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Structural and Functional Properties of the p60 Proteins from Different *Listeria* Species

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The major extracellular protein p60 of *Listeria monocytogenes* seems to be required for this microorganism's adherence to and invasion of 3T6 mouse fibroblasts but not for adherence to human epithelial Caco-2 cells. Western blot analysis with polyclonal antibodies against p60 of *L. monocytogenes* indicated the presence of cross-reacting proteins in the culture supernatants of all *Listeria* species. Protein p60 of *L. monocytogenes* could restore adhesion of the *L. monocytogenes* mutant RIII (impaired in the synthesis of p60) to mouse fibroblasts more efficiently than that of *Listeria grayi*. The amino acid sequences of the p60-related proteins of *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* indicated highly conserved regions of about 120 amino acids at both the N-terminal and the C-terminal ends. The middle portions of these proteins, consisting of about 240 amino acids, varied considerably. These parts include the repeat domain consisting of repetitions of Thr (T) and Asn (N) which was present only, albeit in different arrangements, in the p60 proteins of *L. monocytogenes* and *L. innocua*. The p60-related proteins of *L. grayi*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* each contained an insertion of 54 amino acids which was absent in the p60 proteins of *L. monocytogenes* and *L. innocua*.

The genus *Listeria* comprises six characterized species (12). *Listeria monocytogenes*, a pathogen responsible for opportunistic infections in humans and animals (30, 31), belongs to the facultative intracellular bacteria which can invade, survive, and replicate within nonprofessional phagocytes and also within phagocytic cells such as macrophages and monocytes. Listeriolysin is required for intracellular survival (10, 18, 27). This virulence factor is part of a gene cluster which includes, in addition to the structural gene for listeriolysin (*hly*), genes encoding a phosphatidylinositol-specific phospholipase C (*plcA*), a metalloprotease (*mpl*), a protein involved in actin polymerization (*actA*), and a lecithinase (*plcB*) (5, 6, 14, 21, 24, 25, 32). All of these genes are under the control of a transcriptional activator, PrfA (22, 23).

A major extracellular protein of all *L. monocytogenes* isolates is p60. Synthesis of this protein is not under the control of PrfA. Mutants which show a decreased level of p60 (R mutants) are avirulent (11, 28) and unable to invade the nonprofessional phagocytic 3T6 mouse fibroblast cells (13). Synthesis of the previously described internalin, a surface protein of *L. monocytogenes* which has been shown to be essential for invasion of epithelial cells (9), is not impaired in this mutant (26). This type of R mutant forms long cell chains with unseparated septa between the individual bacterial cells. Partially purified p60 protein disrupts these cell chains and restores the invasiveness of the R mutants (17). Cell chain disruption activity was also present in the supernatants of all other *Listeria* species (1), suggesting that extracellular proteins exhibiting such activity are common to all *Listeria* species.

The gene encoding p60 of *L. monocytogenes* (designated *iap* for invasion-associated protein) was recently cloned and sequenced (16). It was shown that expression of p60 is regulated on the posttranscriptional level (15). The amino acid sequence deduced from the nucleotide sequence showed a high basic amino acid content. In addition, a repeat domain consisting of repetitions of Thr (T) and Asn (N) was found in the middle portion of p60. Southern hybridization data suggested that the repeat domain of p60 is a specific structural element of the *L. monocytogenes* protein (16).

We have previously reported on the reduced invasiveness of the *L. monocytogenes* rough mutant RIII toward mouse 3T6 fibroblasts (17). This mutant synthesizes a significantly lower amount of the major extracellular protein p60 than the wild-type (WT) strain. Reduction in the amount of p60 leads to the formation of long cell chains (17).

The decrease in the invasiveness of this mutant toward the 3T6 cells was due not only to the long cell chains but probably also to the insufficient amount of p60, since addition of this protein at least partially restored the invasiveness of this mutant (17). Interestingly, this effect was not observed when the epithelial human colon carcinoma cell line

TABLE 1. Adhesion of ultrasonicated *L. monocytogenes* RIII to 3T6 cells after treatment with different p60 preparations

Source of p60[a]	Mean no. of bacteria (SD) after addition of the following volumes of p60-containing extracts[b]:		
	1 μl	10 μl	100 μl
L. monocytogenes WT	0.72 (0.22)	1.33 (0.54)	4.45 (1.10)
L. grayi	0.95 (0.28)	0.95 (0.21)	2.07 (0.90)
L. monocytogenes RIII	0.95 (0.25)	0.75 (0.32)	1.05 (0.23)
Control (BHI)[c]	ND[d]	1.00 (0.47)	ND

[a] p60-enriched concentrated supernatant, prepared as described in the text.
[b] 3T6 cells were infected as described in the text. *Listeria* cells were counted in groups of 5 to 10 host cells. The numbers of bacteria per mammalian cell were calculated for each group; the mean values and the standard deviations of 5 to 10 groups are given.
[c] BHI medium was concentrated as described above and used as negative control.
[d] ND, not determined.

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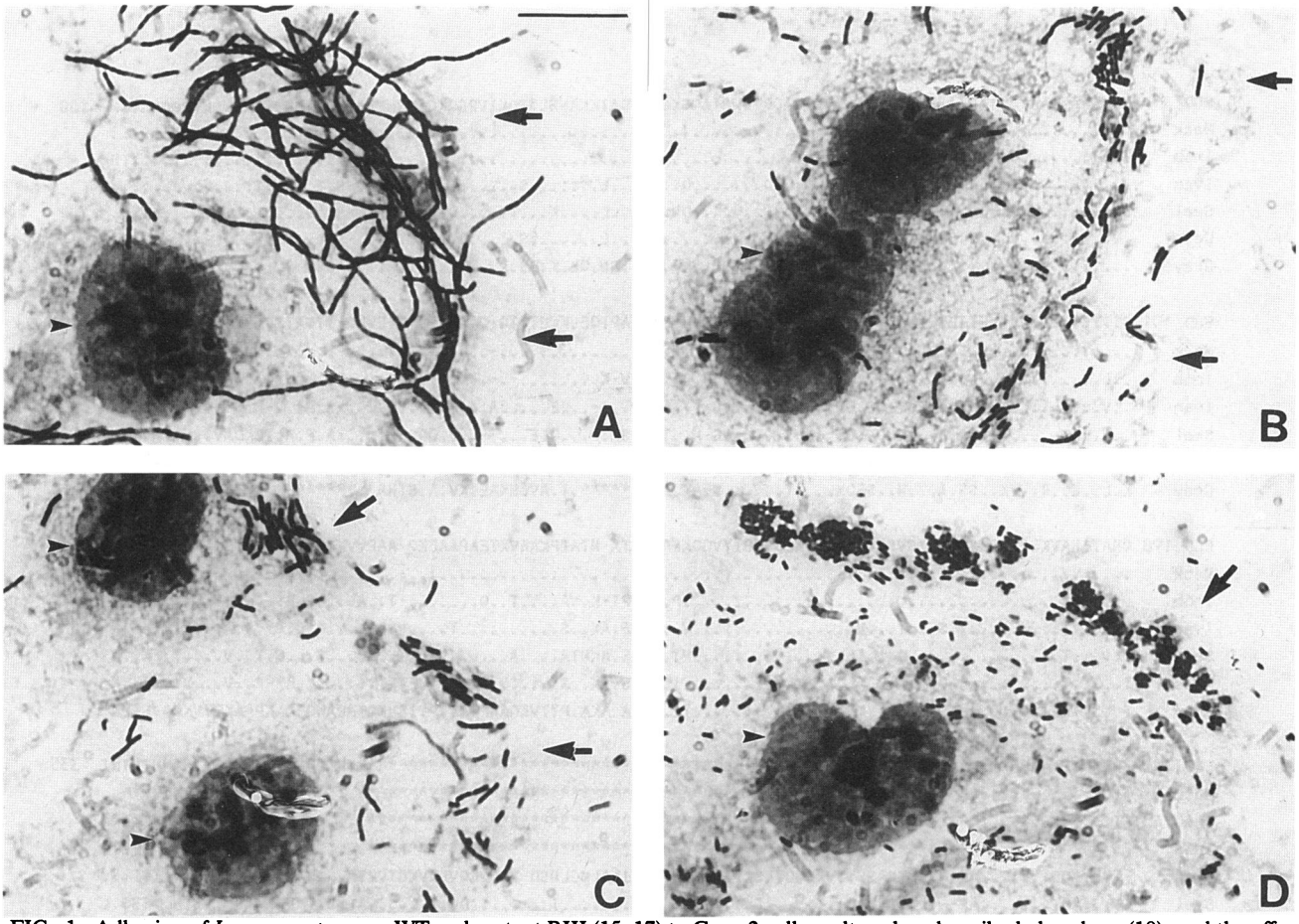


FIG. 1. Adhesion of *L. monocytogenes* WT and mutant RIII (15, 17) to Caco-2 cells, cultured as described elsewhere (10), and the effects of different treatments of mutant RIII. Caco-2 cells (10^5) were seeded in tissue culture plates (60-mm diameter; Greiner) 72 h prior to infection. Tissue culture medium containing 5 μg of tetracycline per ml, p60 preparations, and *L. monocytogenes* strains cultured and prepared for infection as described earlier (17) was added to Caco-2 cells to yield a multiplicity of infection of about 50 bacteria per eucaryotic cell. After centrifugation of the bacteria onto the monolayer as described elsewhere (17), the infected cultures were incubated for 1 h. They were then washed with PBS, and the cells were fixed with methanol for 5 min, dried, and stained with Giemsa (Sigma) directly in the tissue culture plate. (A) *L. monocytogenes* RIII; (B) *L. monocytogenes* RIII ultrasonicated and treated with 100 μl of p60-enriched supernatant from *L. monocytogenes* WT; (C) *L. monocytogenes* RIII ultrasonicated; (D) *L. monocytogenes* WT. Arrowheads point to the nuclei. Each arrow is located to the outer edge of the cytoplasm, which is invisible in each photograph. Bar, 10 μm.

Caco-2 was used as a host for the mutant RIII. As shown in Fig. 1A, efficient adherence even of long chains of the mutant bacterial cells to specific sites of the Caco-2 cells occurred. Disruption of the bacterial cell chains by treatment with p60 from *L. monocytogenes* (Fig. 1B) or with mild ultrasonication (Fig. 1C) led to single cells of similar sizes which adhered to the same region of the Caco-2 cells as the cell chains and the WT bacteria (Fig. 1D).

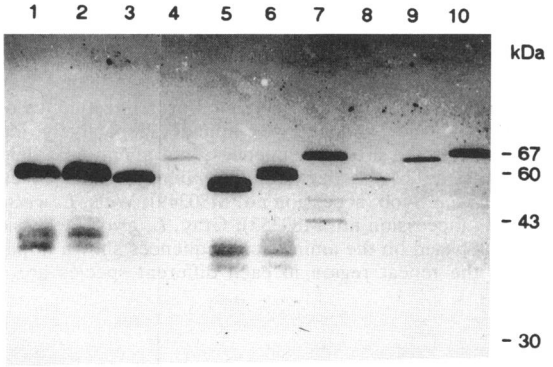


FIG. 2. Identification of the p60 proteins from different *Listeria* species. Proteins from overnight culture supernatants (1 ml) were precipitated with 7% trichloroacetic acid (final concentration) on ice for at least 1 h, and the precipitates were washed once with acetone, solubilized in 20 μl of Laemmli sample buffer (20), and heated to 95°C for 3 min. Protein separation was performed by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel (12.5% polyacrylamide). The proteins were then transferred onto nitrocellulose sheets by semidry electroblotting in a graphite chamber (19). p60 proteins were specifically detected by immunoblot analysis with 1:1,000-diluted polyclonal anti-p60 antiserum (16). Lanes: 1, *L. monocytogenes* Sv1/2a (SLCC5764); 2, *L. monocytogenes* Sv3a (SLCC5015); 3, *L. monocytogenes* Sv4b (SLCC 4013); 4, *L. welshimeri* A; 5, *L. innocua* Sv6a (NCTC 11288); 6, *L. innocua* Sv6b; 7, *L. ivanovii* (ATCC 19119); 8, *L. grayi*; 9, *L. welshimeri* B; 10, *L. seeligeri*. The strains used and the cultivation procedures have been described earlier (2).

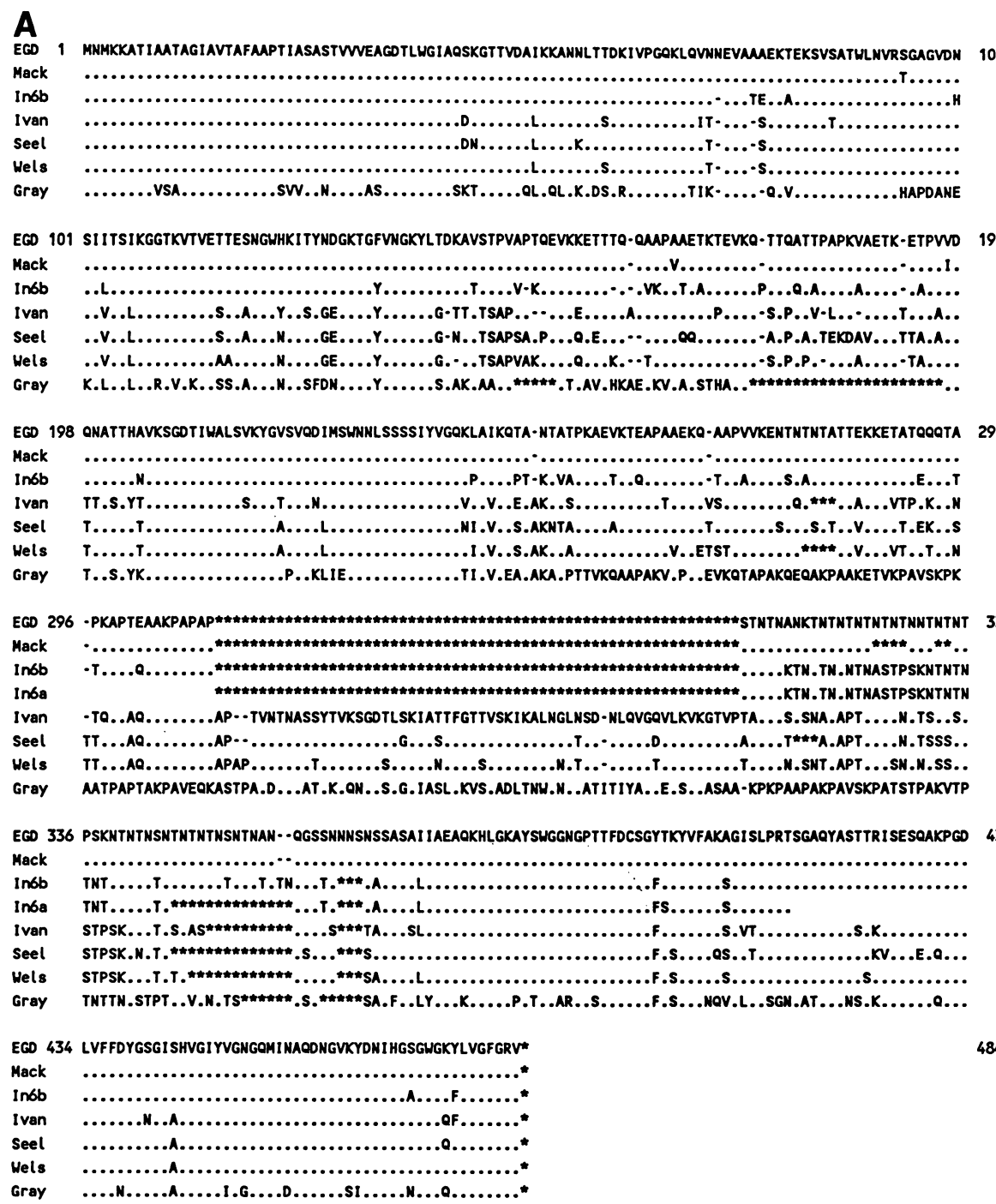
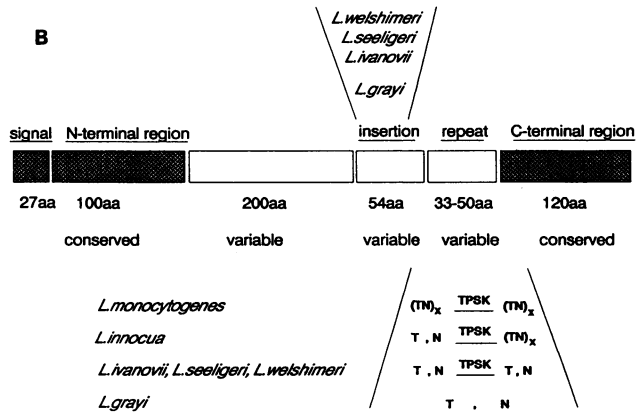


FIG. 3. (A) Comparison between the amino acid sequences of various *Listeria* p60 proteins. The p60-related protein sequence of each *Listeria* species is compared with the p60 sequence of *L. monocytogenes* EGD. Dots, dashes, and asterisks indicate, respectively, identical amino acids, gaps inserted in order to maintain the highest degree of homology among the protein sequences, and amino acid deletions. Abbreviations: EGD, *L. monocytogenes* EGD (gene accession no. X52268 in GenBank data base); Mack, *L. monocytogenes* Mackaness (accession no. M80351); In6a, *L. innocua* Sv6a (accession no. M80347); In6b, *L. innocua* Sv6b (accession no. M80349); Wels, *L. welshimeri* (accession no. M80348); Ivan, *L. ivanovii* (accession no. M80350); Seel, *L. seeligeri* (accession no. M80353); Gray, *L. grayi* (accession no. M95579). (B) Conserved and variable regions in the p60 proteins of *Listeria* species based on the amino acid sequences shown in panel A. *Listeria* species containing an insertion are listed at the top. The structures of the repeat region in each different species are shown schematically below. aa, amino acids.



Whereas for Caco-2 cells invasion of the cell chains by RIII was drastically reduced compared with that by the WT strain, this defect could be completely repaired upon disruption of the bacterial cell chains regardless of the mode of disruption (33).

These data suggested that the reduced invasiveness which this R mutant showed for Caco-2 and 3T6 cells is caused for the second cell line only at least in part by the reduced adherence of this mutant to 3T6 cells. This reduced adherence seems to be directly correlated with the reduced amount of p60 protein synthesized by the mutant *L. monocytogenes* RIII.

Immunoblots of extracellular proteins from other *Listeria* species with polyclonal antibodies against gel-purified p60 of *L. monocytogenes* (16) showed cross-reaction with proteins of all *Listeria* species, as shown in Fig. 2. The sizes of these proteins ranged from 58 kDa (*L. innocua* Sv6a) to 67 kDa (*L. ivanovii*, *L. seeligeri*, and *L. welshimeri*). The apparent sizes of the p60 proteins from *L. monocytogenes* strains belonging to different serovars were similar, whereas the sizes of the p60-related proteins from *L. innocua* strains of different serovars (Sv6a and Sv6b) varied. The intensities of the immunological cross-reactions of the p60-related proteins from *L. grayi* were considerably lower than those of the p60 proteins from the other *Listeria* strains (Fig. 2), although similar amounts of p60 protein in the culture supernatant of *L. grayi* were observed when the supernatant was stained with Coomassie blue (data not shown). This result suggested a lower degree of antigenic similarity for the p60 protein in this *Listeria* species. The cross-reacting protein bands with lower molecular masses visible in the immunoblots (Fig. 2) were probably degradation products of the p60 proteins, since their appearances and intensities varied from preparation to preparation.

The antigenic difference observed between the p60 proteins from *L. grayi* and *L. monocytogenes* seems to reflect a functional difference as well. This was demonstrated by studying adhesion of the ultrasonicated RIII mutant to 3T6 fibroblasts in the presence and absence of p60 proteins from both bacterial species. Supernatant proteins from bacterial overnight cultures were precipitated on ice for 2 h in 60% ammonium sulfate (final concentration). The proteins were pelleted by centrifugation and dissolved in H_2O. After dialysis in phosphate-buffered saline (PBS), the protein was stored in aliquots at −20°C. The 3T6 fibroblasts (5×10^5) were infected as described in the legend to Fig. 1 for Caco-2 cells, and the numbers of bacteria attached to the fibroblasts were counted microscopically.

As shown in Table 1, the concentrated culture supernatant of *L. monocytogenes* which is enriched for protein p60 obviously enhances the adherence of this mutant to the fibroblast cells by increasing the amounts of p60. In contrast, a similar p60 preparation of *L. grayi*, containing an amount of p60 protein approximately equal to that of the p60 preparation from *L. monocytogenes* (see above), caused only a slight and probably insignificant increase in the adherence of RIII bacterial cells to the mouse fibroblasts. Both extracts exhibited activity for the disruption of RIII filaments. Concentrated supernatant from *L. monocytogenes* RIII, which differs from WT supernatant only in the dramatically reduced amount of p60 (17), was unable to restore adhesion of the mutant RIII to 3T6 fibroblasts (Table 1).

To obtain information on the structural differences of the various p60 proteins which might explain the apparent difference in the adhesion-stimulating activities of the p60 proteins from the two different *Listeria* species (and to obtain detailed information on the structural differences), we compared the amino acid sequences of the p60-related proteins from all *Listeria* species.

The corresponding genes of *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* were obtained by polymerase chain reaction (29) from isolated chromosomal DNAs (7) with a 19-nucleotide primer from the 5′ end (sequence, GTCGACTGAATATGAAAAAAGCAAC) and a 19-nucleotide primer from the 3′ end (sequence, GAATTC TATACGCGACCGAAGCCAA) of the *iap* gene of *L. monocytogenes* (16). The polymerase chain reaction products were amplified, cloned, and sequenced as described previously (2). The DNAs and the derived protein sequences were analyzed by the Genetics Computer Group sequence analysis software package 6.2 (4). Figure 3A shows a comparison of the deduced p60 sequences of all *Listeria* species. Several structural features of these p60-related proteins, schematically summarized in Fig. 3B, can be recognized. The N-terminal 120 amino acids and the C-terminal 120 amino acids

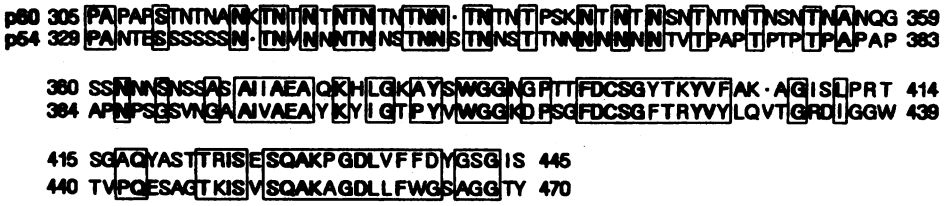


FIG. 4. Comparison of the amino acid sequences of the C-terminal portions of p60 from *L. monocytogenes* EGD and p54 from *E. faecium*. Regions of homology are boxed. Numbers indicate amino acid positions within the protein sequences of p60 and p54.

were highly conserved in all p60 proteins, whereas the middle parts of the p60 proteins, comprising about 240 amino acids, varied considerably. The middle parts consisted of the following three structural elements, as outlined in Fig. 3B. (i) The repeat region, with various numbers of TN repeat units, was present in the p60 proteins of *L. monocytogenes* (two strains of serotype Sv1/2a) and of *L. innocua* serotypes Sv6a and Sv6b. Whereas the repeat regions of the two p60 sequences from *L. monocytogenes* that were determined differed only in the numbers of repeat units (19 for EGD and 16 for Mackaness), however, the p60 proteins from *L. innocua* Sv6a and Sv6b contained only an asymmetric TN repeat starting at the right side of the TPSKN motif and varying in length in the two serotypes. The left-side sequence was also rich in T and N residues, but these amino acids were not arranged in repeat units. Interestingly, the p60 proteins from the other *Listeria* species contained similar compositions of amino acids in this region, mainly N and T, which were, however, not arranged in TN repeat units. However, the TPSKN motif was still observed in all p60 proteins, except in the p60 protein of *L. grayi*. (ii) Directly adjacent to the repeat region (toward the N-terminal end of *L. monocytogenes* p60) was a sequence of 180 amino acids which showed many amino acid exchanges in the corresponding parts of the p60-related proteins from the other *Listeria* species. The p60-related proteins from *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* showed a high sequence homology with each other in this region, which further supports the close phylogenetic relationship among these *Listeria* species (3). (iii) The p60 proteins from *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* and that of *L. grayi* contained, between the two structural elements described above, an insertion of 54 amino acids which was absent from the p60 proteins of *L. monocytogenes* and *L. innocua*. The 54-amino-acid insert seems to be generated by an amplification of a sequence in the variable region of the *iap*-related genes (encoding amino acids at positions 170 to 250, depending on the p60 protein). This part of the p60-related proteins shows 55 to 60% sequence similarity with the amino acid sequence of the insert (data not shown). (iv) The almost identical p60 sequences of *L. grayi* and *L. murrayi* (1) support the idea that both strains are from the same species (12).

The only significant homology between p60 and other proteins was observed with a protein, termed p54, of *Enterococcus faecium* which is composed of 507 amino acids. The function of this protein is unknown, but it is localized on the cell surface (8). The detected homology is limited to a stretch of 140 amino acids at the C-terminal ends of the proteins, as shown in Fig. 4. It is remarkable that the region of p54 corresponding to the p60 repeat also contains mainly T and N residues, which are, however, not arranged in TN repeats, thus more closely resembling the p60 sequences of the *L. ivanovii-L. seeligeri-L. welshimeri* group.

In conclusion, our data indicate that the extracellular protein p60 of *L. monocytogenes* seems to increase binding and invasion of these intracellular bacteria to 3T6 fibroblasts but not to epithelial Caco-2 cells. This activity appears to be rather specific for the *L. monocytogenes* protein. The p60-related proteins observed for all other *Listeria* species differ from that of *L. monocytogenes* particularly in a symmetrically arranged TN repeat region. It remains to be seen whether the observed adhesion property of p60 from *L. monocytogenes* is directly correlated with this repeat domain.

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