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Synthetic Peptides Derived from the *Listeria monocytogenes* p60 Protein as Antigens for the Generation of Polyclonal Antibodies Specific for Secreted Cell-Free *L. monocytogenes* p60 Proteins

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All species of the genus *Listeria* secrete a major extracellular protein called p60. A comparison of the deduced amino acid sequences of all listerial p60 proteins previously indicated there were only a few regions which were unique to the pathogenic, food-borne species *Listeria monocytogenes*. Two of these p60 regions were chosen for the development of antibodies specific for the facultative intracellular species *L. monocytogenes*. Initially, these regions were characterized via epitope mapping, and this led to the development of two different synthetic peptides. Rabbits immunized with these synthetic peptides generated polyclonal antibodies that were then used in Western blot (immunoblot) analyses. Antiserum against peptide A (PepA) recognized the p60 protein in the supernatants collected from most *L. monocytogenes* serotypes except for several strains belonging to serotypes 4a and 4c. No p60-related protein was detected in the supernatants from other *Listeria* species with this anti-PepA antiserum. Antibodies raised against peptide D (PepD) reacted with p60 from all *L. monocytogenes* serotypes, including all 4a and 4c strains that were tested, and also showed no cross-reactivity with supernatant proteins from other *Listeria* species. Both antisera also detected p60 in supernatants of a large number of environmental isolates of *L. monocytogenes*. Besides Western blot analyses, these antisera to PepA and PepD reacted with secreted p60 in an enzyme-linked immunosorbent assay, indicating recognition of the native antigen in addition to the denatured form. These data suggest that synthetic peptides derived from the variable region of the *L. monocytogenes* p60 protein may be useful for the development of an immunological diagnostic assay.

The genus *Listeria* comprises six characterized species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, and *L. grayi* (16). Among these gram-positive, nonsporulating, and motile species that are widespread in nature, only *L. monocytogenes* is a human pathogen and is capable of causing severe infections like septicemia, encephalitis, and meningitis, particularly in immunocompromised individuals, newborns, and pregnant women (13, 33). *L. monocytogenes* is a facultative intracellular bacteria that invades, replicates, and multiplies in a variety of mammalian cells, including macrophages, epithelial cells, and fibroblast cells (11, 20, 26). A number of genes and gene products necessary for the intracellular survival of this pathogen have been previously reviewed (29).

Several outbreaks of listeriosis had been associated with contaminated food such as milk and milk products (8), meat (32), and vegetables (31), in which these bacteria can multiply even at low temperatures (7). Because classical selective enrichments and biochemical analyses for detecting listeriae in foodstuffs are laborious and time-consuming, faster and more reliable methods have to be developed. The generation of antibodies specific for *L. monocytogenes*, which was the goal of this work, could lead to more practicable immunological assays. The previously reported extracellular protein p60 of *L. monocytogenes*, which seems to be required for this organism's adherence to and invasion of fibroblasts but not for adherence to human epithelial Caco-2 cells (5, 19), could be a potential target protein because of its high abundance in the culture supernatants and its high immunogenicity (12). Nevertheless, an antiserum raised against the whole p60 is not suitable for the specific detection of *L. monocytogenes*, since Western blot (immunoblot) analyses have demonstrated cross-reactivity with p60-related proteins in the supernatant of all *Listeria* species (3). The *iap* (invasion-associated protein) gene coding for p60 and the *iap*-related genes of each other *Listeria* species have been cloned, sequenced, and analyzed (3, 17, 18). On the basis of nucleic acid sequence comparison of all listerial *iap* genes, we previously developed a versatile PCR protocol for the unambiguous genus- and species-specific identification of different *Listeria* species (3). Comparisons of the deduced amino acid sequences suggested that there were conserved and variable regions within these proteins (5). It has been shown that the C-terminal conserved region is critical for cell division activity, suggesting the basic function of the p60 protein family may be as a murein hydrolase (39). Portions of the 200 amino acids comprising the variable region near the N terminus appear to be specific for a given *Listeria* species (5).

Because immunizations with short synthetic peptides in a size range of 10 to 15 amino acids could also lead to highly

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reactive antibodies that may recognize both denatured and native proteins (14), we have arbitrarily selected two hydrophilic amino acid sequences derived from the variable region of the *L. monocytogenes* p60 for developing antibodies. Hydrophilicity of a synthetic peptide has been associated with better immunogenicity (14, 37). Besides, antibodies raised against hydrophobic peptides may not recognize the cognate sequence in the native protein because it is likely to be buried and thus inaccessible to the antibody (23).

Initially, the immunogenic character of the selected sequences was analyzed by epitope mapping using an antiserum raised against the whole p60. From these results, two synthetic peptides of 11 and 12 amino acids were then used as antigens for the generation of antibodies in rabbits. These antibodies specifically recognized both denatured and native p60 from culture supernatants. Here, we report on the feasibility of using these antibodies for the development of an immunological assay which may be specific for the food-borne pathogen *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used and their sources are listed in Table 1. *L. monocytogenes* environmental isolates were received from A. Weber (Landesgesundheitsamt Nordbayern, Nürnberg, Germany). Bacterial strains were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C. For enzyme-linked immunosorbent assay (ELISA) studies, all bacterial strains were incubated overnight in Caso-Bouillon (Merck, Darmstadt, Germany) at 35°C, except for *Brochothrix thermosphacta* and *Pseudomonas fluorescens*, which were incubated at room temperature overnight.

Peptide synthesis. Cellulose-bound overlapping peptides for mapping studies were prepared by the spot synthesis technique as described by Frank (9). The peptides are immobilized to the cellulose membrane by their C termini via a β-Ala–β-Ala dipeptide anchor and are N-terminally acetylated. Peptide A (PepA; STPVAPTQEVKK) and peptide D (PepD; QQQTAP KAPTE), derived from p60 of *L. monocytogenes* (accession number P21171 in the SwissProt protein data bank), were synthesized in a peptide synthesizer apparatus (Applied Biosystems, Foster City, Calif.) with the F_{moc}- chemistry with an additional N-terminal cysteine residue for coupling. Purity of the peptides was analyzed by high-performance liquid chromatography (Bio-Rad Laboratories, Richmond, Calif.) and a reverse-phase RP18 column (Macherey und Nagel, Düren, Germany).

Preparation of peptide antisera. Peptides were coupled via the SH group of the N-terminal cysteine residue to glucose dehydrogenase (molecular mass, 30 kDa) from *Bacillus megaterium* (Merck) as a carrier protein by using the linker substance *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Calbiochem Co., La Jolla, Calif.) (37). New Zealand White rabbits (2 to 2.5 kg; Charles River, Kisslegg, Germany) were subcutaneously injected with 750 μg of conjugated peptide emulsified in the oil adjuvant MISA 50 (Seppic, France) for primary immunization, boosted twice with 500 μg of the conjugated peptide, also emulsified in oil adjuvant, on days 14 and 21, and then bled after 30 days. The sera were stored at −20°C. Immunoglobulin G (IgG) fractions from these antisera were prepared by precipitation and removal of serum proteins with octanic acid. IgG-containing supernatants were then precipitated in 45% ammonium sulfate. The antibody pellets were dissolved and dialyzed overnight at 4°C in phosphate-buffered saline (PBS; pH 7.4) (14).

TABLE 1. Bacterial strains used[a]

Species	Strain	Serotype
Listeria monocytogenes EGD		1/2a
L. monocytogenes Mackaness	SLCC 5764	1/2a
L. monocytogenes	SLCC 2755	1/2b
	NCTC 5348	1/2c
	NCTC 5105	3a
	SLCC 5543	3b
	SLCC 2479	3c
	L 99	4a
	SLCC 6999	4a
	SLCC 4561	4ab
	SLCC 4013	4b
	ATCC 19116	4c
	SLCC 4954	4c
	SLCC 6177	4c
	SLCC 6277	4c
	SLCC 6813	4c
	SLCC 6821	4c
	SLCC 6829	4c
	ATCC 19117	4d
	ATCC 19118	4e
	SLCC 2482	7
	NCTC 11288	6a
	NCTC 11289	6b
L. ivanovii	ATCC 19119	5
L. seeligeri	SLCC 3945	1/2b
L. welshimeri	SLCC 5334	6a
L. grayi subsp. *murrayi*		
Bacillus subtilis		
Brochothrix thermosphacta	DSM 20171	
Erysipelothrix insidiosa		
Escherichia coli	ATCC 25922	
Pseudomonas fluorescens	ATCC 13526	
Serratia liquefaciens	ATCC 14460	
Streptococcus faecalis	ATCC 19473	

[a] *L. monocytogenes* serotype (Sv)1/2a EGD was obtained from S. H. E. Kaufmann, University of Ulm, Ulm, Germany. *L. monocytogenes* Sv4a (L 99) was obtained from T. Chakraborty, Institute for Medical Microbiology, Gießen, Germany. *L. grayi* subsp. *murrayi* was obtained from the Special *Listeria* Culture Collection (SLCC) of the Institute of Hygiene and Microbiology at the University of Würzburg, Würzburg, Germany. *B. subtilis* and *E. insidiosa* were taken from our institute's culture collection. Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; NCTC, National Collection of Type Cultures, London, England.

SDS-PAGE and immunoblotting. Proteins from overnight *Listeria* culture supernatants (1 ml) of were precipitated with trichloroacetic acid (final concentration, 7%) on ice for 1 h, washed with acetone, dissolved in Laemmli sample buffer (22), and heated at 95°C for 5 min. Protein separation was achieved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gels as described by Laemmli (22). Transfer to nitrocellulose membranes (Hybond-C; Amersham-Buchler, Braunschweig, Germany) was performed by semidry electroblotting in a graphite chamber (21). For Western blotting, rabbit antisera were diluted as indicated in the figure legends in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20. Blots were developed by using horseradish peroxidase conjugated swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) and 4-chloro-1-naphthol (Sigma).

For immuno-dot blots, anti-p60 antiserum was diluted 1:100 in PBS containing 1% BSA and 0.05% Tween 20. Blots were developed with β-galactosidase-conjugated anti-rabbit antibodies and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Roth, Karlsruhe, Germany) as a substrate.

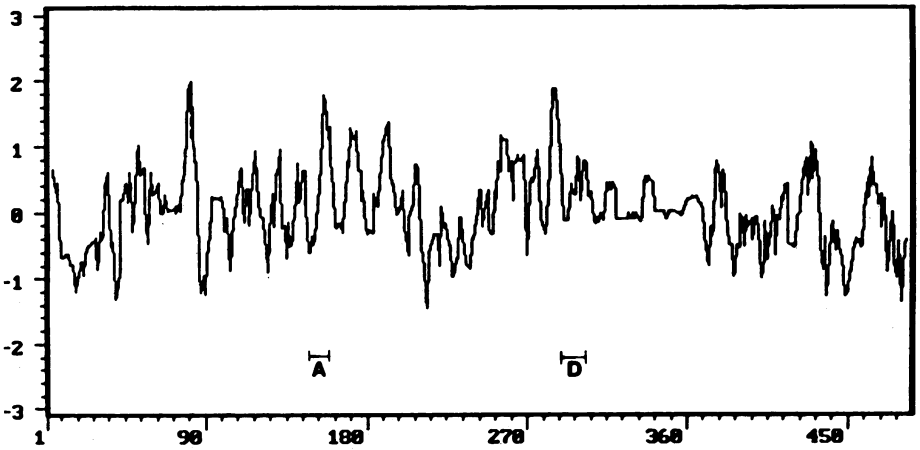

FIG. 1. Hydrophilicity profile of the amino acid sequence of the *L. monocytogenes* p60 protein as described by Hopp and Woods (15). The plot was drawn from average values of six amino acid intervals (positive values = hydrophilic). The positions of the selected peptide regions A and D are indicated with bars.

ELISA. One hundred microliters of cell-free supernatants from overnight cultures was incubated in 96-well microplates (Costar, Cambridge, Mass.) for 2 h at 35°C. After the coated proteins were washed with PBS, wells were blocked for 1 h at room temperature in PBS containing 1% casein–0.05% Tween 20 in PBS. IgG fractions of peptide antisera were used in a concentration of 20 μg/ml. For colorimetric reactions, horseradish peroxidase-conjugated sheep anti-rabbit antibodies (ICN, Costa Mesa, Calif.), diluted 1:500 in PBS, and the substrate 2,2′-azino bis(3-ethylbenzothiazoline-6-sulfonic acid (Pierce, The Netherlands) were used. The optical density (OD) was measured at 405 nm in an ELISA reader apparatus (Dynatech Instruments, Torrance, Calif.). For sandwich ELISA, anti-p60 goat antibodies prepared as described previously (18) were immobilized in microtiter wells and then incubated with 100 μl of supernatant. No blocking step was used, but all other steps were done as described above.

For whole cell ELISA with living and heat-killed bacteria, overnight cultures were centrifuged at 12,000 rpm for 10 min, resuspended in growth medium, and incubated for 1 h at 35°C for coating in the wells. Heat killing was achieved by incubating bacteria for 5 min at 100°C prior to coating. The rest of the procedure was performed as described above.

Amino acid sequence analysis from the various *iap* sequences. The deduced amino acid sequences were analyzed with the ANTIGEN menu of the PC GENE program (Intelligenetics, Palo Alto, Calif.).

RESULTS

Selection of amino acid sequence regions specific for the p60 protein of *L. monocytogenes*. The amino acid sequence comparison of the *Listeria* p60 protein family deduced from genomic DNA sequences indicated that there were only few portions, mainly in the 200 amino acids comprising the variable region, which appeared to be unique to the *L. monocytogenes* p60 (5). To identify potential short antigenic sequences for developing antibodies specific for this species, a hydrophilicity plot of the whole p60 protein was drawn as described by Hopp and Woods (15) (Fig. 1). Two areas that were arbitrarily selected, named A and D, not only showed sequence variability among the p60-related proteins from the other *Listeria* species (Fig. 2) but also exhibited a hydrophilic nature. We chose the latter characteristic in particular because it has been associated with the development of a strong B-cell response when a synthetic peptide is used as an antigen for immunizations (37).

Epitope mapping of the selected p60 regions with polyclonal antiserum raised against the whole p60 of *L. monocytogenes*. To evaluate which amino acid sequences of the arbitrarily selected p60 regions could be potential epitopes, we analyzed several series of overlapping cellulose-bound decapeptides for binding to an antiserum raised against the whole p60 of *L. monocytogenes* Sv1/2a. Generation of this antiserum has been reported earlier (18). Eleven or ten different peptides were synthesized from region A or D, respectively (Fig. 3A and B). Additionally, the homologous sequences to region D of the p60-related protein of *L. innocua* were mapped (Fig. 3C). As shown in Fig. 3A and B, the decapeptides 6 to 11 from region A and 1 to 8 from region D exhibited strong reactions with this antiserum in immuno-dot blot analysis, whereas only two decapeptides from the *L. innocua* portion gave strong positive signals (Fig. 3C). These results indicated that only specific sequences of each region could react with the anti-p60 antibodies. Therefore, we decided to synthesize two peptides (PepA [STPVAPTQEVKK] and PepD [QQQTAPKAPTE]) in a larger quantity for immunizations of rabbits.

Western blot analysis with the antipeptide antisera. Anti-PepA and anti-PepD antisera were tested to determine whether they were suitable for the specific detection of the *L. monocytogenes* p60 protein. For this purpose, we performed Western blot analyses with the supernatant proteins of various *Listeria* species. As shown in Fig. 4, the antisera reacted specifically only with a 60-kDa protein representing the p60 of the homologous *L. monocytogenes* strain, whereas no cross-reaction with the p60-related proteins could be observed.

Next, these antisera were tested to determine the cross-reactivity against the p60 proteins produced by all serotypes of *L. monocytogenes* (Fig. 5A). Supernatants of 13 *L. monocytogenes* strains belonging to all known serotypes were analyzed together with two other *Listeria* species by Western blotting. As shown in Fig. 5B, the anti-PepA antiserum recognized most serotypes except for the low-virulence strains Sv4a and 4c. This difference was probably due to major amino acid sequence variations in this region of their p60 proteins. In contrast, antiserum raised against PepD reacted with the p60 of all *L. monocytogenes* serotypes, including 4a and 4c, like the anti-

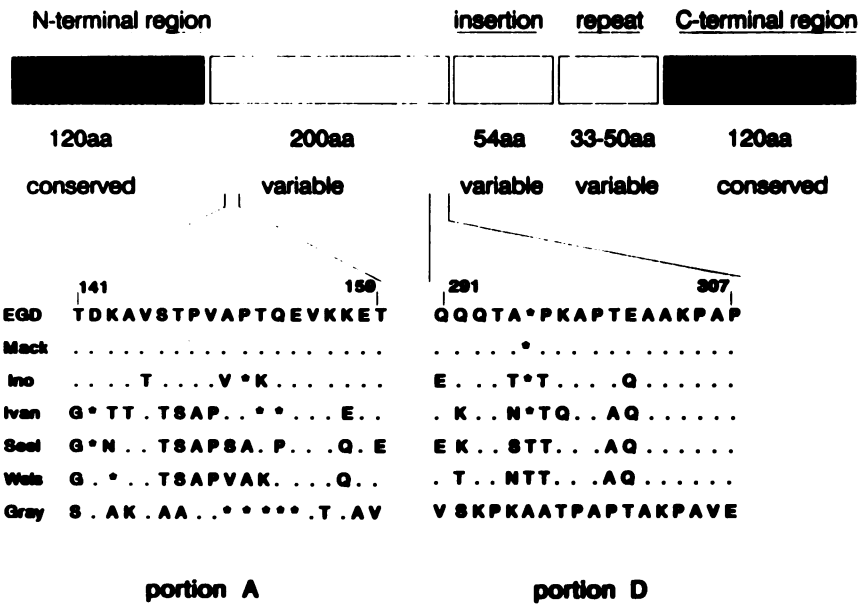

FIG. 2. Amino acid sequence variations of portions of the *Listeria* p60 protein family which were selected for epitope mapping. The p60 proteins are schematically summarized into conserved and variable regions (5). Identical amino acids (aa) are indicated by dots. Asterisks are used to show the highest degree of homology of the protein sequences. The numbers indicate the amino acid position from the N-terminal end of the *L. monocytogenes* p60 protein. Abbreviations: EGD, *L. monocytogenes* EGD; Mack, *L. monocytogenes* Mackaness; Ino, *L. innocua* serovar 6b; Ivan, *L. ivanovii*; Seel, *L. seeligeri*; Wels, *L. welshimeri*; Gray, *L. grayi*.

serum against the whole p60 protein (Fig. 5C), indicating that the amino acid sequence QQQTAPKAPTE is a very conserved region in the p60 protein of all serotypes of this species. The lower bands visible in Fig. 5 represented degradation products of p60, which drastically increased when the supernatant proteins were isolated from late-stationary-phase cultures.

Detection of p60 proteins in the supernatant of *L. monocytogenes* environmental isolates. To confirm the results presented above for various laboratory strains, the supernatants of a number of *L. monocytogenes* environmental strains isolated from food and from feces of zoo animals were analyzed by Western blotting. As shown in Fig. 6, both antipeptide antisera reacted with the p60 protein produced by each isolate. Note that the p60 degradation products in this assay were much lower because the supernatant proteins were harvested from late-logarithmic-phase cultures. In contrast to the 4a strain (see above), the p60 of the environmental isolate belonging to serotype 4a (Fig. 6A, lane 15) was efficiently recognized by the anti-PepA antiserum. Therefore, the supernatants of eight other strains belonging to serotypes 4a and 4c were analyzed with both antipeptide antisera by Western blotting. Whereas anti-PepD antiserum reacted with all produced p60 proteins, the anti-PepA antiserum also recognized the p60 protein from 50% of the examined strains (data not shown).

Detection of native p60 proteins in supernatants by ELISA. To determine whether antibodies raised against PepA and PepD also react with native p60 proteins, IgG fractions of the antisera were tested in ELISAs with supernatant proteins of various *Listeria* species and other bacteria coated onto microtiter wells. Table 2 shows that the anti-PepA antibodies were also able to recognize the epitope of native p60 proteins of all *L. monocytogenes* strains except the two examined 4a and 4c strains, whereas anti-PepD antibodies recognized all serotypes of this species. Detection was specific for p60 of *L. monocytogenes*, and the antibodies showed no cross-reaction with the supernatants of other *Listeria* species or other bacteria tested.

Because coating on microtiter plates can lead to conformational changes or to partial denaturation of proteins (24), the peptide antibodies were also analyzed in sandwich ELISAs. Polyclonal goat antibodies raised against the whole p60 protein of *L. monocytogenes* used as the capture antibodies were immobilized on the plates and incubated with supernatant proteins from a number of *Listeria* species or other bacteria. As shown in Table 2, specific detection of the native *L. monocytogenes* p60 with these peptide antibodies was also observed, albeit the overall readings were lower as in the above-described ELISA. The immobilized antibody in the sandwich ELISA may have blocked recognition sites necessary for the peptide antibody, leading to lower values compared with the standard ELISA.

Detection of surface-bound p60 by ELISA. As reported earlier, p60 can also be detected on the surface of *Listeria* species with a polyclonal antiserum against the whole protein (30). Therefore, the reactivity of anti-PepD antibodies, which recognized all *L. monocytogenes* serotypes, was analyzed with live and heat-killed *L. monocytogenes* Sv1/2a EGD and *L. innocua* Sv6a in ELISA. The values obtained for *L. monocytogenes* (0.26 OD) were not significantly higher than the background value (0.12 OD), which consisted of culture medium. In contrast, antiserum against the whole p60 protein strongly reacted with both live and heat-killed *L. monocytogenes* as well as *L. innocua* (values of >2.7 OD).

DISCUSSION

The standard assay for detection of the pathogen *L. monocytogenes* from polymicrobially contaminated food samples consists of selective cultivation and biochemical analysis, which can take up to 10 days before a clear result is obtained. Other assays such as serotyping or an improved phage typing of *Listeria* species (25) are either time-consuming or not very reliable (7). Another possible method to decrease the detec-

FIG. 3. Epitope mapping by dot blot analysis of the PepA (A) and PepD (B) from the p60 protein of *L. monocytogenes* and from the amino acid sequence of the *L. innocua* p60-like protein homologous to PepD (C). Reactions of the various immobilized decapeptides were performed with anti-p60 antiserum (1:100). Numbers above the dots correspond to the decapeptide numbers from the N-terminal end. The sequences of the various peptides are indicated by bars.

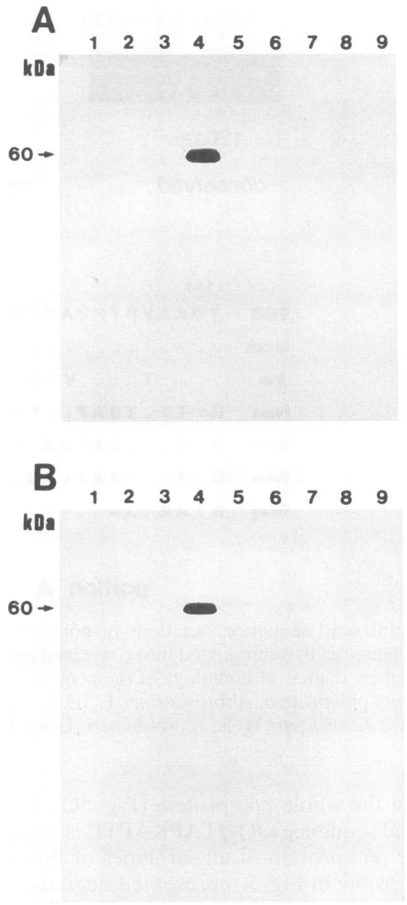

FIG. 4. Western blot analysis of precipitated culture supernatants from 1 ml of overnight cultures from various *Listeria* species and gram-positive bacteria with anti-PepA antiserum (A) and anti-PepD antiserum (B), both diluted 1:500. Lanes: 1, *Bacillus subtilis*; 2, *Brochothrix thermosphacta*; 3, *Enterococcus faecalis*; 4, *L. monocytogenes* EGD; 5, *L. innocua* serovar 6b; 6, *L. ivanovii*; 7, *L. seeligeri*; 8, *L. welshimeri*; 9, *L. grayi* subsp. *murrayi*.

tion time is the use of PCR for the specific identification of *Listeria* species, especially *L. monocytogenes*. Although there have been a number of studies using PCR, only a few showed DNA amplifications specific for all serotypes of *L. monocytogenes* (3, 28). In principle, all serotypes can lead to listeriosis, but serotypes 1/2a, 1/2b, and 4b are the predominant serotypes isolated from infected individuals (7). However, PCR procedures are still limited because some food ingredients can interfere with amplification (38), and a more rapid and efficient method for extracting DNA needs to be developed.

An alternative to PCR technology is the development of an immunological detection assay using antibodies specific for *L. monocytogenes*. Thus far only a few antibodies have been tested, and either they have shown cross-reactions with closely related nonpathogenic *Listeria* or other bacterial species (1, 6, 27, 34) or not all serotypes of *L. monocytogenes* were tested and/or detected (2, 35, 36).

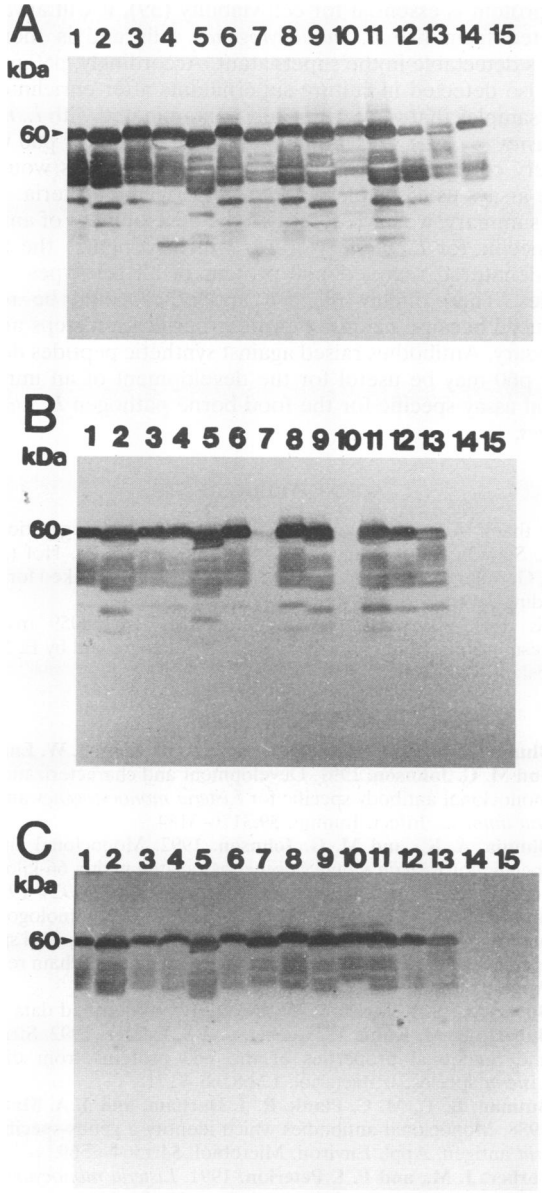

FIG. 5. Western blot analysis of precipitated culture supernatants from all known *L. monocytogenes* serotypes detected with anti-p60 antiserum (A), anti-PepA antiserum (B), and anti-PepD antiserum (C), all diluted 1:250. Lanes: 1, *L. monocytogenes* Sv1/a EGD; 2, *L. monocytogenes* Sv1/2b; 3, *L. monocytogenes* Sv1/2c; 4, *L. monocytogenes* Sv3a; 5, *L. monocytogenes* Sv3b; 6, *L. monocytogenes* Sv3c; 7, *L. monocytogenes* Sv4a (L99); 8, *L. monocytogenes* Sv4ab; 9, *L. monocytogenes* Sv4b; 10, *L. monocytogenes* Sv4c (ATCC 19116); 11, *L. monocytogenes* Sv4d; 12, *L. monocytogenes* Sv4e; 13, *L. monocytogenes* Sv7; 14, *L. innocua* Sv6b; 15, *L. grayi* subsp. *murrayi*.

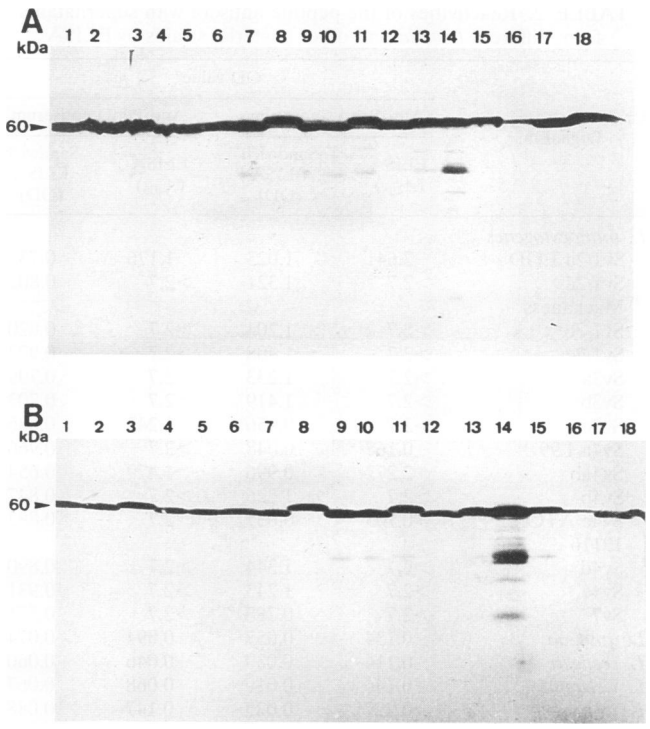

FIG. 6. Western blot analysis of precipitated culture supernatants from *L. monocytogenes* environmental strains which were isolated from feces probes (f.p.) of different zoo animals or from food samples with anti-PepA antiserum (A) and anti-PepD antiserum (B), both diluted 1:250. Lanes: 1, *L. monocytogenes* Sv4b (f.p.); 2, *L. monocytogenes* Sv4b (f.p.); 3, *L. monocytogenes* Sv1/2b (f.p.); 4, *L. monocytogenes* Sv4e (f.p.); 5, *L. monocytogenes* Sv4b (f.p.); 6, *L. monocytogenes* Sv4ab (f.p.); 7, *L. monocytogenes* Sv4ab (f.p.); 8, *L. monocytogenes* Sv1/2b (f.p.); 9, *L. monocytogenes* Sv4ab (f.p.); 10, *L. monocytogenes* Sv4ab (f.p.); 11, *L. monocytogenes* Sv1/2b (f.p.); 12, *L. monocytogenes* Sv4ab (f.p.); 13, *L. monocytogenes* Sv1/2b (beef); 14, *L. monocytogenes* Sv1/2b (raw milk); 15, *L. monocytogenes* Sv4a (duck meat); 16, *L. monocytogenes* Sv1/2a (minced meat); 17, *L. monocytogenes* Sv4ab (pork); 18, *L. monocytogenes* Sv1/2a EGD.

In this report, we describe the development of antibodies specific for the native and denatured secreted p60 protein of *L. monocytogenes*. This protein was selected because antisera from patients with listeriosis and healthy individuals had high titers of anti-p60 antibodies, indicating a highly and predominant immunogenic character (12). We began by epitope mapping of short amino acid sequences in the p60 protein with an anti-p60 antiserum. Selection of peptides from the 200 amino acids comprising the variable region were chosen for three reasons: (i) this portion of p60 showed a relatively higher hydrophilicity profile, which may be necessary for eliciting a strong B-cell response (37); (ii) comparative sequence and immunological studies between the p60-related proteins of two *L. welshimeri* isolates indicated that the N- and C-terminal regions, which are highly conserved in all listerial p60 proteins, do not seem to be very immunogenic (4); and (iii) immunizations with synthetic peptides from the Thr-Asn repeat region of p60 did not lead to an acceptable antibody titer (4).

The epitope mapping suggested that only certain regions would be capable of eliciting a sufficiently high antibody response. Both antipeptide antisera recognized denatured as well as native p60 proteins from different serotypes of *L. monocytogenes*. Antibodies raised against PepA did not react with the p60 protein from several strains belonging to serotypes 4a and 4c in Western blot or ELISA analyses. This may be attributed to major amino acid sequence variations in this portion of their respective p60 proteins. These results are similar to those of other investigations in which PCR amplification and DNA-DNA hybridizations failed to give signals when this region of the *iap* gene from an isolate belonging to serotype 4a was analyzed (10). The conclusion that the gene is missing in this serotype is refuted by our Western blot data

TABLE 2. Reactivities of the peptide antisera with supernatants from different *Listeria* and other bacterial strains by ELISA

Organism	OD value[a]			
	Anti-PepA antiserum		Anti-PepD antiserum	
	ELISA (A_{405})	Sandwich ELISA (OD)	ELISA (A_{405})	Sandwich ELISA (OD)
L. monocytogenes				
Sv1/2a EGD	2.641	1.023	1.176	0.738
Sv1/2a Mackaness	>2.7	1.321	>2.7	0.802
Sv1/2b	>2.7	1.204	>2.7	0.820
Sv1/2c	>2.7	1.408	>2.7	0.922
Sv3a	>2.7	1.233	>2.7	0.709
Sv3b	>2.7	1.419	>2.7	0.703
Sv3c	>2.7	0.956	1.243	0.686
Sv4a L99	0.167	0.048	>2.7	0.966
Sv4ab	2.272	0.996	1.479	0.654
Sv4b	>2.7	1.226	>2.7	0.847
Sv4c ATCC 19116	0.105	0.037	>2.7	0.883
Sv4d	>2.7	1.344	>2.7	0.890
Sv4e	>2.7	1.243	>2.7	0.931
Sv7	>2.7	0.798	>2.7	0.771
L. innocua	0.134	0.057	0.094	0.074
L. seeligeri	0.114	0.030	0.046	0.060
L. welshimeri	0.146	0.019	0.068	0.067
L. ivanovii	0.227	0.025	0.147	0.088
Non-*Listeria* sp.[b]	<0.150	<0.050	<0.150	<0.050

[a] Antibodies were used in a concentration of 20 μg/ml. Values higher than 1 in ELISA and 0.6 in sandwich ELISA were considered positive.

[b] Supernatants of *Streptococcus faecalis*, *Brochothrix thermosphacta*, *Erysipelothrix insidiosa*, *Pseudomonas fluorescens*, *Escherichia coli*, and *Serratia liquefaciens* were also analyzed and showed OD values below 0.15 in ELISA and 0.05 in sandwich ELISA.

with anti-p60 antisera and prior DNA amplification results (3). Taken together, our data demonstrate that a *iap* gene is present and a p60 protein is secreted by serotype 4a. Furthermore, antibodies raised against PepD detected denatured and native secreted, but not cell-associated, p60 proteins from all *L. monocytogenes* strains tested, including all 4a and 4c strains. This finding indicated that the latter portion of the variable region is highly conserved. However, as the whole cell ELISA with live and heat-killed *L. monocytogenes* Sv1/2a EGD indicated, this region apparently is not accessible to the antibody when p60 is cell associated.

Western blot analyses demonstrated that p60 proteins tended to degrade into relative stable protein fragments when isolated from stationary-phase cultures. Because p60 was identified as an autolysin (39), it can be assumed that this effect could be an inactivation mechanism, although no experimental evidence exists thus far. The presented Western blot data demonstrated that degradation products could also be detected by the antipeptide antisera.

The immunological detection of *L. monocytogenes* by using the p60 protein as a target molecule could have the following advantages. (i) p60 is secreted in large concentrations by all serotypes of *L. monocytogenes*, which can lead to a sensitive assay. (ii) Although amino acid sequence comparisons exhibited homologies between portions of two streptococcal proteins and small regions of p60 proteins (5, 39), production of p60 or p60-like proteins seems to be restricted to all members of the genus *Listeria*. No cross-reactivity with anti-p60 antiserum was detected against a battery of gram-positive and gram-negative bacteria with anti-p60 antiserum (30). (iii) Since p60 protein is essential for cell viability (39), it will always be secreted from live *L. monocytogenes* cells and is therefore always detectable in the supernatant. Accordingly, p60 protein was also detected in culture supernatants after enrichment of food samples that were artificially contaminated with *L. monocytogenes* (4). (iv) The immunological detection of p60 would be very convenient because cell-free supernatants would replace assays using whole potential pathogenic bacteria.

In summary, we have described the development of antibodies specific for *L. monocytogenes* which recognize the native and denatured secreted p60 protein of all serotypes of this species. These highly reactive antibodies could be readily produced because extensive protein purification steps are not necessary. Antibodies raised against synthetic peptides derived from p60 may be useful for the development of an immunological assay specific for the food-borne pathogen *L. monocytogenes*.

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