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Identification of an Extracellular Protein of *Listeria monocytogenes* Possibly Involved in Intracellular Uptake by Mammalian Cells

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Mutants of *Listeria monocytogenes* were recently isolated which are impaired in the synthesis of a major extracellular protein (p60). As shown in this investigation, the p60 mutants have lost the capability of invading nonprofessional phagocytic 3T6 mouse fibroblast cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of supernatant proteins of these mutants indicated that no other extracellular protein was altered in these mutants. The p60 mutants formed long cell chains which disaggregated to normal-sized single bacteria upon treatment with partially purified p60. These disaggregated bacterial cells were able to invade 3T6 cells. Physical disruption of the cell chains by ultrasonication produced similar single cells which were, however, noninvasive. Treatment of these ultrasonicated mutant cells with wild-type p60 restored their ability to invade 3T6 cells.

Listeria monocytogenes are gram-positive, rod-shaped, facultatively intracellular bacteria that can cause severe infections in immunocompromised humans and animals, with symptoms such as septicemia and meningitis. The hemolytic phenotype of *L. monocytogenes* is due to an SH-activated cytolysin termed listeriolysin. All hemolytic *L. monocytogenes* strains are virulent, and direct evidence for the involvement of listeriolysin in virulence was recently provided by using nonhemolytic mutants (4, 10). Although nonhemolytic mutants are still able to invade nonprofessional phagocytic cells, such as mouse fibroblast 3T6 cells or Caco-2 cells (3, 12), they are unable to survive within mouse professional macrophages (12). These data indicate that hemolysin is not involved in invasion and thus cannot represent the only virulence factor of *L. monocytogenes*. This conclusion is further suggested by the occurrence of the hemolytic species *Listeria ivanovii* and *Listeria seeligeri*, which are essentially noninvasive (3, 12) and avirulent (or only weakly virulent) for humans (17, 19).

The molecular mechanisms leading to invasion of intracellular bacteria have been studied primarily in the gram-negative intracellular bacteria *Shigella flexneri* (18), *Shigella dysenteriae* (6), *Salmonella typhimurium* (2), *Yersinia enterocolitica* (7, 8, 14), and *Yersinia pseudotuberculosis* (1).

The requirements for this decisive step in the intracellular bacterial life cycle appear to be quite different among these species. Whereas a plasmid-borne stretch of DNA comprising at least six genes has been identified in *Shigella* sp. (18), only a single chromosomal gene appears to be necessary for the same step in *Yersinia* sp. (7). In the latter case, the product of the *inv* gene, termed invasin, was characterized (8) and shown to act as a trigger for endocytosis after binding of the bacteria to the target cells (probably by a specific host receptor). There is circumstantial evidence that a similar adhesinlike substance may be responsible for the uptake of *S. typhimurium* by nonphagocytic host cells. Whereas these invasins may first act to bring about binding of the bacteria to suitable target cells, it should be emphasized that the adhesinlike property of the invasins is different from that of adhesins of extracellular bacteria, which also bind bacteria to target cell receptors but do not trigger endocytosis.

It is unknown whether similar mechanisms are responsible for the invasion of gram-positive intracellular bacteria. We have investigated this question in *L. monocytogenes* and identified a protein with a relative molecular mass of 60 kilodaltons (kDa) (p60). This protein is secreted in relatively large amounts by all invasive *L. monocytogenes* strains, whereas noninvasive mutants exhibit an impaired synthesis of p60. Our data suggest that p60 may be involved in the uptake of *L. monocytogenes* by nonprofessional phagocytic cells.

MATERIALS AND METHODS

Bacterial strains and growth media. The *Listeria* strains (Table 1) used in this study were, if not otherwise indicated, derived from the *Listeria* strain collection (Special Listeria Culture Collection [SLCC]) of H. P. R. Seeliger, Würzburg, Federal Republic of Germany. The nonhemolytic transposon Tn*916* mutant used in this study has been described elsewhere (10). Strain SLCC 5779, a rough variant, was originally obtained from J. Potel (Institute for Medical Microbiology, Medical Academy, Hannover, Federal Republic of Germany). This variant was derived from a smooth hemolytic strain of serovar 1/2a (16). *Listeria* cells were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with or without 0.5% glucose at 37°C with aeration. For invasion assays, bacteria were harvested in the logarithmic growth phase and stored at −70°C in phosphate-buffered saline (PBS) with 20% glycerol (vol/vol) until used.

Invasion assay. Invasion assays were performed essentially as described elsewhere (7, 12). 3T6 cells (10^6 per well, 12-well dishes, type 3512; Costar, Cambridge, Mass.) in RPMI 1640 medium supplemented with 10% fetal calf serum and L-glutamine were infected with bacteria grown and stored as described above. After centrifugation of the bacteria onto the monolayer for 1 min at 1,000 rpm at 20°C in an RT 6000 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), the infected cultures were incubated at 37°C in a 5% CO_2 atmosphere. After the indicated times, nonadherent bacteria were removed by three washes with 3 ml of PBS, and the cultures were further incubated in RPMI 1640 medium containing 50 μg of gentamicin per ml. The cells were then washed twice with 3 ml of PBS, and intracellular bacteria were released by the addition of 2 ml of 1% Triton X-100. Viable cells were determined by plating the lysate.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-

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TABLE 1. *Listeria* strains used

Strain	Serovar	Listeriolysin	Invasion	Virulence	SLCC no.	Origin
L. monocytogenes Mackaness	1/2a	+	+	+	5764	
L. monocytogenes EGD	1/2a	+	+	+		S. H. E. Kaufmann, Ulm, Federal Republic of Germany
L. innocua	6a	−	−	−	3379	
L. innocua	6b	−	−	−	3423	
L. seeligeri		+	−	−	18/100	
L. grayi		−	−	−		
L. monocytogenes M3 Hly$^-$	1/2a	−	+	−		S. Kathariou
L. monocytogenes RI[a]		+	−	−		S. Kathariou
L. monocytogenes RII[b]		+	−	−		S. Kathariou
L. monocytogenes RIII		+	−	−	5779	J. Potel
L. monocytogenes RIV[a]		+	−	−		S. Kathariou

[a] Derived from *L. monocytogenes* Mackaness (SLCC 5764).
[b] Derived from *L. monocytogenes* EGD.

polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% acrylamide gels as described by Laemmli (13) was used for protein separation. Transfer of proteins from SDS-PAGE onto nitrocellulose and identification by specific antisera were performed according to the method of Towbin et al. (20).

Concentration of supernatants. A 10-ml sample from an overnight culture of each *Listeria* strain grown in brain heart infusion medium was pelleted by centrifugation at 5,000 rpm and 4°C for 15 min. The cell-free supernatant was mixed with 100% ice-cold trichloroacetic acid to a final concentration of 7% trichloroacetic acid, and protein precipitation was accomplished by incubation of the mixture on ice for 1 h. The precipitated protein was collected by centrifugation and suspended in 60 μl of SDS-PAGE sample buffer (50 mM Tris hydrochloride [pH 6.2], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.05% bromphenol blue). Saturated Tris solution (20 μl) was added to neutralize the sample. Alternatively, to 50 ml of cell-free supernatant from cultures grown as described above, solid ammonium sulfate was added to a final concentration of 50% with stirring at 4°C. After 1 h, the precipitated protein was collected by centrifugation, suspended in 1 ml of PBS, and dialyzed twice against a 1,000-fold volume of H_2O for 1 h each time and against PBS overnight. The concentrate was then filter sterilized and stored at −20°C.

Separation of proteins by glycerol gradients. Glycerol gradients (10 to 30% glycerol) in PBS with a total volume of 5.2 ml were prepared in polycarbonate tubes. Protein solution (200 μl) was added, and the gradient was run in an SW50 rotor for 44 h at 35,000 rpm and 4°C. The gradient was then separated into 250-μl fractions.

Biological assay for p60. PBS plus concentrated supernatant (20 μl each) was incubated at 37°C together with 10^7 cells of *L. monocytogenes* SLCC 5779 (in PBS containing 20% [wt/vol] glycerol) for up to 45 min. At the indicated time points, 10 μl of the mixture was examined by phase-contrast microscopy to evaluate the length of the bacteria. Activity was standardized as follows: at a relative activity of 100, cell appearance was essentially like that of wild-type cells; at a relative activity of 50, cells were shortened but still significantly longer than wild-type cells; at a relative activity of 0, the shape of the long cell chains formed by *L. monocytogenes* SLCC 5779 was not altered (see Fig. 6).

IEF. Isoelectric focusing (IEF) was performed as described previously (15).

(i) Sample preparation. An overnight culture of each *Listeria* strain was pelleted by centrifugation. The cell-free supernatant was precipitated with trichloroacetic acid as described above. The precipitated protein was collected by centrifugation, washed once with acetone, and suspended in 90 μl of IEF sample buffer containing 9.5 M urea, 2% Nonidet P-40, 100 mM dithiothreitol, and 2% ampholine solution (pH 3.5 to 10). Tris solution (1 M) was added to neutralize the sample.

(ii) Gel preparation and running conditions. A 2-mm-thick IEF gel was prepared from a deaerated solution containing 9 M urea, 5% acrylamide, 0.15% bisacrylamide, 2% Nonidet P-40, 5% ampholine solution (pH 3.5 to 10), and 0.05% ammonium persulfate. After the gel was placed on the cooling plate of the 2117 electrophoresis system (LKB Instruments, Inc., Rockville, Md.) and electrode strips were positioned with the appropriate buffer (cathode, 1 M NaOH; anode, 1 M H_3PO_4), the gel was prefocused with 500 V · h. After the sample (30 μl) was applied, focusing was achieved with 3,500 V · h overnight. For immunoblotting, the gel was washed three times for 5 min each in a solution containing 100 mM Tris and 3% SDS.

Ultrasonication. *Listeria* cells (10^9 ml^{-1} in PBS) were sonicated in short intervals (5 s) at 60 W for 1 min in a Sonifier (Branson Sonic Power Co., Danbury, Conn.). Temperature was controlled during sonication and did not exceed 4°C.

Electron microscopy. Cells were grown to mid-log phase and washed once with PBS before application to Formvar-coated grids. After the grid was dried, one drop of a solution containing 3% tungstophosphoric acid and 0.3% sucrose (pH 6.8 to 7.4) was added. The solution was removed after 30 to 60 s, and the grid was dried and examined on a Zeiss-10 A transmission electron microscope.

Preparation of p60 antiserum. The methods used for preparation of the specific polyclonal p60 antiserum will be described in detail in a subsequent publication (S. Köhler and W. Goebel, manuscript in preparation).

RESULTS

R mutants of *L. monocytogenes* are noninvasive. We have previously reported on the isolation of spontaneously occurring mutants with rough colony appearance (R mutants) from *L. monocytogenes* Mackaness 1/2a and EGD 1/2a (9). These two strains are equally virulent in the mouse model system but secrete different amounts of listeriolysin and other extracellular proteins (11). The R mutants of both strains exhibited a similar phenotype; they tend to form cell chains in which septum formation between the individual cells still

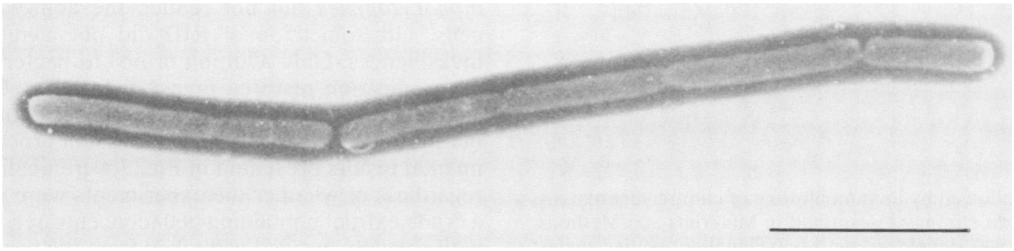

FIG. 1. Electron micrograph of *L. monocytogenes* RII showing septum formation in the cell chains. Bar, 1 μm.

occurs but the cells do not separate (Fig. 1). All four R mutants tested showed a considerably reduced ability to invade embryo mouse fibroblast cells (3T6 cell line) under conditions which were appropriate for the efficient uptake of the two wild-type *L. monocytogenes* strains (Fig. 2).

R mutants are still hemolytic but are impaired in the synthesis of a p60. All four R mutants were hemolytic and synthesized levels of listeriolysin comparable to those of the corresponding wild-type strains (Fig. 3). The only apparent difference in the patterns of extracellular proteins between the R mutants and their parental *L. monocytogenes* strains was the altered amount of a protein (p60) with a molecular mass of 60 kDa, migrating in SDS-PAGE directly above listeriolysin (58 kDa). The amount of this protein was considerably reduced in three of the four R mutants analyzed, whereas the fourth mutant (designated RIV) seemed to synthesize a wild-type level or an even larger amount of this protein. As previously shown, p60 is present in roughly equal amounts in all *L. monocytogenes* strains. Immunologically cross-reacting proteins are present (often with altered sizes) in most other *Listeria* species, including *L. ivanovii*, *Listeria innocua* Sv6a and Sv6b, and *L. seeligeri*. Proteins cross-reacting with anti-p60 antiserum are poorly detectable in *L. grayi* and *L. welshimeri* (5). IEF of supernatants in the presence of 9 M urea followed by immunoblotting indicated an isoelectric point (pI) for p60 of about 9.3 in all *L. monocytogenes* strains (wild type and R mutants). The cross-reacting proteins of *L. innocua* Sv6a and Sv6b showed a slightly altered pI of approximately 9.5 (Fig. 4).

Partially purified p60 restores invasiveness of the R mutants. The nonhemolytic mutant M3 lacks the 58-kDa listeriolysin but produces a wild-type level of p60 and is invasive (12). This strain was used as a source for the enrichment and purification of p60 by glycerol gradients (Fig. 5). Concentrated supernatant and fractions from the glycerol gradient were tested for the ability to restore the invasiveness of RIII, which virtually lacks p60. Addition of p60 to this mutant (suspended in PBS) led to a rapid decay of the cell chains to

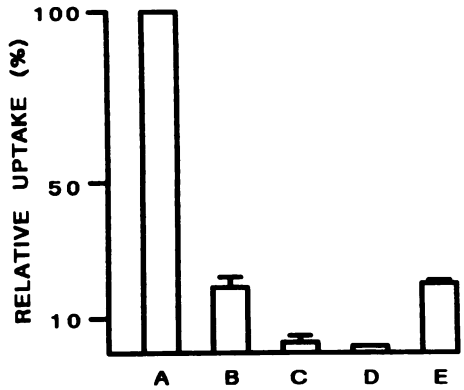

FIG. 2. Uptake of different *Listeria* strains by 3T6 fibroblast cells. Bacteria were added to 3T6 monolayers at a ratio of one bacterial cell to one fibroblast cell and treated further as described elsewhere (12). The strains used were *L. monocytogenes* Mackaness (SLCC 5764) and *L. monocytogenes* EGD. The efficiency of uptake of these strains was set to 100% (A), and uptake of *L. monocytogenes* RI (B), RII (C), RIII (SLCC 5779) (D) and RIV (E) was determined relative to this value. Each column represents the average value of two independent experiments.

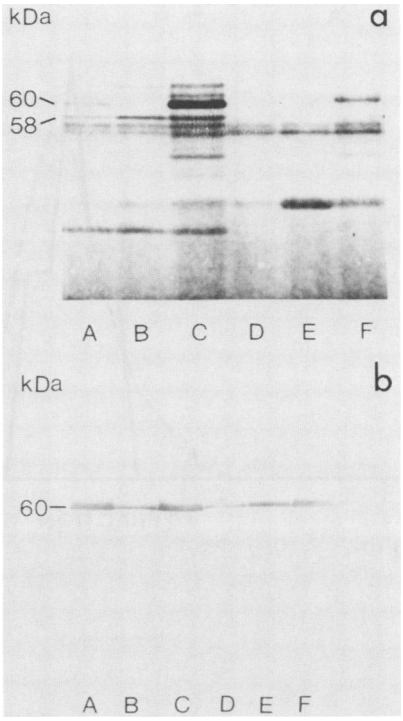

FIG. 3. (a) SDS-PAGE of culture supernatants from different wild-type strains and rough mutants of *L. monocytogenes*; 12.5% SDS-polyacrylamide gels were used and stained with Coomassie blue. (b) Proteins visualized by immunoblotting, using polyclonal antibodies raised against purified p60. Lanes: A, *L. monocytogenes* Mackaness (SLCC 5764); B, *L. monocytogenes* RI; C, *L. monocytogenes* RIV; D, *L. monocytogenes* RIII (SLCC 5779); E, *L. monocytogenes* RII; F, *L. monocytogenes* EGD. Note the low level of listeriolysin (58 kDa) in lanes D and E. This result arises from the facts that these mutants derive from *L. monocytogenes* EGD, which produces little hemolysin under normal growth conditions compared with *L. monocytogenes* Mackaness (10), and the bacteria were grown in glucose-containing medium. Under these conditions, listeriolysin synthesis is diminished (M. Kuhn, unpublished results).

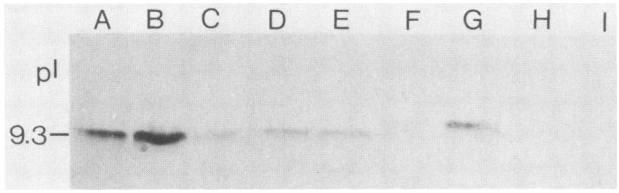

FIG. 4. IEF followed by immunoblotting of culture supernatants of different *Listeria* strains as described in Materials and Methods. The immunoblot was developed with polyclonal antibodies against p60. Lanes: A, *L. monocytogenes* Mackaness (SLCC 5764); B, *L. monocytogenes* RIV; C, *L. monocytogenes* RI; D, *L. monocytogenes* RII; E, *L. monocytogenes* RIII; F, *L. innocua* Sv6a; G, *L. innocua* Sv6b; H, *L. grayi*; I, *L. seeligeri*.

normal-size single cells (Fig. 6). The p60-treated RIII cells also regained the ability to invade 3T6 cells in a concentration-dependent manner (Fig. 7). To determine whether p60 participates directly in the uptake mechanism or whether the decay of the cell chains is sufficient for rendering the arising single cells invasive, we sonicated RIII under conditions which led to a similar decay of the cell chains and the generation of apparent single cells (Fig. 6). Whereas ultrasonication of the wild-type (p60 containing) strains of *L. monocytogenes* did not reduce the ability to invade 3T6 cells, ultrasonication of RIII did not significantly restore invasiveness. Only addition of p60 to bacteria disrupted by ultrasonication restored invasiveness (Fig. 7). This finding suggests that p60 not only causes the decay of the cell chains but participates actively in the invasion process. The experimental results presented in Fig. 7 were qualitatively similar regardless of whether the experiments were performed with a crude extract containing p60 (concentrated supernatant) or with partially purified p60 taken from the peak fractions of a glycerol gradient.

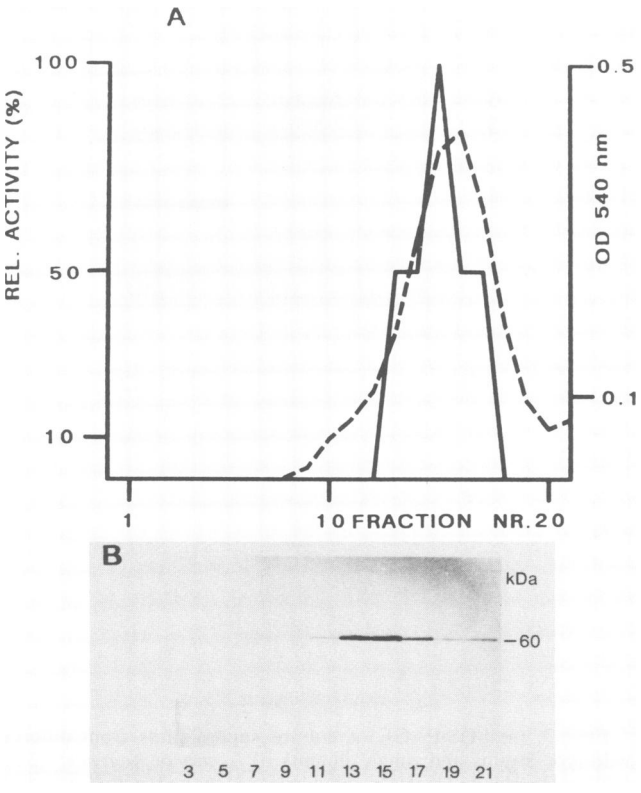

FIG. 5. Purification of p60 by glycerol gradient centrifugation. (A) Concentrated supernatant from Hly$^-$ mutant M3 was run on a glycerol gradient as described in Materials and Methods. Each fraction was tested for activity on *L. monocytogenes* RIII (SLCC 5779) (——). Hemoglobin (relative molecular size, 64 kDa) was run as an external molecular weight marker, and hemoglobin concentration was measured photometrically at 540 nm (– – –). Proteins from each fraction were analyzed by SDS-PAGE, transferred to nitrocellulose, and developed with p60 antiserum as described above. Numbers on the bottom of the immunoblot (B) correspond to the fraction numbers of the glycerol gradient.

As mentioned above, RIV produces large amounts of a p60-like protein with a molecular mass similar to that of the wild-type p60. Treatment of *L. monocytogenes* RIV with p60 of wild-type *L. monocytogenes* or ultrasonication did not lead to a decay of the cell chains. But, interestingly, addition of p60 (concentrated supernatant) restored the invasiveness to a significant extent in a concentration-dependent manner (Fig. 8).

L. innocua Sv6a (SLCC 3379) is unable to invade 3T6 cells (12) but produces relatively large amounts of a protein which is similar in size and cross-reacts immunologically with p60 of *L. monocytogenes*. Partially purified p60 of *L. innocua* was unable to restore invasiveness of RIII. Treatment of the *L. innocua* strain with p60 of *L. monocytogenes* also did not render this noninvasive microorganism invasive for 3T6 cells (data not shown). These data suggest that p60 is necessary but not sufficient to induce uptake of noninvasive *Listeria* strains by 3T6 cells.

DISCUSSION

L. monocytogenes, like many other intracellular bacteria, use professional phagocytic cells such as macrophages and monocytes as host cells. Uptake of the bacteria by professional phagocytes presumably occurs by the normal route of phagocytosis and may even be similar to the mechanism used for the ingestion of extracellularly growing bacteria. There is, however, growing evidence that most (if not all) intracellular bacteria are also invasive for nonprofessional phagocytic cells which are normally unable to take up extracellular bacteria. Thus, it has been suggested that intracellular bacteria may possess special properties which enable them to stimulate endocytosis by nonprofessional phagocytes, presumably by recognizing a receptor(s) on the surface of the host cells. The recent identification of invasins in various *Yersinia* species (8,14) is in line with this assumption. These invasins are proteins that are localized on the bacterial surface and seem to cause adherence to the host cells. In this report, we have identified a protein (p60) in *L. monocytogenes* which may serve a similar function. Most p60 is found in the supernatant. However, we cannot exclude the possibility that a smaller portion remains attached to the bacterial surface. Therefore, it is difficult to determine whether the extracellular or the cell-bound form of p60 is essential for invasion. Our data indicate that cell-free p60, when added to mutants which are impaired in the synthesis of this protein, can restore the ability of the mutants to invade 3T6 cells. Spontaneous mutants which form colonies of a rough appearance (R phenotype) arise at a relatively high frequency (about 1 in 10,000 colonies). All R mutants are impaired in the synthesis of p60. Most R mutants still synthesize low amounts of p60, which suggests that most of the mutations leading to the R phenotype affect regulatory sites rather than the coding region of the gene for p60. One R mutant was isolated which synthesized a wild-type level of

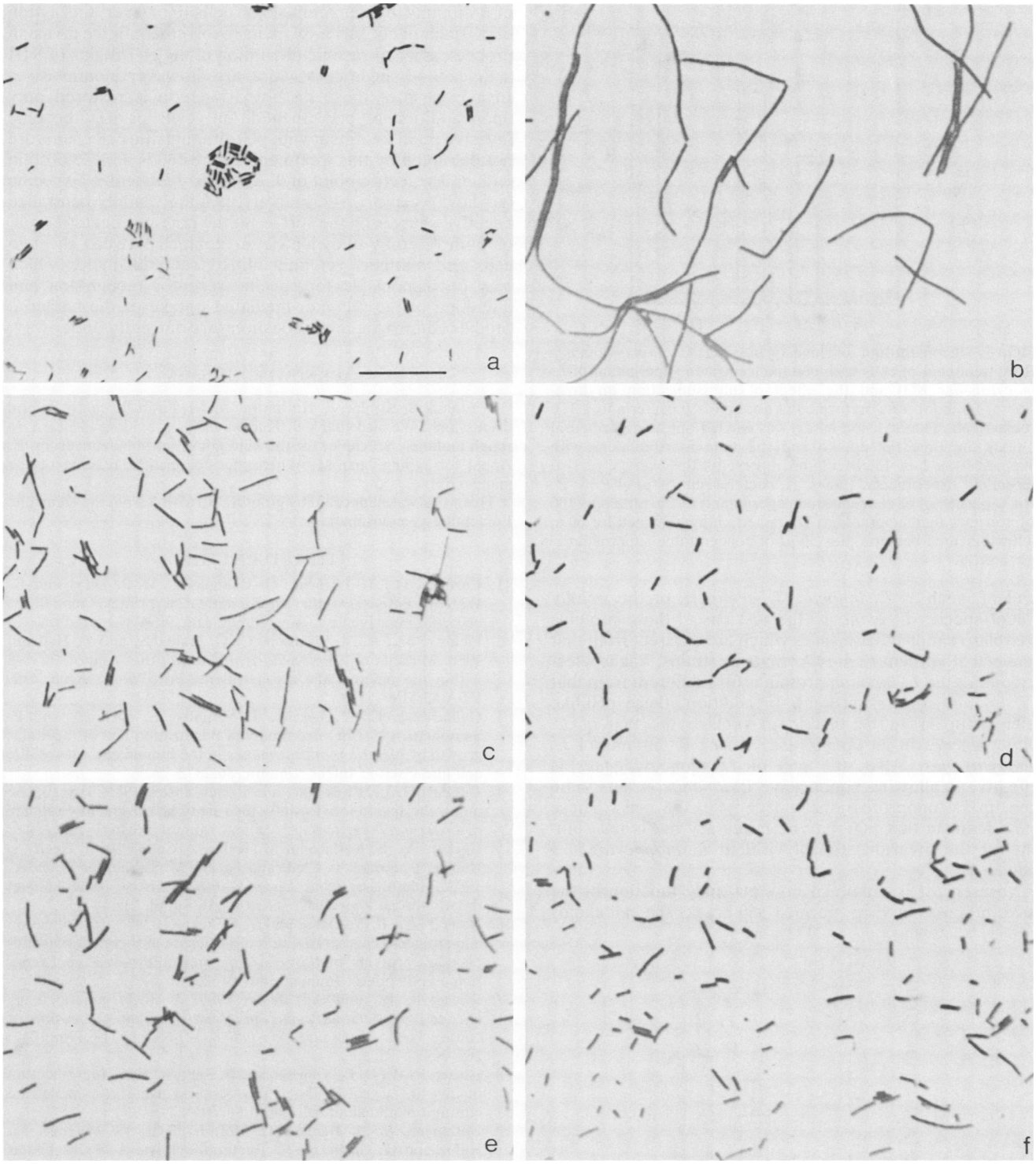

FIG. 6. Gram stain of *L. monocytogenes* Mackaness (a) and *L. monocytogenes* RIII (b). Panels c to f show the decay of the cell chains of *L. monocytogenes* RIII after treatment with 5 (c) and 20 (d) μl of concentrated supernatant containing p60 as well as the effect of ultrasonication for 60 (e) and 120 (f) s. Bar, 10 μm.

an apparently altered p60. The extracellular growth rate of these mutants does not appear to be different from that of wild-type *L. monocytogenes*, but the mutants tend to form cell chains. Although septum formation still occurs, separation of the divided cells does not take place. Since our invasion assay measures viable intracellular bacteria only, the data suggest that p60 may not be necessary for intracellular survival but for uptake only. We have no experimental evidence, however, as to whether multiplication of the intracellular bacteria occurs in the absence of p60.

Extracellular proteins cross-reacting with p60 can be detected in other *Listeria* species, even in those which are

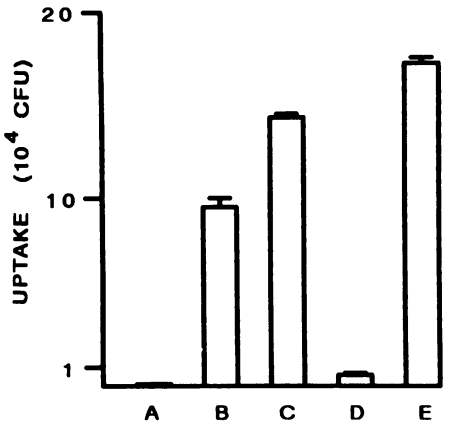

FIG. 7. Reconstitution of invasiveness of *L. monocytogenes* RIII by treatment of cells with p60 (concentrated supernatant of *L. monocytogenes* M3) and the effect of ultrasonication on invasiveness. Infection was performed at a ratio of 10 bacteria to 1 3T6 fibroblast cell. The invasion assay was carried out as described in the text; the time for uptake and the time for treatment with gentamicin was 1 h. Each column represents the average of two independent experiments. Lanes: A, bacteria plus 50 μl of PBS; B, bacteria plus 50 μl of concentrated supernatant; C, bacteria plus 100 μl of concentrated supernatant; D, bacteria ultrasonicated for 60 s; E, ultrasonicated bacteria plus 50 μl of concentrated supernatant.

avirulent, such as *L. innocua*, *L. seeligeri*, or the weakly virulent species *L. ivanovii*. In most cases, these proteins have relative molecular masses which are different from that of the p60 of virulent *L. monocytogenes* strains. The isoelectric point of the *L. innocua* protein is also different from that of *L. monocytogenes* protein. In addition, these p60-like proteins appear to be unable to replace the p60 of *L. monocytogenes* in an uptake assay using R mutants of *L. monocytogenes*. Also, the p60 of *L. monocytogenes* is ineffective in allowing noninvasive *L. innocua* strains to be taken up by nonprofessional phagocytic 3T6 cells. These results indicate that p60 may be necessary but is not sufficient for the invasion step of virulent *L. monocytogenes* strains.

At present, it is unknown how p60 may function in the uptake process. In vitro, purified p60 breaks the cell chains

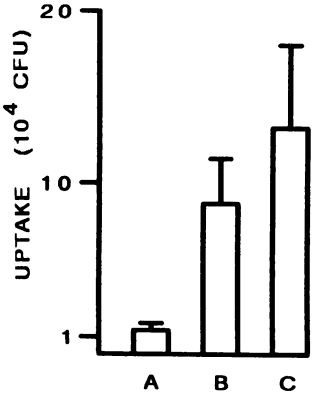

FIG. 8. Effect of p60 on invasiveness of *L. monocytogenes* RIV. Infection was performed as described in the legend to Fig. 7. Lanes: A, untreated *L. monocytogenes* RIV; B, bacteria plus 50 μl of concentrated supernatant; C, bacteria plus 100 μl of concentrated supernatant. Each column represents the average of two independent experiments.

of R mutants into apparent single cells. However, this action of p60 cannot entirely explain its involvement in the invasion process, since ultrasonic disruption of the cell chains of RIII also leads to single cells which are, however, noninvasive. Invasiveness of these disrupted bacteria is restored only upon addition of p60. In addition, cells of RIV become invasive upon treatment with p60 although the cell chains are not disrupted by this treatment. Since p60 is a basic protein with an isoelectric point of 9.3, p60 may recognize a specific (acidic?) receptor. Alternatively, p60 may simply neutralize negative charges on the surface of the host, the bacterial cell, or both, thereby allowing better contact between the two cells and another, yet unidentified bacterial factor which may be responsible for specific receptor recognition and induction of the receptor-mediated uptake of the bacteria. Studies addressing these questions are currently under way.

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