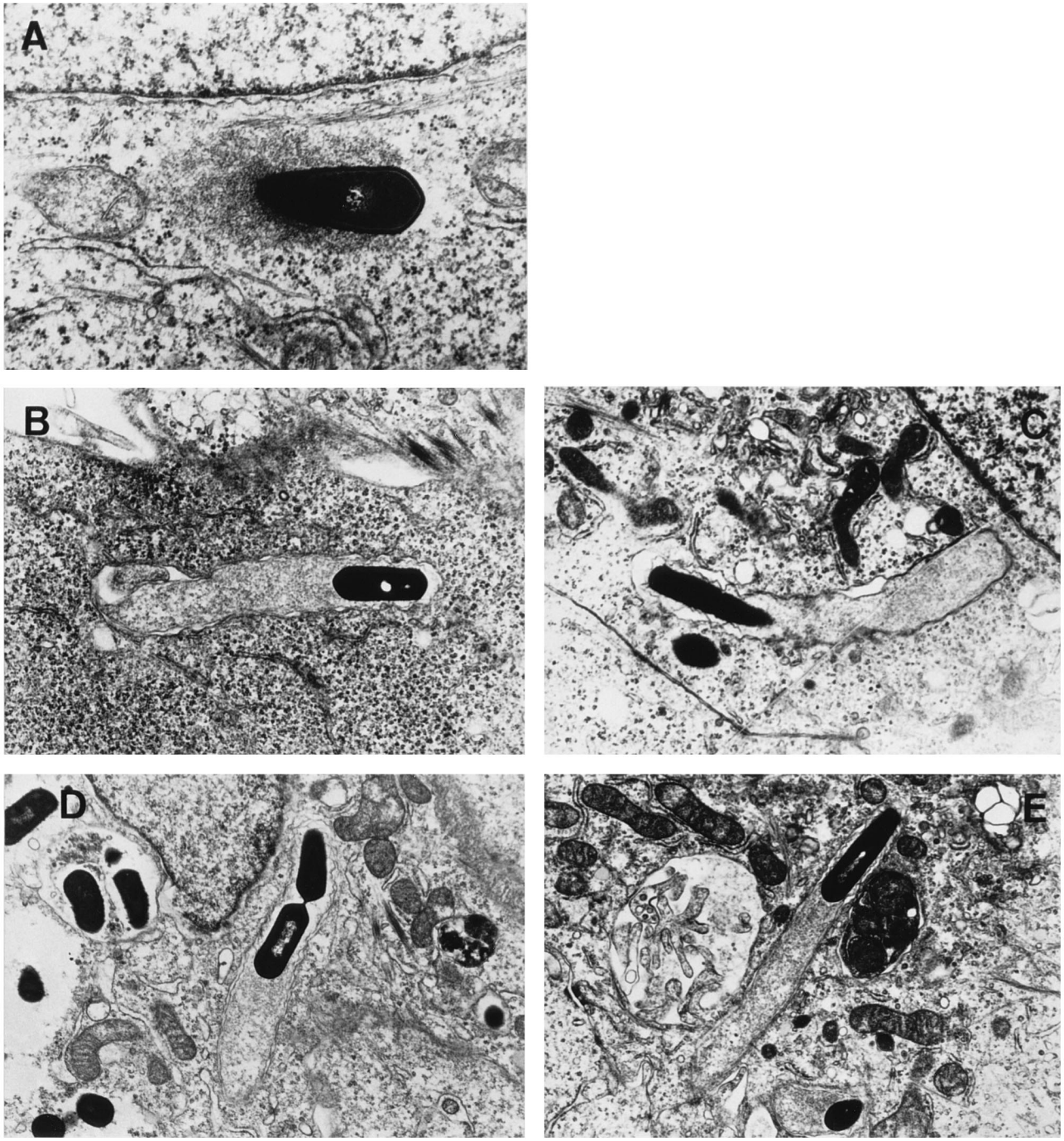

FIG. 4. Thin sections of Caco-2 cells infected with *L. monocytogenes* EGD (A) and strains of serotypes 4a (B and C) and 4c (D and E). After an incubation period of 6 h in gentamicin-containing medium, most bacteria of serotypes 4a and 4c carry an actin tail and are surrounded by a double membrane which is not observed for the control strain EGD. Magnifications, ×30,000 (A) and ×15,000 (B to E).

the transcriptional level, we determined the concentrations of the corresponding transcripts.

Transcription of the PrfA-regulated genes in the serogroup 4 strains after a shift to MEM. The concentrations of the transcripts of the known PrfA-regulated genes synthesized by these strains after a shift from BHI into MEM were determined by Northern blot analysis with specific gene probes. The serotype 1/2a strain EGD again was taken as a control, and equal amounts of total RNA (based on the same level of 16S rRNA) were loaded on each gel lane. As shown in Fig. 6A, the amounts of the *prfA*-derived mono- and bicistronic mRNAs were similar in strains 4a, 4b[1], 4c, and 4d but were smaller in all of these strains than in the control strain EGD. Only strain 4ab reached a level of *prfA* transcripts comparable to that of strain EGD. In strain 4e, the amounts of both mono- and bicistronic *prfA* transcripts were very small in MEM and did

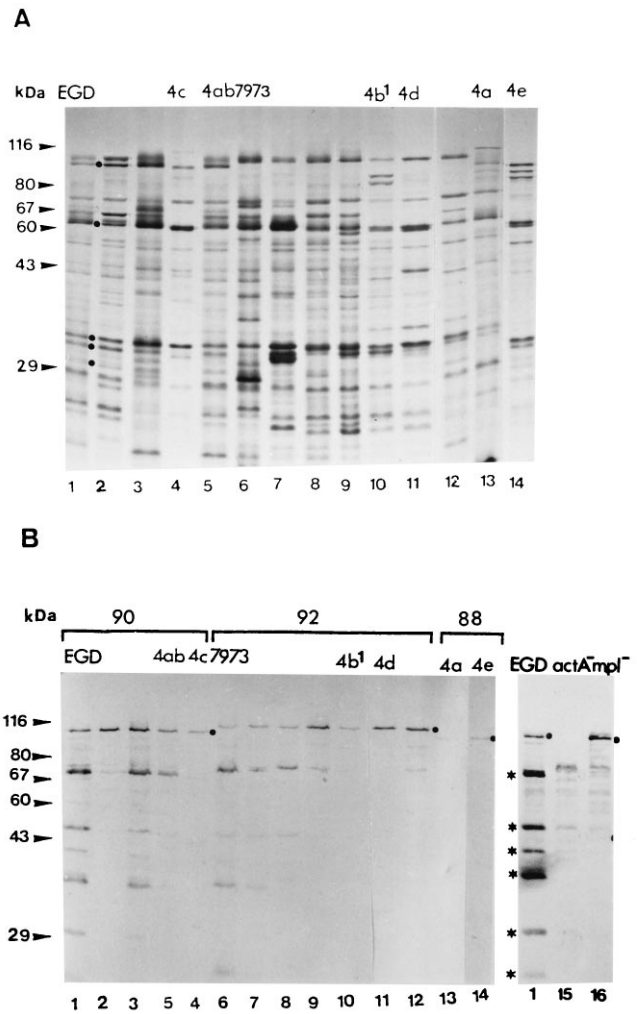

FIG. 5. (A) Surface-associated proteins of different *L. monocytogenes* serotypes expressed in MEM. Bacterial cells were labelled with [^{35}S]methionine for 30 min at a cell density of 2×10^9 to 3×10^9 cells ml^{-1}. The positions of ActA protein, listeriolysin, and PlcA and PlcB (32 and 29 kDa) are marked by black dots. Marker proteins are indicated on the left. Lane 1, *L. monocytogenes* EGD (serotype 1/2a); lane 2, serotype 1/2c; lane 3, serotype 3c; lane 4, serotype 4c; lane 5, serotype 4ab; lane 6, NCTC 7973 (serotype 1/2a), lane 7, serotype 3a; lane 8, serotype 1/2b; lane 9, serotype 3b; lane 10, serotype 4b^1; lane 11, serotype 4d; lane 12, serotype 7; lane 13, serotype 4a; lane 14, serotype 4e. (B) Immunoblot of the surface-associated proteins after labelling with anti-ActA antiserum. Lanes 1 to 14 are the same as in the panel A. The proteins of wild-type strain EGD (lane 1) in comparison with those of the *actA* and *mpl* insertion mutants (lanes 15 and 16) are shown on the right. The ActA proteins of 90, 92, and 88 kDa are marked by black dots, and the degradation products of ActA are marked with asterisks.

not seem to be induced at all. The major *prfA* transcript in all of these strains corresponded to the bicistronic *plcA-prfA* message (2.1 kb). Monocistronic *prfA* mRNA (0.9 kb) was slightly increased in strain 4ab and in the control strain EGD (Fig. 6A).

The amounts of the bicistronic *actA-plcB* transcript (2.9 kb) were similar in strains 4b^1 and 4d but smaller than in the control strain when determined with an *actA* gene probe. Again, only strain 4ab exhibited transcription of these genes comparable to that in the control strain EGD, whereas the amounts of the *actA-plcB* transcripts of strains 4a and 4c were considerably smaller, and only a very low level of this transcript was detectable in strain 4e.

The level of the *hly*-specific mRNA was again highest in strain 4ab, but all other strains showed a relatively large amount of this transcript. In almost all serogroup 4 strains, induction of the monocistronic *plcA* mRNA was comparable to that in strain EGD. An exception was strain 4e, which showed a lower level of this transcript. The amount of the bicistronic *plcA* mRNA (which includes the *prfA* message) was, however, smaller in all serogroup 4 strains (with the exception of the 4ab strain) than in the control strain EGD.

The transcription of *inlA* in the serogroup 4 strains showed a rather complex pattern. In MEM, transcription of *inlA* seems to be induced in strain 4ab while being drastically reduced in the 4a and 4c strains. The 4e strain showed a surprisingly high level of *inlA* transcript in MEM despite very low-level transcription of the *prfA* gene in this medium. Recent data indicate, however, that synthesis of *inlA* transcripts occurs in BHI-growing EGD bacteria from three promoters (P1, P2, and P3), only one of which (P2) is regulated by PrfA (3, 14, 33). Transcription of *inlA* from P2 still occurred in MEM but, in contrast to the case for most of the other PrfA-dependent genes, at an uninduced (or even slightly reduced) rate. Transcription of *inlA* from P1 and P3 was turned off in MEM. This led to a reduction of the total amount of *inlA*-specific transcripts in EGD when this strain was shifted from BHI medium into MEM (3). Assuming that the PrfA-dependent promoter(s) of the *inlA* gene in the serogroup 4 strains is also active in BHI and MEM, whereas the PrfA-independent promoter(s) of *inlA* is active in BHI but inactive in MEM, the data obtained suggest that the transcription of *inlA* in these strains may be controlled by promoters different from those in EGD. The observed differences in invasiveness of the serogroup 4 strains are therefore difficult to correlate with the amounts of *inlA* transcripts synthesized under either BHI or MEM culture conditions.

The virulence of the strain 4b^1 was surprisingly low in the mouse model compared with that of the control strain EGD (Fig. 1), and the amounts of the *prfA* and *actA-plcB* transcripts were also rather small after a shift of the culture into MEM. We therefore analyzed two additional clinical isolates belonging to serotype 4b with regard to the expression of the PrfA-dependent genes *prfA*, *plcA*, and *actA-plcB*. The two strains 4b^2 and 4b^3 already had exhibited a much higher virulence in the mouse model than strain SLCC 4013 (4b^1) (Fig. 1). As shown in Fig. 6B, all three 4b strains induced transcription of *prfA* after a shift into MEM. The levels of monocistronic *prfA* transcripts of the 4b strains were, however, lower than that for the control strain EGD. Despite the high-level transcriptional induction of *prfA* in strain 4b^1, the amount of the *actA-plcB* transcript was considerably smaller in this strain than in strains EGD, 4b^2, and 4b^3, suggesting that strain 4b^1 might be impaired in the induction of the bicistronic *actA-plcB* transcript. Our previous results indicate that PrfA undergoes an activation in MEM, and this activated PrfA seems to be necessary for the transcriptional induction of *actA-plcB* (3, 4). We therefore suggest that this activation of PrfA is impaired in strain 4b^1.

Table 2 summarizes the data on the synthesis of PdPs and their transcripts in the *L. monocytogenes* strains after a shift into MEM.

The smaller sizes of the ActA proteins in the strains of serotypes 4a and 4e are due to a deletion in the proline-rich repeats. The amino acid sequences of the ActA proteins, determined for the ActA proteins of strains EGD and LO28 (13, 51), contain a repeat region consisting of two long and three shorter proline-rich sequences (13). In order to test whether the size differences observed among the ActA proteins of the various *L. monocytogenes* strains shown in Fig. 5B are due to

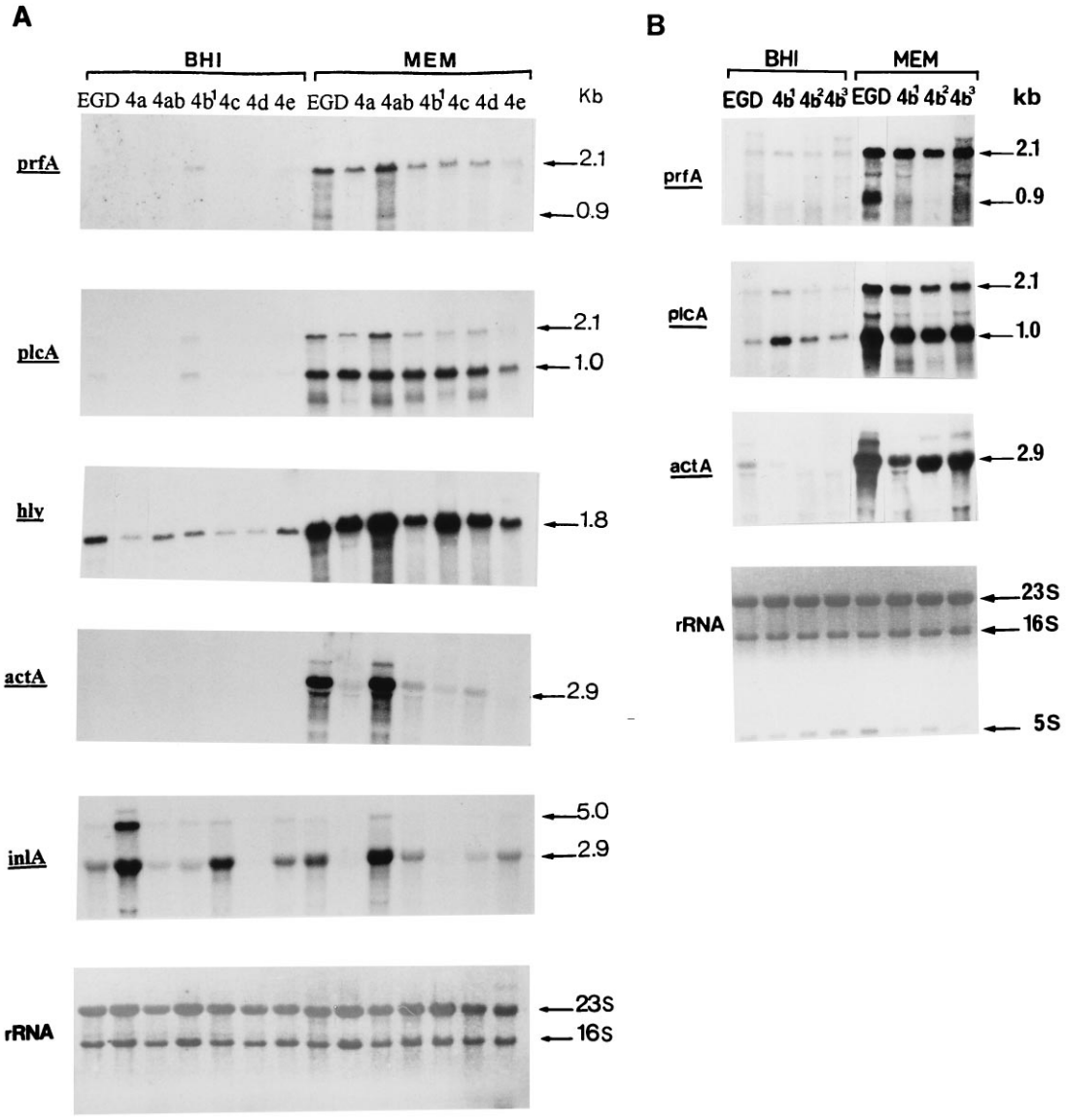

FIG. 6. (A) Northern blot analysis of PrfA-regulated genes from different *L. monocytogenes* strains. Total RNAs were isolated at log growth phase directly from BHI and after a shift to MEM. Transcriptional analysis was performed on strains EGD (serotype 1/2a), 4a, 4ab, $4b^1$, 4c, 4d, and 4e. The sizes of transcripts are indicated on the right, and the probes used are indicated on the left. The same transcriptional patterns were observed with *actA* and *plcB* probes. (B) Northern blot analysis of the *prfA*, *plcA*, and *actA-plcB* genes from three different *L. monocytogenes* 4b strains. The BHI-grown (log growth phase) strains EGD, $4b^1$, $4b^2$, and $4b^3$ were shifted into MEM, and the total RNAs were isolated before and after the shift to MEM. The sizes of transcripts are indicated on the right, and the probes used are indicated on the left.

changes in this repeat region, this region was amplified by PCR from the *actA* gene of some serogroup 4 strains which possess ActA of one of the three different size classes. The results shown in Fig. 7 indicate that strains 4a and 4e possess an ActA protein which lacks one long and one short sequence compared with the ActA sequence of the control strain EGD. Interestingly the ActA proteins of strains 4d and $4b^1$ (and also the serotype 1/2a strain NCTC 7973) show the same composition of the repeat region as strain EGD, although the molecular mass of the former ActA proteins appears to be slightly higher (92 kDa) than that of strain EGD (90 kDa) (Fig. 5B). There was no difference in the sizes of the PCR products obtained from the 5′- and 3′-terminal halves of the *actA* genes from strains EGD, NCTC 7973, $4b^1$, 4c, and 4d (data not shown). This suggests that the observed difference in the sizes of the corresponding ActA proteins is not caused by an alteration in the number of repeats.

ActA synthesis by strains 4a, $4b^1$, 4c, 4d, and 4e grown in J774 cells. We have recently shown (4) that ActA was the major de novo-synthesized surface-associated protein when *L. monocytogenes* was grown in the mouse macrophage cell line J774. The data shown in Fig. 5 and 6 suggest that the levels of transcription of *actA* and synthesis of the ActA proteins are lower in the 4a, $4b^1$, 4c, 4d, and 4e strains than in the control strain EGD and the 4ab, $4b^2$, and $4b^3$ strains after a shift of the bacterial cultures into MEM. We therefore tested whether the expression of ActA in these strains is also reduced during growth in J774 cells. For this purpose, the bacterial proteins were labelled with [^{35}S]methionine in the presence of cycloheximide 5 h postinfection. We chose 4 h postinfection as the

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L.m. EGD  CCGCCACCAC  CTACGGATGA  AGAGTTAAGA  CTTGCTTTGC  CAGAGACACC  AATGCTTCTT
L.m. 4a   CCGAGACCAC  CTACGGATGA  GGAGTTAAGA  CTCGCTTTGC  CAGAGACGCC  AATGCTTCTT
L.m. 4e   CCGACACCAC  CTACGGATGA  AGAGTTAAGA  CTTGCTTTGC  CAGAGACACC  AATGCTTCTT

L.m. EGD  GGTTTTAATG  CTCCTGCTAC  ATCAGAACCG  AGCTCATTCG  AATTTCCACC  ACCACCTACG
L.m. 4a   GGTTTTAATG  CTCCTGCTAC  ATCGGAACCG  AGCTCATTCG  AATTT.....  .....
L.m. 4e   GGTTTTAATG  CTCCTGCTAC  ATCGGAACCG  AGCTCATTCG  AATTT.....  .....

L.m. EGD  GATGAAGAGT  TAAGACTTGC  TTTGCCAGAG  ACGCCAATGC  TTCTTGGTTT  TAATGCTCCT
L.m. 4a   .....  .....  .....  .....  .....  .....
L.m. 4e   .....  .....  .....  .....  .....  .....

L.m. EGD  GCTACATCGG  AACCGAGCTC  GTTCGAATTT  CCACCGCCTC  CAACAGAAGA  TGAACTAGAA
L.m. 4a   .....  .....  .....  CCGCCGCCTC  CAACAGAAGA  TGAACTAGAA
L.m. 4e   .....  .....  .....  CCACCACCTC  CAACAGAAGA  TGAACTAGAA

L.m. EGD  ATCATCCGGG
L.m. 4a   ATTATGCGGG
L.m. 4e   ATTATGCGGG
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FIG. 7. Nucleotide sequence (bp 996 to 1246) of the proline-rich region in the *actA* gene. In comparison with the *actA* sequence of *L. monocytogenes* (L.m.) EGD (13), those of strains 4a and 4e exhibit a deletion (dotted lines) of 105 bp. Base exchanges in the sequences of strains 4a and 4e are in boldface.

time point for the labelling of ActA since our previous data (4) had shown that ActA is very efficiently synthesized at this time, when all bacteria have reached the cytoplasm and are actively moving within the infected host cell and into neighboring host cells. The labelled surface proteins of equal numbers of these bacteria (based on viable bacterial counts) were isolated and analyzed on SDS-polyacrylamide gels. Figure 8 shows that the major labelled proteins were at 92 kDa ($4b^1$ and 4d), 90 kDa (EGD and 4c), and 88 kDa (4a and 4e). The sizes of these proteins correlate with the expected sizes of the ActA proteins in these strains. The amount of ActA in each strain was determined by the amount of radioactivity incorporated into the ActA protein by an equal number of viable bacteria. It was also shown that the specific radioactivities of the total protein (counts per minute per microgram of protein) were quite similar in all of these strains. On the basis of this determination, the amount of ActA protein was smaller in all tested serogroup 4 strains (4a, $4b^1$, 4c, 4d, and 4e) than in the control strain EGD. However, the ActA protein was still the major labelled surface protein in all of these strains, indicating that the intracellular (cytoplasmic) environment is a more potent trigger for the induction of ActA than MEM. This was particularly significant for strain 4e, which showed a relatively large amount of ActA protein under these conditions compared with the extremely low level synthesized in MEM. The proteins at 32, 34, 45, and 60 kDa have not yet been identified.

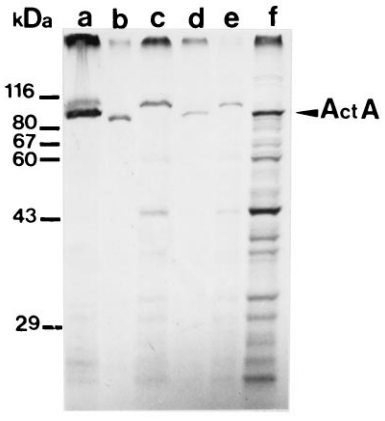

FIG. 8. Expression of ActA proteins in *L. monocytogenes* 5 h postinfection of J774 cells. Lane a, strain EGD (serotype 1/2a); lane b, strain 4a; lane c, strain $4b^1$; lane d, strain 4c; lane e, strain 4d; lane f, strain 4e. Labelling was carried out for 60 min. No proteins were labelled in cycloheximide-treated macrophages or in gentamicin-treated bacteria (data not shown). The position of ActA is indicated on the right, and those of the marker proteins are indicated on the left.

DISCUSSION

In this study we have tried to correlate the in vivo virulences of *L. monocytogenes* isolates belonging to different subtypes of serogroup 4 with their abilities to invade and multiply in the Caco-2 epithelial cell line and the macrophage cell line J774. We also analyzed the expression of the known PdPs on the transcriptional and translational levels in these strains. Most of the PdPs were previously shown to be involved in virulence (for reviews, see references 28, 40, and 41). The *L. monocytogenes* strains of serogroup 4 are of particular interest since members of subtype 4b are, after serotypes 1/2a and 1/2b, the most common clinical isolates. However, there are several subtypes in serogroup 4, e.g., 4a, 4c, 4d, and 4e strains, which are only rarely found in human or animal listeriosis. Some of these latter strains have been already shown to possess low levels of virulence in mice (9, 27).

In our studies, using an intraperitoneal infection of mice by serogroup 4 strains of *L. monocytogenes*, the number of viable listeriae, while increasing in mice infected with sublethal doses of strains 4ab, $4b^1$, $4b^2$, and $4b^3$ and the serotype 1/2a control strain EGD, decreased within the observed 3 days in mice infected by the 4a, 4c, 4d, and 4e strains. There are, however, significant differences in the virulence of the three tested 4b strains. Whereas SLCC 4013, a 4b strain obtained from a culture collection, exhibits a rather low level of virulence, the virulence of two other 4b strains (LL 195 and LL 201, derived from the Swiss epidemic of 1987) in this mouse model is comparable to that of the 1/2a control strain EGD.

There is a good correlation between this in vivo virulence of the serogroup 4 strains and their invasiveness into Caco-2 cells, which is significantly lower for the 4c and 4d strains than for

the 4ab, 4b[1], and 1/2a strains. Invasiveness is lowest for the 4a and 4e strains. Interestingly, there is not much difference in the rate of intracellular replication between the various serogroup 4 strains. Since low numbers of bacteria per host cell were used in this study, escape from the phagosome and intracellular multiplication within the infected host cells occur during this time, but no or little cell-to-cell spread occurs. This is confirmed by light microscopy, which shows that the strains exhibiting low levels of invasiveness are present in large numbers in the few infected cells. The data therefore suggest that the virulence factors required for phagosomal escape and intracellular replication, mainly listeriolysin and PlcA (7, 20, 42), are provided equally well by all serogroup 4 strains.

Actin polymerization around the bacterial cells was also observed by light and electron microscopy for all of these strains, indicating that recruitment of cellular F-actin seems to occur with similar efficiencies. However, the strains exhibit significant differences with respect to the rearrangement of the polymerized actin into tails. Whereas the tail formation of the 4ab and 4b[1] strains is comparable to that of the control strain EGD (most bacterial cells being equipped with an actin tail 4 h postinfection), only a small percentage of the cells of the other serogroup 4 strains carry such tails. In addition, the few tailed bacteria are frequently surrounded with membranous material, as shown for strains 4a and 4c in Fig. 4, which probably derives from the double membrane formed when the listeriae enter the neighboring host cell (51), suggesting that not only actin tail formation but also cell-to-cell spread is inhibited.

The inefficient actin tail formation and the observed low concentration of PlcB, particularly in the 4a, 4c, and 4d strains, correlate with the inefficient cell-to-cell spread of these strains in Caco-2 and J774 host cells. In addition, the amount of the metalloprotease Mpl, which seems to be required for the processing of the PlcB precursor to the mature, enzymatically active PlcB form, seems to be very small in all serogroup 4 strains as judged by the small amount of *mpl*-specific transcripts (data not shown).

Transcription of the PrfA-regulated genes (with the exception of *inlA*) is as low in all serogroup 4 strains as in the control strain EGD when these strains are grown in BHI. Upon a shift into MEM, transcription of the *prfA* gene is induced in all serogroup 4 strains, but only in the 4ab strain does it reach a level similar to that in the control strain EGD. An exception is the 4e strain, which shows low levels of transcription of *prfA* in BHI and in MEM. The induced *prfA*-specific transcripts correspond in size to the *plcA-prfA* bicistronic mRNA which is positively regulated by PrfA (36), whereas the level of the monocistronic *prfA* transcript is low in all strains. The amount of the *hly* and *plcA* transcripts, comparably as small as in the control strain EGD when grown in BHI, is induced in MEM. Interestingly, even the amounts of the induced *hly* transcript and the monocistronic (but not the bicistronic) *plcA* transcript of the 4e strain are relatively large, suggesting that the induction of these two genes requires very low concentrations of PrfA (17). These data nicely explain the almost equal capabilities for phagosomal release and intracellular replication by all serogroup 4 strains in Caco-2 and J774 cells.

The amounts of the bicistronic *actA-plcB* transcript (2.9 kb), determined by hybridization with an *actA*-specific gene probe, differ significantly among these strains. The synthesis of this transcript is low in all of these bacteria grown in BHI and in MEM is induced only in the 4ab strain to the same level as in the control strain EGD. The amount of this transcript is already considerably smaller in the 4a, 4b[1], 4c, and 4d strains and is very small in the 4e strain. This observation correlates with the similarly small amounts of *prfA* transcripts synthesized by these strains. The low levels of *actA* and *plcB* expression in the 4b strain SLCC 4013 after a shift into MEM agrees with the low level of virulence of this strain in the mouse model. In contrast, the two other 4b strains (LL 195 and LL 201), which exhibit high levels of virulence in mice (as does the control strain EGD), also show higher levels of *actA* and *plcB* expression in MEM. Since all three 4b strains induce similar levels of *prfA* transcripts, it is likely that the activation of PrfA which occurs in MEM and seems to be required for the induced synthesis of the bicistronic *actA-plcB* mRNA (3, 4) may not properly function in strain SLCC 4013. According to our previous data (4, 49), the amount of synthesized PdPs in MEM follows essentially the amount of the corresponding transcripts in MEM, suggesting that the amount of ActA protein synthesized in MEM by strain SLCC 4013 may be also smaller than that synthesized by the two other 4b strains. Surprisingly, the actin tail formation by this strain in Caco-2 cells is comparable to that by control strain EGD. Although this microscopic analysis does not allow any conclusions concerning the rate of actin polymerization within these host cells (which may be dependent on the concentration of ActA), it is also possible that in some strains the virulence genes are more strongly induced under in vivo conditions than in vitro (e.g., in MEM). Alternatively, the quality of the virulence factors (e.g., ActA) may be strain dependent, and either larger or smaller amounts of the same virulence factor may be required for performing the same process in different strains.

The observation that the ActA protein is the major bacterial surface protein synthesized in the cytoplasm of J774 host cells by all serogroup 4 strains tested (4a, 4b[1], 4c, 4d, and 4e) and by the control strain EGD is in line with previous data (4, 6) showing that this protein is highly induced once the bacteria have reached the host cell cytoplasm and are in the process of active movement within the infected cell and into neighboring cells. On the basis of our semiquantitative measurements, which are based on equal numbers of viable bacteria and similar specific radioactivities of the labelled total bacterial protein in all of these strains, the amount of ActA appears to be smaller in all serogroup 4 strains than in the control strain EGD. Interestingly, the amount of ActA protein in strain 4e is considerably larger in J774 cells than in MEM, again suggesting that PrfA-dependent expression of *actA* in MEM may not be as efficient as it is inside these phagocytic cells. Nevertheless, the reduced actin tail formation observed on the bacterial surface of strains 4a, 4c, 4d, and 4e replicating in Caco-2 and J774 cells may be caused, at least in part, by the apparently reduced amount of ActA observed inside these cells.

In addition to the smaller amount of ActA (compared with that in strains EGD and 4ab), the sequences of the ActA proteins of the 4a and 4e strains indicate the loss of a long and a short repeat in the proline-rich repeat domain compared with the published sequences of the ActA proteins from *L. monocytogenes* EGD and LO 28 (13, 51). It has recently been shown (8) that the repeat domain of ActA probably represents the binding site for the host factor VASP, which is involved in the formation of cellular actin microfilaments, while the site where cellular F-actin is thought to bind to ActA is located at the N-terminal part of ActA (18, 39). Although the N-terminal parts of the different ActA proteins have not been determined, the altered ActA sequence in the proline-rich repeat regions of strains 4a and 4e may explain the reduced ability of these strains to rearrange the actin filaments into tails. This may also explain the observation of reduced actin tail formation of strain 4e despite the only slightly reduced cell-to-cell spreading in comparison with that in the control strain EGD. Interestingly, the amino acid sequences of the repeat domains in those

ActA proteins of the serogroup 4 strains which show molecular masses apparently higher than that of ActA from strain EGD are not altered (1a). The reason for the apparently higher molecular masses of these ActA proteins remains to be determined.

In summary, our data show that the considerable differences in virulence and in the uptake and intracellular multiplication in epithelial and phagocytic host cells of *L. monocytogenes* strains belonging to the various subtypes of serogroup 4 can be correlated only in part with the different expression of the *prfA* gene and the known PrfA-regulated virulence genes under in vitro conditions. The data suggest that the regulation of these genes inside mammalian host cells is different from what we see in the in vitro studies and/or that additional virulence genes may have to be considered to fully explain the virulence of *L. monocytogenes*.

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Z. Sokolovic and S. Schüller contributed equally to this paper.

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