INFECTION AND IMMUNITY, Sept. 1991, p. 3176–3184
0019-9567/91/093176-09$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 59, No. 9

Development and Characterization of a Monoclonal Antibody Specific for *Listeria monocytogenes* and *Listeria innocua*

ARUN K. BHUNIA,* PETER H. BALL, AYRIANA T. FUAD, BEN W. KURZ, JOEL W. EMERSON, AND MICHAEL G. JOHNSON

Department of Food Science and Arkansas Biotechnology Center, University of Arkansas, 272 Young Avenue, Fayetteville, Arkansas 72703

Received 19 November 1990/Accepted 2 July 1991

BALB/c mice were immunized with crude cell surface proteins of *Listeria monocytogenes* V7. Approximately 1,680 hybridomas were generated after two fusions, and the monoclone C11E9 was selected and used for further characterization. The monoclonal antibody (MAb) produced by C11E9 was immunoglobulin subclass G2b with κ light chains. Dot and colony blot results indicated that MAb C11E9 was reactive to all the *L. monocytogenes* (34 of 34) and *Listeria innocua* (6 of 6) isolates without any cross-reaction to other organisms tested. Western blot (immunoblot) analysis of crude cell surface proteins in native polyacrylamide gel electrophoresis (PAGE) indicated that MAb C11E9 reacts with a single band in each species, with a molecular mass of 174 kDa for *L. monocytogenes* and 182 kDa for *L. innocua*. The MAb reacted with one major protein band in Western blot from acid-urea PAGE for both *L. monocytogenes* and *L. innocua*. Isoelectric focusing results indicated two immunoreactive protein bands with pIs of 8.1 and 7.4 for *L. monocytogenes*. Sodium dodecyl sulfate (SDS)-PAGE and Western blot analysis indicated several proteins with molecular masses of 76, 66, 56, and 52 kDa for *L. monocytogenes* and 66, 56, and 52 kDa for *L. innocua*. Reaction of MAb C11E9 to washed live cells indicated the possible binding of antibody to cell surface antigen. These cell surface antigens could be removed by 1 N HCl plus 9 M urea, 2% SDS–0.5% β-mercaptoethanol, or 4 M guanidine-HCl. The epitope of MAb C11E9 binding site was shown to be protein in nature. Periodic acid-Schiff staining and glycoprotein immunoassay indicated that carbohydrate was absent in the epitope. The cellular locations of the MAb C11E9-reactive antigens were calculated to be 76 and 90% outside and 24 and 10% inside the cell membranes of *L. monocytogenes* and *L. innocua*, respectively, for 12- to 14-h cultures.

Listeria monocytogenes infection through contaminated food is well documented in the literature. Because of its disease-producing abilities and fatal prognosis in humans, several detection systems have been developed. Among these techniques, isolation and identification of *L. monocytogenes* by selective culture media are very effective (8, 13, 23–26, 28, 35). Similarly, DNA-based assay systems to detect *Listeria* spp. in food have also been reported (3, 9, 19, 21). However, these techniques take about 2 to 4 days for detection.

Monoclonal antibody (MAb)-based enzyme-linked immunoassays (ELISA) and dot blot assays have been reported to identify these organisms in food (6, 12, 18, 27, 30, 36) and clinical samples (29, 31, 32). However, some of these MAbs react with all species of *Listeria* (6, 12, 27, 40) or react with *L. monocytogenes* and some of the other nonpathogenic *Listeria* spp. Siragusa and Johnson (36) reported a MAb, P5C9, which reacted with *L. monocytogenes* as well as *Listeria innocua* and *Listeria welshimeri*.

The purpose of this study was to develop MAbs which react with the cell surface antigens of *L. monocytogenes* which will remain present in the bacteria irrespective of their growth environment and life-style. MAb raised against flagellar antigen (11) or listeriolysin (22) may not give positive results when *Listeria* spp. are grown in unfavorable environments (33, 34) in which these antigens are not produced. Besides developing a MAb against stable cell surface antigen, we attempted to develop MAbs specific for *L. monocytogenes*.

* Corresponding author.

In this study, we report a MAb which reacts with a stable cell surface antigen of *L. monocytogenes* and *L. innocua* and shows no cross-reactions with other gram-positive or gram-negative organisms. Furthermore, we characterized this antibody using ELISA, dot blot, colony blot, and Western blot (immunoblot) techniques. We also looked at the response of the epitope to several proteolytic enzymes and solvents and the distribution of MAb C11E9-reactive antigens among various cell fractions.

MATERIALS AND METHODS

Cultures and media. The cultures of *Listeria* spp. and other gram-positive and gram-negative organisms mentioned in Table 1 were maintained in tryptic soy broth (TSB; Difco Laboratories) containing yeast extract (0.5%) and Difco agar (1.5%) slants (TSA-YE) at 25°C. The fresh cultures for all the experiments were obtained by inoculating slant cultures into TSB containing yeast extract (0.5%) (TSB-YE) at 37°C for 16 to 18 h. The protein A-negative *Staphylococcus aureus* strain (Sansorbin) was obtained from Calbiochem Co., La Jolla, Calif.

Extraction of *Listeria* surface protein. *L. monocytogenes* V7 and *L. innocua* LA-1 were grown separately in 10 liters of TSB-YE at 37°C for 16 to 18 h. The cells were harvested by centrifugation (6,000 × *g*, 10 min) and washed three times with phosphate-buffered saline (PBS), pH 7.0 (PBS is 20 mM sodium phosphate–0.85% NaCl). The washed cell pellets were resuspended with 100 ml of SDS buffer (2% sodium dodecyl sulfate, 0.5% β-mercaptoethanol, PBS, pH 7.0) and incubated at 37°C for 30 min. After centrifugation (10,000 × *g*, 10 min), the cell extracts were dialyzed exhaustively

TABLE 1. Reactivity of MAb C11E9 to different organisms

Organism	Reaction		
	Dot blotting		Colony blotting
	Live	Heat killed[a]	
Listeria monocytogenes			
V7	+	+	+
Scott A	+	+	+
Brie 1	+	+	+
F1057	+	+	+
F5069	+	+	+
F2379	+	+	+
F1109	+	+	+
LCDC 81-861	+	+	+
ATCC 15313	+	+	+
ATCC 35152	+	+	+
171	+	+	+
USDA-RB	+	+	+
V37CE	+	+	+
Murray B	+	+	+
Listeria innocua			
LA-1	+	+	+
ATCC 33090	+	+	+
Listeria ivanovii			
KC1714	−	−	−
ATCC 19119	NT[b]	NT	−
Listeria grayi ATCC 19120	−	−	−
Listeria welshimeri ATCC 35897	−	−	−
Listeria seeligeri SE 31	−	−	−
Listeria murrayi ATCC 25401	−	−	−
Enterococcus faecalis ATCC 344	−	−	−
Enterococcus pyogenes ATCC 19615	NT	NT	−
Staphylococcus aureus ATCC 25923	+	+	+
Staphylococcus aureus (Sansorbin)	NT	−	NT
Staphylococcus epidermidis ATCC 12228	NT	NT	−
Bacillus cereus ATCC 14597	−	−	−
Bacillus subtilis	−	−	−
Micrococcus luteus	−	−	−
Citrobacter freundii ATCC 3624	−	−	−
Rhodococcus equii	NT	NT	−
Lactobacillus plantarum ATCC 8014	NT	NT	−
Escherichia coli			
ATCC 9002	−	−	−
ATCC 25922	NT	NT	−
Salmonella typhimurium ATCC 14028	−	−	−
Klebsiella pneumoniae ATCC 13883	NT	NT	−
Enterobacter cloacae ATCC 23355	NT	NT	−
Serratia marcesens ATCC 8100	NT	NT	−
Proteus vulgaris ATCC 13315	NT	NT	−
Pseudomonas aeruginosa ATCC 19142	−	−	−
Pseudomonas putrefacens P-19	−	−	−

[a] 16- to 18-h-grown cultures were subjected to heat at 90°C for 20 min.
[b] NT, not tested.

against deionized water at 25°C for 48 h and lyophilized for future use; the extracts were designated crude cell surface proteins (CCSP). After SDS buffer extraction, the cells were checked under a phase-contrast microscope to assess any cell lysis. The protein concentration of the CCSP was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Mouse immunization and hybridoma production. Five-week-old BALB/c mice were injected intraperitoneally over a 12-week period with CCSP from *L. monocytogenes* V7 cells (200 μg per mouse) with or without alum precipitation (14) over a 12-week period. At least 6 days before fusion, the animals were immunized daily with the same antigen with poly(A) · poly(U) as an adjuvant (36).

The hybridoma cells were produced according to the basic procedure of Kohler and Milstein (20). Equal numbers of murine myeloma P3/Ns1/1-Ag4-1 (NS1) cells and spleen cells from immunized mice were fused and maintained in 96-well tissue culture plates containing Iscove's modified Dulbecco's medium (J.R. Scientific, Inc., Woodland, Calif.) with 10% fetal bovine serum (Hyclone, Logan, Utah) and the selective agents hypoxanthine, aminopterin, and thymidine (all from Sigma). Screening and cloning of hybridomas were achieved by the method described by Siragusa and Johnson (36). The monoclone C11E9 was recloned by limiting dilution to obtain a single clone per well in 96-well tissue culture plates and allowed to grow for 10 to 12 days. Wells which contained single colonies were further propagated in Iscove's modified Dulbecco's medium plus fetal bovine serum, and cell supernatants were tested by ELISA and Western blotting.

Screening of hybridoma for anti-*Listeria* antibody by ELISA. Several 96-well flat-bottom microtiter plates (Immulon 1, Dynatech Lab) were coated with either CCSP from *L. monocytogenes* V7 cells (100 μg per well) or washed whole cells (10^8 cells per well) by using 0.05 M carbonate coating buffer, pH 9.6, for 12 to 14 h at 4°C. After the plates were washed four times with PBS–0.5% Tween 20 (PBS-T), 0.1 ml of hybridoma culture supernatants were added to each well and incubated for 1 h at 37°C in a shaker incubator. Plates were then washed four times with PBS-T. A goat anti-mouse immunoglobulin (IgG) (heavy and light chain) horseradish peroxidase conjugate (Sigma) in PBS (100 μl per well) was added, and the mixture was incubated for 1 h. After four PBS-T washes, 100 μl of substrate solution (1 mg of *O*-phenyldiamine per ml of 0.1 M sodium citrate buffer containing 0.4 ml of 30% H_2O_2, pH 4.3) was added per well. The reaction was stopped by the addition of 2 N H_2SO_4 (100 μl per well), and the color reaction was measured with a plate reader at 490 nm.

Ascites production, immunoelectrophoresis, and immunoglobulin isotyping. Two weeks prior to the start of ascites production, 20 8-week-old mice (IRCF-1 strain; Simonsen Lab Inc., Gilroy, Calif.) were injected with pristane (0.5 ml per mouse) (Sigma). Log-phase C11E9 hybridoma cells were injected into pristane-primed mice to yield 1×10^6 to 3×10^6 cells per mouse. The ascites fluid containing MAb was collected after the onset of ascites tumors and centrifuged ($1,200 \times g$), filtered with cheesecloth, and partially purified by ammonium sulfate precipitation (17).

The presence of MAb in ascites fluid was quantitated by serum protein electrophoresis (14). The immunoglobulin isotyping was accomplished in a microtiter plate which was previously sensitized with CCSP of *L. monocytogenes* V7. After addition of MAb (10^{-4}) to the plate, the immunoglobulin subclass and heavy and light chain types were determined by using the Bio-Rad Mouse Typer (Bio-Rad).

Dot and colony blotting. The spectrum of activity of MAb from C11E9 towards different *Listeria* spp. and other gram-positive and gram-negative organisms was tested by dot and colony blot analyses. Different organisms mentioned in Table 1 were inoculated into 5 ml of TSB-YE media and grown for 16 to 18 h. The cells were harvested by centrifu-

gation and resuspended in 5 ml of sterile PBS. For dot blotting, 100 μl of each culture was placed in a designated well in a dot blot manifold apparatus (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) containing an Immobilon P membrane (Millipore Corp., Bedford, Mass.). The membrane containing cells was air dried and soaked in 30 ml of methanol containing H_2O_2 (1%) for 30 min at 25°C to remove the endogenous bacterial peroxidases (15).

For colony blot analysis the cultures were stabbed onto TSA-YE plates with an inoculation needle and incubated at 37°C for 4 to 6 h. A piece of Immobilon P membrane was then placed over the stabbed cultures, and incubation was continued for another 12 h. The membrane was removed, air dried, and treated with methanol-H_2O_2. Both dot blot and colony blot membranes were blocked with 2% bovine serum albumin in PBS for 1 h and probed with MAb C11E9 (2×10^{-4} dilution in PBS) for 1 h. The blots were washed twice for 10-min periods with PBS-T, and then goat anti-mouse horseradish peroxidase-conjugated IgG (heavy and light chain) was added. After incubation for 1 h, the blots were washed as before and reacted with 20 ml of substrate solution containing 10 mg of 3,3'-diaminobenzidine tetrahydrochloride–20 ml of 0.05 M Tris, pH 7.6–1 ml of 8% nickel chloride–0.1 ml of 30% H_2O_2 (Sigma). The color reaction was stopped by washing the membrane in water (5, 36).

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting. (i) Native PAGE. The lyophilized CCSP of *L. monocytogenes* V7 and *L. innocua* cells were dissolved to give a concentration of 2 mg/ml in sample solvent (20% glycerol, 1.5% Tris, 1% bromophenol blue, pH 6.8). A 0.03-ml volume of each sample and a 0.015-ml volume of molecular mass standards for use under nondenaturing conditions (molecular masses, in kilodaltons: apoferritin, 900 [dimer] and 450 [monomer]; jack bean urease, 545 [hexamer]; bovine serum albumin, 132 [dimer] and 66 [monomer]; and chicken egg albumin, 45; Sigma) were loaded onto each of several wells of 10 to 20% or 5 to 10% discontinuous gradient nondenaturing gels (without any SDS) (39). At the end of electrophoresis (25 mA for 3.5 h), part of the gel was stained with Coomassie blue R-250 for protein stain and the other part was transblotted to a Immobilon-P membrane by using the Bio-Rad transblot apparatus (Bio-Rad) (38). The blotted membrane containing the protein bands were either stained with fast green (0.1%) or immunoprobed with MAb C11E9 as described for dot blotting with the modification that an alkaline phosphatase-conjugated IgG (heavy and light chain) was used as the secondary antibody. The substrate mixture contained 0.33 mg of Nitro Blue Tetrazolium per ml and 0.165 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml of alkaline phosphatase buffer, pH 9.5 (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$).

(ii) SDS-PAGE. For SDS-PAGE the CCSP were dissolved in sample solvent, previously described, to which 4.6% SDS and 10% β-mercaptoethanol were added. Similarly, SDS was added to the gel and the running buffer. Protein samples and molecular weight standards (MW-SDS-7; Sigma) were loaded onto several wells of 10 to 15% gradient gel and electrophoresed at 25 mA for 3.5 h (4, 39). The gel sections either were stained with Coomassie blue (for protein stain) or periodic acid-Schiff stain (for glycoprotein), using glyceraldehyde 3-phosphate dehydrogenase as positive control (11), or were transblotted to Immobilon P membranes. Subsequently, the blotted membranes were either stained for glycoprotein by an enzyme immunoassay (DIG glycan detection kit; Boehringer Mannheim Co., Indianapolis, Ind.) or immunoprobed with MAbs as before.

(iii) Acetic acid-urea PAGE. The CCSP from *L. monocytogenes* V7 or *L. innocua* cells were also analyzed by acetic acid-urea PAGE (37). The proteins (2 mg/ml) were dissolved in sample solvent containing 1 ml of HCl (1 M), 0.5 ml of β-mercaptoethanol, 5.4 g of urea, 0.5 ml of pyronin Y (0.4% [wt/vol] in H_2O), and 4.5 ml of H_2O. A 0.03-ml volume of each sample and 0.01-ml volumes of molecular marker (phosphorylase *b*, bovine serum albumin, and carbonic anhydrase) were loaded onto several wells and electrophoresed (180 V, 5 h) in a vertical slab gel (Bio-Rad) with the cathode at the bottom end of the gel. At the end of the electrophoresis, the gel was either stained or transblotted to Immobilon P membrane for immunoassay.

Isoelectric focusing and immunoblotting. The CCSP from *L. monocytogenes* and *L. innocua* cells were focused in an 8% acrylamide gel containing ampholyte (pH 3 to 10) according to the Pharmacia isoelectric focusing manual (Pharmacia Fine Chemicals, Uppsala, Sweden). A 0.012-ml volume of the sample along with 0.004 ml of protein standards (IEF Standards; 161-0310; Bio-Rad) were loaded on to the gel and focused for 3 h at 6 W (constant current). The pH gradient of the gel was determined at intervals by using a blunt probe electrode and digital pH meter (Fisher Scientific, Pittsburgh, Pa.). One part of the gel was stained with Coomassie blue R-250, and the other part was transblotted to an Immobilon P membrane and immunoprobed with C11E9.

Removal of surface proteins by different chaotropic agents. A 5-ml volume each of freshly grown cultures of *L. monocytogenes* V7 and Scott A and *L. innocua* LA-1 was centrifuged (12,000 × *g*, 5 min), and cell pellets were washed once with 1 volume of PBS. The cells were suspended in 5 ml of one of four solutions: (i) an acid-urea solution containing 1 ml of HCl (1 N), 5.4 g of urea, 0.5 ml of β-mercaptoethanol, and 5 ml of H_2O; (ii) 2% SDS–0.5% β-mercaptoethanol in PBS, pH 7.0; (iii) 1 M NaCl; or (iv) 4 M guanidine-HCl (Gu-HCl), pH 7.0. After incubation for 30 min, the cells were harvested by centrifugation, resuspended with the same volume of PBS, and examined under a phase-contrast microscope for cell lysis. Both extracted whole cells and cell-free supernatant preparations were then tested by dot blotting for reactivity with MAb C11E9. Unextracted samples of whole cells of *L. monocytogenes* and *L. innocua* served as positive controls, while *L. welshimeri* was used as a negative control.

Epitope characterization of C11E9-reactive proteins. A 2-mg quantity of CCSP from *L. monocytogenes* V7 or *L. innocua* cells was dissolved in 1 ml of 0.05 M carbonate buffer, pH 9.6. A 0.05-ml aliquot of each protein was added with 0.05 ml of trypsin type 1, γ-chymotrypsin type III, or pronase E (protease type XXV) at a concentration of 1 mg/ml in PBS (Sigma). After 1 h of incubation at 37°C, 0.05 ml of trypsin inhibitor (1 mg/ml) was added to all the tubes, and the mixture was incubated for 40 min. Similarly, 0.05 ml of each protein sample was mixed with 0.1 ml of acetone, methanol, hexane, or ethanol (50% [vol/vol]), and the mixture was incubated for 1 h at 37°C. The solvents were removed by evaporation, and the final volume of each sample was adjusted to 0.2 ml with carbonate buffer, including the control samples, which did not receive any enzyme or solvent treatments. The samples were then used to sensitize ELISA plates in duplicate wells. The ELISA was continued with MAb C11E9.

Localization of MAb C11E9-reactive proteins in cell fractions. MAb-reactive proteins in *L. monocytogenes* and *L. innocua* cell fractions were determined by the method described by Jarvis and Dworkin (16) with some modifications.

The cells were grown in 10 ml of TSB-YE for 12 to 14 h. The cell-free supernatants were obtained by centrifugation (10,000 × *g* for 10 min at 10°C) and membrane filtration (0.45-μm-pore-size filter). The cell pellets were resuspended and washed four times with PBS, and all the PBS extracts were saved for further analysis. The PBS-washed cell pellets were then resuspended with 10 ml of PBS containing 5 mM EDTA and disrupted at 4°C with a cell sonifier disrupter (model 200; Branson Sonic Power Co., Canbury, Conn.) following the manufacturer's instructions. The cell wall and the intracellular materials were separated by centrifugation (10,000 × *g* for 20 min at 10°C). The cell wall pellets were resuspended with 10 ml of PBS and were used to coat microtiter plates along with cell-free TSB-YE culture supernatant, PBS-washed whole cells, the PBS washings, and the soluble intracellular fractions. The ELISA was done with MAb C11E9.

RESULTS

MAbs. Approximately 1,680 hybridomas were generated after two fusions. Only 36 hybridomas produced antibodies which showed positive reactions when tested in ELISA against CCSP from *L. monocytogenes* cells. Eight of these 36 hybridomas showed positive reactions when tested against PBS-washed whole cells of *L. monocytogenes*, indicating that the antibody produced by these hybridomas recognize cell surface-associated antigens. Cloning of one (2C11) of these eight hybridomas by limiting dilution generated several monoclones, of which two, C11F3 and C11E9, were selected on the basis of their positive reactions with *L. monocytogenes* V7 and Scott A and *L. innocua* and negative reactions with *L. ivanovii* and *L. welshimeri* cells. Recloning of C11E9 by limiting dilution generated about 25 monoclones. All these clones gave identical ELISA and Western blot results when tested against CCSP from *L. monocytogenes* and *L. innocua* cells. C11E9 was used for all the subsequent studies.

The protein concentration of partially purified MAb C11E9 from ascites fluid was shown to be 12.6 mg/ml, and the MAb showed as a clear gamma globulin band upon immunoelectrophoresis (data not shown). Both monoclones C11F3 and C11E9 produced IgG, subclass G2b, with κ light chains.

Dot and colony blots. The MAb C11E9 reacted in dot blots with both live and heat-killed cells of all the *L. monocytogenes* and *L. innocua* isolates tested and gave no cross-reactions with other *Listeria* or non-*Listeria* organisms tested (Table 1 and Fig. 1). Similar results were obtained in colony blot analyses with only live cells. In addition, the following strains gave positive dot and colony blotting results with MAb C11E9: *L. monocytogenes* transposon mutants Tn*590*, Tn*476*, Tn*585*, Tn*543*, Tn*104035*, Tn*571*, Tn*433*, Tn*434*, Tn*534*, Tn*570*, and Tn*524* (obtained from R. Buchanon, U.S. Department of Agriculture, Agricultural Research Services, Philadelphia, Pa.); cattle isolates of *L. monocytogenes* $C12_1$-2(L) and *L. innocua* $C9_1$-2(L) and $C3_1$-3(L) (obtained from G. R. Siragusa, U.S. Department of Agriculture, Agricultural Research Service, Clay Center, Nebr.); and human clinical isolates of *L. monocytogenes* F4393, F4233, F4264, F4262, F4243, F4263, F4244, and F4260 and *L. innocua* F4247 and F4248 (obtained from B. Swaminathan, Centers for Disease Control, Atlanta, Ga.). *Staphylococcus aureus* gave a nonspecific positive reaction because of the binding of surface protein A to the MAb, whereas a protein A-negative preparation of *S. aureus* did not show any reaction (Table 1).

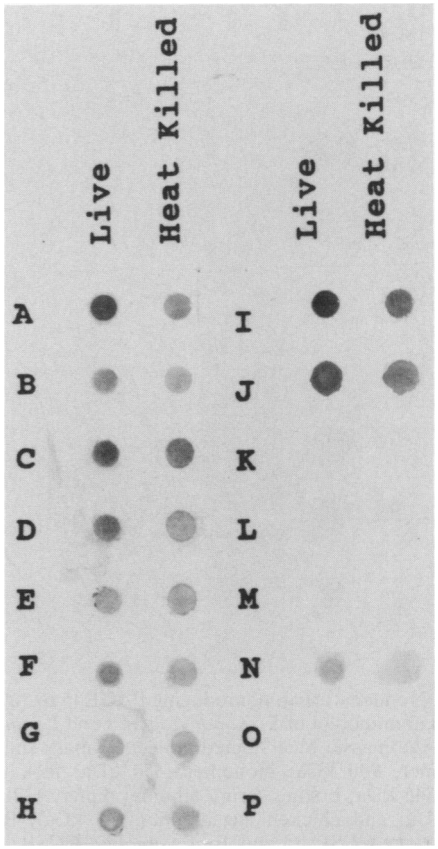

FIG. 1. Dot blot analyses of live and heat-killed *Listeria* spp. with MAb C11E9. The organisms in blot, by row, are *L. monocytogenes* V7 (A), Scott A (B), F1057 (C), F5069 (D), LCDC 81-861 (E), ATCC 15313 (F), ATCC 35152 (G), Murray B (H), V37CE (I), and Brie 1 (J); *L. ivanovii* (K); *L. grayii* (L); *L. welshimeri* (M); *L. innocua* (N); *L. seeligeri* (O); and *L. murrayi* (P).

PAGE and immunoblotting. The native PAGE (10 to 20% polyacrylamide) analysis of CCSP from *L. monocytogenes* V7 and *L. innocua* cells under nondenaturing and nonreducing conditions showed the presence of several protein bands upon Coomassie blue staining. After immunoblotting, the MAb C11E9-reactive proteins were detected in the interface of stacking and running gel (data not shown). For improved resolution, CCSP were analyzed in 5 to 10% PAGE under the same conditions, and one MAb C11E9-reactive protein band of 174 kDa for *L. monocytogenes* (Fig. 2, lane D) and 182 kDa for *L. innocua* (Fig. 2, lane E) was noticed.

In acid-urea PAGE (10% polyacrylamide) the CCSP were separated on the basis of the total charge and the mass. Immunoblotting with MAb C11E9 yielded one strongly reactive protein band and a second minor protein band for each *L. monocytogenes* (Fig. 3, lane D) and *L. innocua* (Fig. 3, lane E) isolate.

Isoelectric focusing gels after immunoblotting with MAb C11E9 indicated that there were two protein bands with isoelectric points (pIs) of 8.1 and 7.4 in CCSP from *L. monocytogenes* (Fig. 4, lane E), whereas *L. innocua* showed a wide protein band with a pI of about 7.4 (Fig. 4, lane D).

SDS-PAGE (10 to 15% polyacrylamide) analysis of CCSP from *L. monocytogenes* V7 and *L. innocua* cell showed the

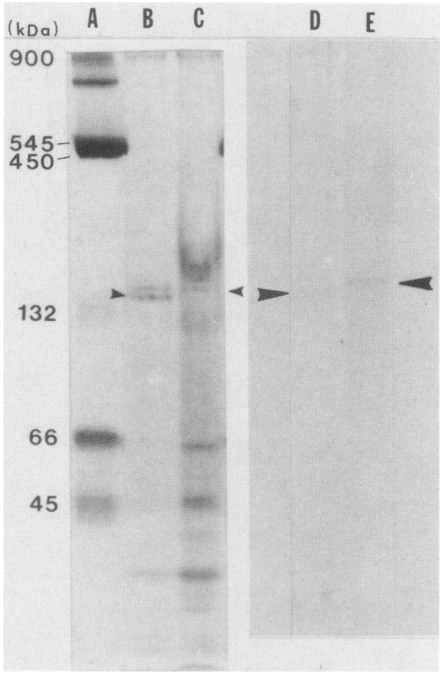

FIG. 2. Nondenaturing, nonreducing PAGE (5 to 10% polyacrylamide) and immunoblot of *L. monocytogenes* and *L. innocua* CCSP. Lanes: A, Coomassie blue-stained molecular mass standards (apoferritin dimer, 900 kDa; monomer, 450 kDa; jack bean urease hexamer, 545 kDa; bovine serum albumin dimer, 132 kDa; monomer, 66 kDa; and chicken egg albumin, 45 kDa); B and D, *L. monocytogenes* CCSP; C and E, *L. innocua* CCSP. Single MAb C11E9-reactive bands of 174 kDa for *L. monocytogenes* (lane B and D) and 182 kDa for *L. innocua* (lane C and E) are shown (see arrows).

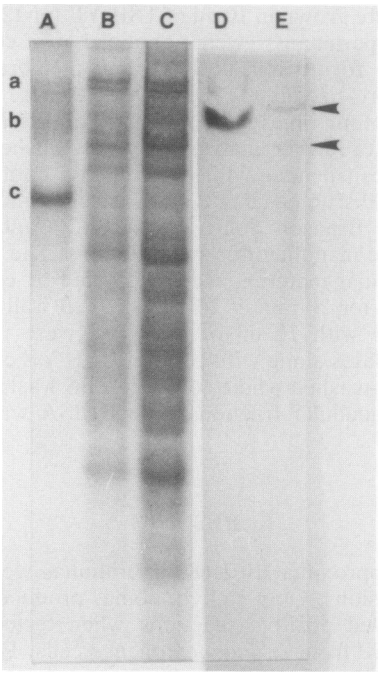

FIG. 3. Acid-urea PAGE (10% polyacrylamide) and immunoblot of CCSP from *L. monocytogenes* and *L. innocua*. Lanes: A, Coomassie blue-stained molecular markers (row a, phosphorylase *b*; row b, bovine serum albumin; row c, carbonic anhydrase); B and D, *L. monocytogenes* CCSP; C and E, *L. innocua* CCSP. Lanes D and E are immunoblots of lanes B and C, respectively. The arrows indicate the reaction of MAb C11E9 to one major and one minor protein band of both *L. monocytogenes* and *L. innocua*.

presence of several protein bands ranging from 10 to 105 kDa (Fig. 5, lanes A and B) upon Coomassie blue staining. After transblot to Immobilon-P membrane, the proteins were immunoprobed with MAb C11E9 (lane D and E). The molecular masses of the MAb-reactive major proteins in CCSP from *L. monocytogenes* cells were calculated to be approximately 76, 66, 56, and 52 kDa with several minor protein bands (lane D). Similarly, in *L. innocua*, the MAb-reactive proteins were 66, 56, and 52 kDa (lane E). Staining of *L. monocytogenes* and *L. innocua* CCSP in SDS-PAGE gel- or blot-transferred proteins with periodic acid-Schiff stain or glycoprotein enzyme immunoassay did not indicate the presence of any carbohydrate with those immunoreactive protein bands while the positive control glycoprotein (glyceraldehyde 3-phosphate dehydrogenase) gave a positive reaction (data not shown).

Removal of surface proteins by chaotropic agents. Dot blot analyses of *L. monocytogenes* V7 and Scott A and *L. innocua* cells after treatment with acid-urea, NaCl, Gu-HCl, or SDS–β-mercaptoethanol showed that the MAb-reactive proteins were removed to various degrees from the cell surface (Fig. 6). Among these, NaCl and Gu-HCl caused partial and complete removal of the protein(s), respectively, and the acid-urea and SDS–β-mercaptoethanol treatments caused intermediate removal, as was demonstrated in dot blotting. Microscopic examination of cells after the treatments did not show any apparent cell lysis. The cells were still motile after treatment with SDS–β-mercaptoethanol or NaCl but were nonmotile after acid-urea or Gu-HCl treatment. Furthermore, treatment with acid-urea but not the three other treatments caused self-agglutination of *L. monocytogenes* V7 and Scott A and *L. welshimeri* cells (data not shown).

Epitope characterization. Treatment of CCSP from *L. monocytogenes* V7 and *L. innocua* cells with various proteolytic enzymes and solvents interfered with the binding of MAb C11E9 to the epitope. The epitope binding activity determined by ELISA was destroyed in the presence of trypsin, chymotrypsin, and pronase E. Similarly, reduced epitope binding activity was noticed for CCSP of *L. monocytogenes* and *L. innocua* in the presence of methanol (0.48 and 0.52%, respectively), acetone (8.12 and 7.86%, respectively), and ethanol (2.03 and 2.35%, respectively), whereas hexane (60.63 and 66.89%, respectively) had very little effect. The control was considered to be 100%.

Localization of MAb-reactive proteins in cell fractions. The MAb C11E9 reaction to different cell fractions was monitored in ELISA at A_{490}. The data in Table 2 indicate that some of the C11E9-reactive proteins are present extracellularly (about 0.003% for *L. monocytogenes* and 0.004% for *L. innocua*). After PBS wash, the antigens which were apparently loosely attached to the cells and extractable with PBS were calculated to be 19% for *L. monocytogenes* and 63% for *L. innocua*. The intracellular fractions or cell lysates of *L. monocytogenes* and *L. innocua* contained about 24 and 10%, respectively, of the reactive protein antigens. Similarly, cell wall fractions contained about 57 and 27% of the reactive protein antigens for *L. monocytogenes* and *L. innocua*, respectively (Table 2). This result indicates that

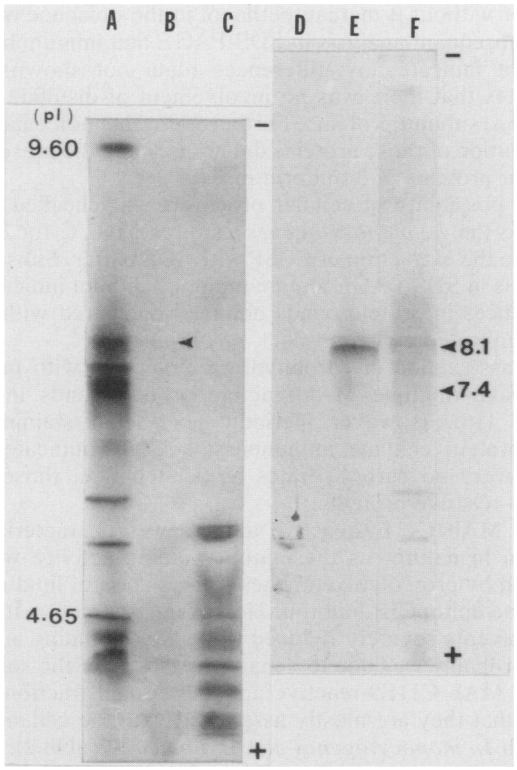

FIG. 4. Isoelectric focusing gel and immunoblotting of CCSP from *L. monocytogenes* and *L. innocua*. Lane A, Coomassie blue-stained isoelectric focusing protein standards. The uppermost protein was cytochrome C (pI, 9.6), and the lowermost was phycocyanin (pI, 4.65). The ampholyte established a pH gradient of 3.9 to 9.65. Lanes B and C, Coomassie blue-stained CCSP from *L. monocytogenes* and *L. innocua* cells, respectively. Lanes D and E, immunoblots of lanes C and B, respectively. Lane F, fast green-stained transblot membrane with isoelectric focusing protein standards. The CCSP from *L. monocytogenes* gave C11E9-reactive bands with pIs of about 8.1 and 7.4 (lane E), whereas *L. innocua* showed a wide protein band with a pI of about 7.4 (lane D).

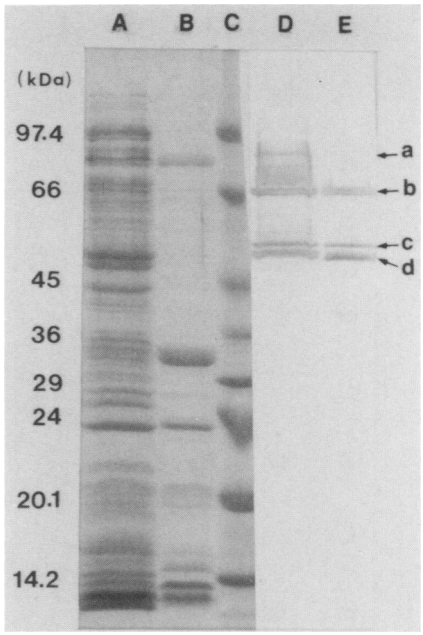

FIG. 5. SDS-PAGE (10 to 15% polyacrylamide) and immunoblot of CCSP from *L. monocytogenes* and *L. innocua*. Lanes A, B, and C, Coomassie blue-stained *L. monocytogenes* V7, *L. innocua*, and molecular mass standards (97.4, 66, 45, 36, 29, 24, 20.1 and 14.2 kDa), respectively. Lanes D and E are immunoprobed (MAb C11E9) blots of *L. monocytogenes* and *L. innocua* CCSP, respectively. The molecular masses for immunoreactive protein bands with their respective arrows were about 76 (a), 66 (b), 56 (c), and 52 (d) kDa for *L. monocytogenes* and 66 (b), 56 (c), and 52 (d) kDa for *L. innocua*.

C11E9-reactive antigens are not similarly distributed between these two bacterial species. *L. innocua* apparently expresses most of its C11E9-specific antigens on the cell surface, part of which are apparently loosely attached to the cell wall. Conversely, *L. monocytogenes* appears to express the majority of its C11E9-specific antigens, which are tightly bound, in the cell wall.

DISCUSSION

MAb specificity. MAb C11E9, described in this study, was shown to react with live or heat-killed *L. monocytogenes* and *L. innocua* cells without any cross-reaction to any other *Listeria* spp. or gram-positive or gram-negative organisms, as shown by dot and colony blot immunoassays.

MAb C11E9 was also shown to react with the CCSP of *L. monocytogenes* and *L. innocua*. Cells of *L. monocytogenes* or *L. innocua* extracted with acid-urea, SDS–β-mercaptoethanol, NaCl, or Gu-HCl to remove cell surface antigens can no longer bind MAb C11E9. These extracted cells nevertheless remained intact and motile in some cases, as was determined by phase-contrast microscopy.

MAb C11E9 is an entirely different antibody than any previously published and has an immunoglobulin isotype of IgG2b. The genus-specific MAbs described by Farber and Speirs (12) and by Butman et al. (6) had isotypes which were either uncharacterized or IgG1 and IgG2a, respectively. The more specific MAbs described by McLauchlin and coworkers (29, 32) had isotypes of IgG1, IgG3, or IgM, while MAb P5C9 of Siragusa and Johnson (36) had isotype IgG1.

MAb C11E9-reactive antigen characterization. When the CCSP samples were analyzed in native PAGE (5 to 10% polyacrylamide) single C11E9-reactive bands were noticed for both *L. monocytogenes* and *L. innocua* cells with molecular masses of 174 and 182 kDa, respectively (Fig. 2). However, when the same CCSP were analyzed in acid-urea PAGE (10% polyacrylamide) (Fig. 3) two protein bands were noticed, indicating that the C11E9-reactive protein in native PAGE is actually composed of two different proteins with variable masses and charges. Similarly, isoelectric focusing of the CCSP showed that the C11E9-reactive protein consisted of two proteins with pIs of 8.1 and 7.4 (Fig. 4). In SDS-PAGE (10 to 15% polyacrylamide) the CCSP gave four major protein bands with molecular masses of 76, 66, 56, and 52 kDa for *L. monocytogenes* and three major bands with molecular masses of 66, 56, and 52 kDa for *L. innocua* cells (Fig. 5). This indicates that the C11E9-reactive native proteins with molecular masses of 174 and 182 kDa were extensively denatured in the presence of SDS and each of the denatured proteins contained some epitopes for MAb C11E9. The SDS treatment that preceded SDS-PAGE probably allowed the protein aggregates to unfold and expose some of the epitopes which were previously not recognized by C11E9 in the native state of the protein. The multiple

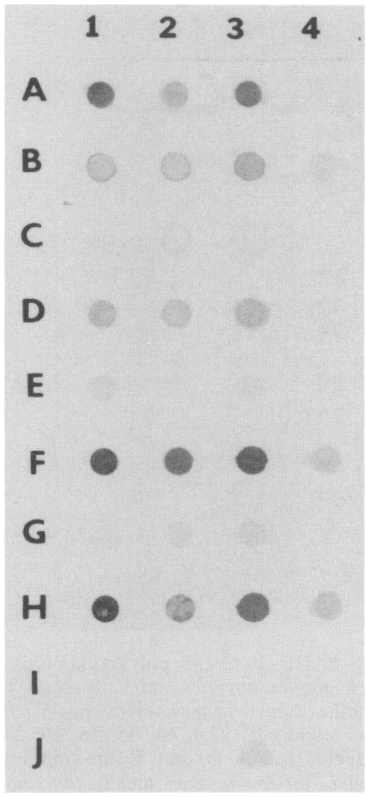

FIG. 6. Dot blot analyses of *Listeria* sp. after treatment with different chaotropic agents and SDS–β-mercaptoethanol (see text for details). The organisms were *L. monocytogenes* V7 (lane 1), Scott A (lane 2), *L. innocua* (lane 3), and *L. welshimeri* (lane 4). Rows: A, PBS-washed cells (control); B and C, acid-urea-extracted cell-free materials and cells, respectively; D and E, SDS–β-mercaptoethanol extracted materials and cells, respectively; F and G, NaCl-extracted materials and cells, respectively; H and I, Gu-HCl-extracted materials and cells, respectively; J, cell-free TSB-YE supernatants.

C11E9-reactive protein bands in SDS-PAGE were also thought to be subunit proteins or breakdown products of cellular proteases. To eliminate the possibility of subunit proteins, we extracted CCSP from *L. monocytogenes* cells with or without β-mercaptoethanol in the presence of SDS, and subsequent analysis in SDS-PAGE and immunoblotting did not indicate any differences (data not shown). This indicates that there was no involvement of disulfide bonds for those subunit proteins. Furthermore, the molecular mass distribution of those proteins did not indicate the presence of subunit proteins of a uniform mass.

The possibility of cellular proteases was checked for by heating the *L. monocytogenes* cultures at 82°C for 20 min prior to the extraction of CCSP with SDS buffer. Subsequent analysis in SDS-PAGE and immunoblot did not indicate any differences in protein bands obtained compared with those from unheated cell controls.

An association of carbohydrate molecules with proteins also gave multiple MAb-reactive protein bands in SDS-PAGE (16). However, periodic acid-Schiff staining and glycoprotein enzyme immunoassay results indicated that there were no carbohydrates associated with those MAb C11E9-reactive proteins.

The MAb C11E9-reactive antigen was characterized as protein in nature, as the epitope-binding activity was destroyed by proteolytic enzymes. Association of lipid moiety with the epitope is doubtful. However, treatment with various solvents severely reduced the epitope binding activity. This probably was due to denaturing effects of the solvents.

The MAb C11E9-reactive antigens in cell fractions indicated that they are mostly associated with the cell surfaces of both *L. monocytogenes* and *L. innocua* (Table 2).

Collectively, our data suggested that MAb C11E9 reacted with a protein complex or an aggregate (native state) consisting of two or more proteins with different molecular weights and charges and an entirely different group of proteins from those previously published and reviewed below.

Since MAb C11E9 reacts to several protein bands (in SDS-PAGE), one can speculate that the organisms might produce some related but different extracellular proteins which possess a common epitope. One such candidate is listeriolysin, which we prepared by the method of Barclay et al. (1, 2), ran in SDS-PAGE, transferred, and immunoblotted. Our immunoblot results (data not shown) indicated that MAb C11E9 did not react with a band where the listeriolysin (58 kDa) was located. Immunoblots of our MAb also showed there was little or no reaction with proteins of 60-kDa molecular mass reported as "invasin proteins" by Kuhn and

TABLE 2. Distribution of MAb C11E9-reactive proteins in cell fraction

Fraction[a]	Mean (± SEM)[b] ELISA A_{490} with MAb (C11E9)		% of total[c]	
	L. monocytogenes	*L. innocua*	*L. monocytogenes*	*L. innocua*
Cell-free culture supernatant	0.004 ± 0	0.009 ± 0	0.003	0.004
PBS washings[d]				
1st	0.096 ± 0.004	0.521 ± 0.028		
2nd	0.059 ± 0.002	0.444 ± 0.010	19	63
3rd	0.033 ± 0.002	0.431 ± 0.010		
4th	0	0.262 ± 0.008		
Intracellular (cytoplasm)	0.247 ± 0.004	0.255 ± 0.008	24	10
Cell wall[e]	0.583 ± 0.025	0.724 ± 0.009	57	27
Whole cells[f]	0.483 ± 0.025	0.768 ± 0.011		

[a] About 3×10^8 cells/ml were used.
[b] Mean of three experiments, each in triplicate.
[c] Calculated by dividing individual A_{490} values by the sum of A_{490} values of all the fractions.
[d] Cell pellets were suspended in PBS and washed four times to remove loosely attached antigens.
[e] Cell wall fraction obtained after mechanical disintegration; also contains cell membrane.
[f] After PBS washes, the whole cells were also used for ELISA.

Goebel (22). Carlier et al. (7) and Delvallez et al. (10) reported their MAb reacted with a surface antigen (antigen 2), shared by several *Listeria* spp., which had a molecular mass of about 160 kDa. The MAb P5C9 reported by Siragusa and Johnson (36) reacted with cell surface proteins of 18.5 kDa from *L. monocytogenes*, *L. innocua*, and *L. welshimeri*. Finally, Butman et al. (6) reported their MAbs reacted with genus-specific *Listeria* antigens of 30 to 38 kDa.

Considering the unique properties of MAb C11E9 and its specific reaction to *L. monocytogenes* and *L. innocua*, this antibody should be useful for rapid detection of these organisms in food or clinical samples either by dot blotting (36) or microcolony immunoblotting (3a).

ACKNOWLEDGMENTS

We thank John A. Thoma, Lawrence Raymond, Bruce R. Hamaker, and Dana K. Becker for helpful discussions; Alice Jernigan, Lesley Boaz, Sabrina Pudlas, Wee Goh, Sheila Sevenstar, and Alice Obenshein for excellent technical assistance; and Mary Neighbors for preparing the manuscript.

This work was supported by grants from the USDA Special Consortium Grant for Food Safety and the Southeastern Poultry and Egg Association.

REFERENCES

1. **Barclay, R., D. R. Threlfall, and I. Leighton.** 1989. Separation and properties of the haemolysins and extracellular enzymes of *Listeria monocytogenes* and *L. ivanovii*. J. Med. Microbiol. **30**:119–127.
2. **Barclay, R., D. R. Threlfall, and I. Leighton.** 1989. Haemolysins and extracellular enzymes of *Listeria monocytogenes* and *L. ivanovii*. J. Med. Microbiol. **30**:111–118.
3. **Bessesen, J. T., Q. Luo, H. A. Rotbart, M. J. Blaser, and R. T. Ellison III.** 1990. Detection of *Listeria monocytogenes* by using the polymerase chain reaction. Appl. Environ. Microbiol. **56**: 2930–2932.

3a. **Bhunia, A. K., P. H. Ball, G. R. Siragusa, and M. G. Johnson.** 1990. Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, P-44, p. 285.

4. **Bhunia, A. K., M. C. Johnson, and B. Ray.** 1987. Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Ind. Microbiol. **2**:319–322.
5. **Bhunia, A. K., M. C. Johnson, B. Ray, and E. L. Belden.** 1990. Antigenic property of pediocin AcH produced by *Pediococcus acidilactici* H. J. Appl. Bacteriol. **69**:211–215.
6. **Butman, B. T., M. C. Plank, R. J. Durham, and J. A. Mattingly.** 1988. Monoclonal antibodies which identify a genus-specific *Listeria* antigen. Appl. Environ. Microbiol. **54**:1564–1569.
7. **Carlier, Y., D. Bout, A. Capron, M. Delvallez, G. Martin, G. Strecker, and T. Duriez.** 1980. Physicochemical characteristics of *Listeria* specific antigen 2. J. Gen. Microbiol. **116**:549–552.
8. **Cassiday, P. K., and R. E. Brackett.** 1989. Methods and media to isolate and enumerate *Listeria monocytogenes*. A review. J. Food Prot. **52**:207–214.
9. **Datta, A. R., B. A. Wentz, D. Shook, and M. W. Trucksess.** 1988. Synthetic oligodeoxyribonucleotide probes for detection of *Listeria monocytogenes*. Appl. Environ. Microbiol. **54**:2933–2937.
10. **Delvallez, M., Y. Carlier, D. Bout, A. Capron, and G. R. Martin.** 1979. Purification of a surface-specific soluble antigen from *Listeria monocytogenes*. Infect. Immun. **25**:971–977.
11. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electro-phoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
12. **Farber, J. M., and J. I. Speirs.** 1987. Monoclonal antibodies directed against the flagellar antigens of *Listeria* species and their potential in EIA-based methods. J. Food Prot. **50**:479–484.
13. **Fraser, J. A., and W. H. Sperber.** 1988. Rapid detection of *Listeria spp* in food and environmental samples by esculin hydrolysis. J. Food Prot. **51**:762–765.
14. **Hudson, L., and F. C. Hay.** 1976. Alum precipitation of antigens, p. 10. *In* L. Hudson and F. C. Hay (ed.), Practical immunology. Blackwell Scientific Publications Ltd., Oxford.
15. **Ison, C. A., B. Kolator, J. H. Reid, E. Dermott, J. Clark, and C. S. F. Easmon.** 1989. Characterization of monoclonal antibodies for detection of *Mobiluncus spp.* in genital specimens. J. Med. Microbiol. **30**:129–136.
16. **Jarvis, B. W., and M. Dworkin.** 1989. Purification and properties of *Myxococcus xanthus* cell surface antigen 1604. J. Bacteriol. **171**:4655–4666.
17. **Johnstone, A. P., and R. Thorpe.** 1987. Immunochemistry in practice. 2nd ed., p. 30–109. Blackwell Scientific Publications Ltd., Oxford.
18. **Kamisango, K., M. Nagaoka, H. Fuji, and I. Azuma.** 1985. Enzyme immunoassay of teichoic acids from *Listeria monocytogenes*. J. Clin. Microbiol. **21**:135–137.
19. **King, W., S. Raposa, J. Warshaw, A. Johnson, D. Halbert, and J. D. Klinger.** 1989. A new colorimetric nucleic acid hybridization for *Listeria* in foods. Int. J. Food Microbiol. **8**:225–232.
20. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
21. **Kohler, S., M. Leimeister-Wachter, T. Chakraborty, F. Lottspeich, and W. Goebel.** 1990. The gene coding for protein p60 of *Listeria monocytogenes* and its use as a specific probe for *Listeria monocytogenes*. Infect. Immun. **58**:1943–1950.
22. **Kuhn, M., and W. Goebel.** 1989. Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. Immun. **57**:55–61.
23. **Lee, W. H., and D. McClain.** 1986. Improved *Listeria monocytogenes* selective agar. Appl. Environ. Microbiol. **52**:1215–1217.
24. **Loessner, M. J., R. H. Bell, J. M. Jay, and L. A. Shelef.** 1988. Comparison of seven plating media for enumeration of *Listeria* spp. Appl. Environ. Microbiol. **54**:3003–3007.
25. **Lovett, J.** 1987. Listeria isolation, p. 29.01–29.12. *In* FDA bacteriological analytical manual, supplement to 6th ed. Association of Official Analytical Chemists, Arlington, Va.
26. **Lovett, J.** 1988. Isolation and identification of *Listeria monocytogenes* in dairy products. J. Assoc. Off. Anal. Chem. **71**:658–660.
27. **Mattingly, J. A., B. T. Butman, M. C. Plank, R. J. Durham, and B. J. Robinson.** 1988. Rapid monoclonal antibody-based enzyme-linked immunosorbent assay for detection of *Listeria* in food products. J. Assoc. Off. Anal. Chem. **71**:679–681.
28. **McClain, D., and W. H. Lee.** 1988. Development of USDA-FSIS method for isolation of *Listeria monocytogenes* from raw meat and poultry. J. Assoc. Off. Anal. Chem. **71**:660–664.
29. **McLauchlin, J., A. Black, H. T. Green, J. Q. Nash, and A. G. Taylor.** 1988. Monoclonal antibodies show *Listeria monocytogenes* in necropsy tissue samples. J. Clin. Pathol. **41**:983–988.
30. **McLauchlin, J., and P. N. Pini.** 1989. The rapid demonstration and presumptive identification of *Listeria monocytogenes* in food using monoclonal antibodies in a direct immunofluorescence test (DIFT). Lett. Appl. Microbiol. **8**:25–27.
31. **McLauchlin, J., and D. Samuel.** 1989. The diagnosis of *Listeria monocytogenes* Serogroup 4 infections in humans by the detection of soluble antigen in cerebrospinal fluid by ELISA. Serodiagn. Immunother. Infect. Dis. **3**:17–26.
32. **McLauchlin, J., and A. G. Taylor.** 1989. The use of monoclonal antibodies in the characterization and purification of the cell surface antigens of *Listeria monocytogenes* serogroup 4. Acta Microbiol. Hung. **36**:457–463.
33. **Peel, M. N., W. Donachie, and A. Shaw.** 1988. Physical and antigenic heterogeneity in flagellins of *Listeria monocytogenes* and *L. ivanovii*. J. Gen. Microbiol. **134**:2593–2598.
34. **Peel, M. N., W. Donachie, and A. Shaw.** 1988. Temperature-dependent expression of flagella of *Listeria monocytogenes* studies by electron microscopy, SDS-PAGE and western blotting. J. Gen. Microbiol. **134**:2171–2178.
35. **Rodriguez, L. D., G. S. Fernandez, J. F. F. Garayzabal, and E. R. Ferri.** 1986. New methodology for the isolation of *Listeria* microorganisms from heavily contaminated environments.

Appl. Environ. Microbiol. **47**:1188–1190.
36. **Siragusa, G. R., and M. G. Johnson.** 1990. Monoclonal antibody specific for *Listeria monocytogenes*, *Listeria innocua*, and *Listeria welshimeri*. Appl. Environ. Microbiol. **56**:1897–1904.
37. **Smith, B. J.** 1984. Acetic acid-Urea polyacrylamide gel electrophoresis of proteins, p. 63–73. *In* J. M. Walker (ed.), Methods in molecular biology, vol. 1. Proteins. Humana Press, Clifton, N.J.
38. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
39. **Walker, J. M.** 1984. Gradient SDS polyacrylamide gel electrophoresis, p. 57–61. *In* J. M. Walker (ed.), Methods in molecular biology, vol. 1. Proteins. Humana Press, Clifton, N.J.
40. **Ziegler, H. K., and C. A. Orlin.** 1984. Analysis of *Listeria monocytogenes* antigens with monoclonal antibodies. Clin. Invest. Med. **7**:239–242.