APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1989, p. 2251–2256
0099-2240/89/092251-06$02.00/0

Vol. 55, No. 9

Cloning of a Gene Encoding a Major Secreted Polypeptide of *Listeria monocytogenes* and Its Potential Use as a Species-Specific Probe

ROBERT K. FLAMM,[1]† DAVID J. HINRICHS,[2] AND MICHAEL F. THOMASHOW[3]*

Department of Microbiology, Washington State University, Pullman, Washington 99164[1]; Research Service 151-S, Veterans Administration Medical Center, Portland, Oregon 97207[2]; and Department of Crop and Soil Science and Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824-1325[3]

Received 27 February 1989/Accepted 21 June 1989

A gene, designated *msp*, that encodes a major secreted polypeptide with a molecular mass of approximately 60 kilodaltons (kDa) was cloned from *Listeria monocytogenes* 10403. DNA hybridization analysis indicated that the *msp* gene was highly conserved among 15 independent *L. monocytogenes* isolates and that each of 5 isolates tested secreted a 60-kDa polypeptide that was immunologically related to the *msp* gene product. DNA sequences related to *msp* were not detected in any other *Listeria* species or in strains of *Bacillus cereus*, *Bacillus thuringiensis*, *Streptococcus pyogenes*, or *Streptococcus pneumoniae* when standard stringent DNA hybridization conditions were used. Under nonstringent conditions, related sequences were detected in *Listeria ivanovii*, *Listeria seeligeri*, and *Listeria innocua*, and immunoblot analysis indicated that these strains secreted polypeptides of about 60 kDa that were immunologically related to the *msp* gene product. The possibility of using the *msp* gene as a probe for the detection of *L. monocytogenes* and the potential functions of the *msp* gene product are discussed.

Listeria monocytogenes-contaminated food and dairy products have been the sources of disease outbreaks leading to severe illness and death (4, 7). A rapid and sensitive method to screen for the presence of *L. monocytogenes* would aid in the prevention of such outbreaks. Currently, the primary method of identifying food products contaminated with *L. monocytogenes* is in vitro culture. However, there are a number of problems associated with this method. It is both time consuming and cumbersome. In addition, although *L. monocytogenes* may be present, it may not grow or may grow only with the use of cold enrichment (1). Finally, culture identification is complicated by the fact that there are a number of different *Listeria* species. A DNA probe specific for *L. monocytogenes* could potentially overcome these problems. Such a probe, in conjunction with polymerase chain reaction technology (26), could provide a very sensitive assay for the detection of low numbers of bacteria and even nongrowing organisms.

Secreted polypeptides, such as hemolysins, toxins, and siderophores, often play a role in survival of an organism and/or its ability to cause disease. In addition, secreted polypeptides often play a significant role in the elicitation of the host immune response. Thus, genes encoding major extracellular products could have important roles in the interaction of *L. monocytogenes* with host cells and could potentially provide a useful marker for identification of *L. monocytogenes*.

Here, we report the cloning of a gene, *msp*, that encodes a major secreted polypeptide of *L. monocytogenes*. Hybridization experiments indicated that *msp* is highly conserved among *L. monocytogenes* isolates and that under stringent hybridization conditions, homology is observed only with this species of *Listeria*.

* Corresponding author.
† Present address: Squibb Institute for Medical Research, Princeton, NJ 08540.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media. *Escherichia coli* strains were grown in LB (18) broth, on LB agar, or on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 5% (vol/vol) sheep blood (SBA). All *Listeria* species were grown in brain heart infusion (BHI) (Difco) broth or on SBA plates.

DNA isolation and transformation. Procedures for isolating total DNA (6), lambda bacteriophage DNA (8), and plasmid DNA (2) were as previously described. *E. coli* was transformed by the $CaCl_2$ heat shock method (14).

Restriction enzyme analysis. Samples of DNA were digested with restriction endonucleases as recommended by the supplier (New England BioLabs, Inc., Beverly, Mass.), and the DNA fragments were fractionated by agarose gel electrophoresis as previously described (6).

Southern blot analysis. DNA digests were fractionated by agarose gel electrophoresis, and the DNA fragments were transferred to nitrocellulose filters by the method of Southern (31). The filters were hybridized with DNA probes that had been labeled in vitro by the nick translation reaction (15). Hybridization conditions were as described previously (32). Blots were washed under stringent conditions with a buffer consisting of 0.3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 68°C or under nonstringent conditions with a buffer composed of 3× SSC, 0.2% SDS, and 5 mM EDTA at 55°C.

Molecular cloning procedures. An *L. monocytogenes* gene bank was constructed by using the lambda vector EMBL3A (8). Total DNA isolated from *L. monocytogenes* 10403 was partially digested with *Sau*3A, the restriction fragments were separated by agarose gel electrophoresis, and the fragments 15 to 23 kilobases (kb) in size were collected and ligated to *Bam*HI-digested EMBL3A DNA by using T4 DNA ligase. The DNA mixture was then packaged in vitro into phage (11) and amplified in *E. coli* NM535. Subcloning of the *L.*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype[a]	Source or reference
E. coli		
LE392	*supE44 supF58*	14
JM105	Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} ZΔM15)	35
SM10	Integrated RP4-2 (Tc::Mu)	29
NM535	P2cox3	8
L. monocytogenes		
10403	Hemolytic	6
ATCC 19111	Hemolytic	22
EGD	Weakly hemolytic	19
Scott A	Hemolytic	D. Shah
DA3	Hemolytic	D. Shah
SH-12	Hemolytic	6
Li89	Hemolytic	6
3A	Hemolytic	6
611536	Hemolytic	6
286-2	Hemolytic	6
209	Hemolytic	A. Ward
35152	Hemolytic	L. Meyer
1b	Hemolytic	WSU[b]
307	Hemolytic	A. Ward
78-Li89	Hemolytic	6
81-Li63	Hemolytic	6
L. denitrificans ATCC 14870	Nonhemolytic	30
L. murrayi ATCC 25401	Nonhemolytic	34
L. grayi ATCC 19120	Nonhemolytic	30
L. innocua ATCC 33090	Nonhemolytic	27
L. ivanovii ATCC 19119	Hemolytic	28
L. seeligeri ATCC 35967	Weakly hemolytic	25
Plasmids		
pBR325	Amp^r Cam^r Tet^r	24
pUC8	Amp^r	33
PLc2833	Amp^r	29
pRF102	Amp^r	This study
pRF106	Amp^r	This study
pRT291	Kan^r Tet^r	15
pPH1JI	Gen^r	10

[a] Abbreviations: Amp, ampicillin; Cam, chloramphenicol; Gen, gentamicin; Kan, kanamycin; Tet, tetracycline.
[b] Department of Microbiology, Washington State University.

monocytogenes DNA into plasmid vectors was accomplished by standard methods (15).

Preparations of *L. monocytogenes* extracellular polypeptides. Concentrated BHI broth (4×) was dialyzed overnight at 4°C to remove large proteins (the average pore size of the dialysis tubing was 24 nm). The broth outside the dialysis bag was brought to a 1× concentration and sterilized. *L. monocytogenes* was grown overnight in 2 liters of the dialyzed BHI broth, and the bacteria were removed by centrifugation (10,400 × *g* for 10 min). The culture supernatant was adjusted to 10 mM EDTA–0.02% sodium azide. Crystalline ammonium sulfate was added slowly to a final concentration of 70% (wt/vol), and proteins were allowed to precipitate overnight at 4°C. The precipitate was collected by centrifugation (10,400 × *g* for 10 min), suspended in distilled water, and dialyzed against 100 mM Tris hydrochloride (pH 7.2).

Protein fractionation and immunoblotting. Samples were suspended in lysis buffer (125 mM Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 0.7% 2-mercaptoethanol, 0.003% bromphenol blue), incubated at 100°C for 10 min, and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (13). Polypeptides were visualized by staining with Coomassie blue. In the immunoblotting experiments, the separated proteins were transferred to nitrocellulose by electrophoresis at 500 mA for 3 h (3) and treated with antiserum raised to a gel-purified sample of the 60-kilodalton (kDa) polypeptide. Antibody binding was visualized by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and then with hydrogen peroxide and 4-chloro-1-naphthol (9).

Preparation of antisera. Antisera were raised against a preparation of the 60-kDa extracellular polypeptide and against a gel-purified sample of the 60-kDa extracellular polypeptide (a preparation was fractionated by SDS-PAGE and briefly stained with Coomassie blue, and the desired band was excised and minced into small pieces). The protein preparations for the initial injections were emulsified in Freund complete adjuvant, while subsequent booster injections were prepared in incomplete Freund adjuvant. Rabbits were injected subcutaneously at multiple sites with approximately 500 mg of the total preparation or 200 mg of gel-purified protein at 2-week intervals and bled 1 week after the third injection. Antibody was precipitated twice in 40% (vol/vol) saturated ammonium sulfate (pH 7.5) and dialyzed against normal saline.

Generation of Tn*phoA* inserts. Plasmid pRT291 is a derivative of pRK290 carrying an insert of Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*) (16); it encodes both kanamycin resistance (from Tn*phoA*) and tetracycline resistance (from pRK290). The plasmid was used to generate Tn*phoA* inserts in pRF102 as follows. Plasmid pRT291 was conjugated into *E. coli* LE392 containing pRF102 by selecting for transconjugants that were ampicillin, kanamycin, and tetracycline resistant. Plasmid pRT291 was "bumped" from the transconjugants by introducing pPH1JI, an incompatible plasmid (10). Plasmid DNA was isolated from the transconjugants and used to

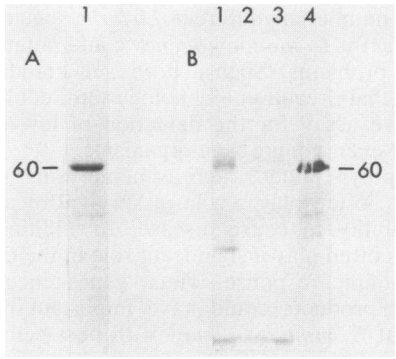

FIG. 1. Detection of the 60-kDa polypeptide in culture supernatants of *L. monocytogenes* and in *E. coli* harboring the *msp* gene. (A). A preparation of *L. monocytogenes* extracellular polypeptides (see the text) was fractionated on a 10% SDS-PAGE gel and stained with Coomassie blue to detect polypeptides. (B). *E. coli* harboring pRF102 (lane 1), pBR325 (lane 2), or no plasmid (lane 3) was grown overnight at 37°C on BHI plates (the medium was supplemented with ampicillin at 100 μg/ml for the plasmid-containing strains). Cells were harvested, and the proteins were fractionated on 10% SDS-PAGE gels and analyzed by immunoblotting, using the antiserum raised to the extracellular polypeptide preparation. Lane 4 contains a sample of the preparation from an *L. monocytogenes* culture supernatant. Molecular mass is indicated in kilodaltons.

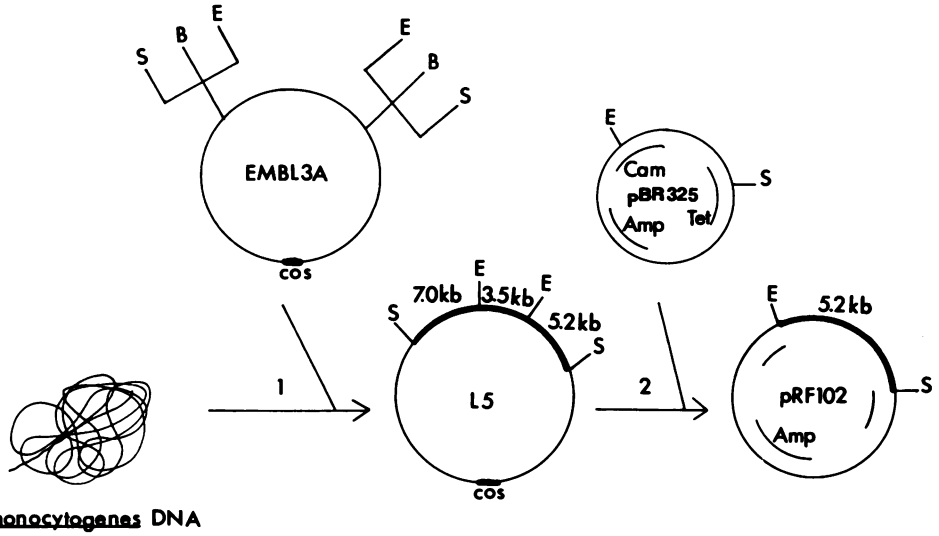

FIG. 2. Cloning of the *L. monocytogenes* gene encoding the 60-kDa polypeptide. (1) Total DNA isolated from *L. monocytogenes* was partially digested with *Sau*3A and fractionated by agarose gel electrophoresis, and the 15- to 23-kb fragments were collected. These DNA fragments were ligated to *Bam*HI-*Eco*RI-digested EMBL3A DNA and packaged in vitro into phage. A recombinant phage, L5, containing a 15.7-kb fragment of *L. monocytogenes* DNA, was identified by the plaque-lift technique as encoding the 60-kDA protein. (2) pRF102 was constructed by inserting the 5.2-kb *Sal*I-*Eco*RI fragment of the *L. monocytogenes* DNA insert into *Sal*I-*Eco*RI-digested pBR325. pRF102 was shown to encode the listerial 60-kDa extracellular protein by immunoblotting (see the text and Fig. 1B). Abbreviations: E, *Eco*RI; S, *Sal*I; B, *Bam*HI.

transform plasmidless *E. coli* LE392, selecting for both ampicillin and kanamycin resistance. These transformants presumably contained pRF102 with Tn*phoA* inserts. This was confirmed by DNA restriction endonuclease analysis. The transformants were also screened for the production of blue colonies on LB plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). This molecule can be cleaved by alkaline phosphatase to form a blue dye. Alkaline phosphatase, however, will only function outside the cytoplasmic membrane. Thus, only those *E. coli* colonies that can transport or secrete alkaline phosphatase turn blue on XP-containing media.

RESULTS

Molecular cloning of *msp*. Growth of *L. monocytogenes* 10403 in dialyzed BHI broth resulted in the production of a major secreted polypeptide(s) with a molecular mass of approximately 60 kDa (Fig. 1A). To clone the gene, *msp* (major secreted polypeptide), encoding this polypeptide, we first constructed a gene bank of *L. monocytogenes* 10403 in *E. coli* by using the lambda vector EMBL3A (Fig. 2). Recombinant phages encoding *msp* were then identified by the plaque-lift technique, using an antiserum raised to a gel-purified sample of the 60-kDa polypeptide. A number of plaques were found to react with the antiserum, and the phage from one of the plaques, L5, was chosen for further study.

Restriction enzyme analysis indicated that the size of the *Listeria* DNA insert in L5 was approximately 15.7 kb (Fig. 2). The general map position of *msp* was determined by subcloning fragments of the *Listeria* DNA into pBR325, transforming the plasmids into *E. coli* LE392, and then assaying the strains for the production of the 60-kDa polypeptide by the immunoblotting technique. The data indicated that *msp* was encoded by the 5.2-kb *Eco*RI-*Sal*I fragment contained in plasmid pRF102 (Fig. 1B and 2).

Location and direction of transcription of *msp*. To determine more accurately the location of *msp* and to determine its direction of transcription, we used the Tn*5* derivative Tn*phoA* (16). This transposon contains most of the *E. coli* alkaline phosphatase gene inserted into IS*50*$_L$ of Tn*5* but lacks the *phoA* promoter and signal peptide sequences (Fig. 3A). When the transposon inserts itself into a gene in the proper orientation and reading frame, a hybrid protein consisting of the target-gene product fused to alkaline phosphatase is created. If the target gene encodes a transported or secreted protein, the fusion protein may also be transported or secreted. Bacteria producing such a gene fusion product can be identified on a medium containing the chromogenic substrate XP: the colonies are blue on this medium (see Materials and Methods).

Since the 60-kDa polypeptide was a secreted protein in *L. monocytogenes*, it was possible that fusions of the gene to Tn*phoA* would result in proteins that were transported or secreted in *E. coli*. This was the case. Of approximately 800 Tn*phoA* inserts into pRF102, 8 resulted in *E. coli* that produced blue colonies. Restriction enzyme analyses indicated that each of these eight inserts was in the 5.2-kb *Eco*RI-*Sal*I fragment of *Listeria* DNA and that they were all in the same orientation; the 5′-to-3′ orientation of the *phoA* gene was from the *Eco*RI site to the *Sal*I site. Thus, the direction of transcription of the 60-kDa polypeptide was from the *Eco*RI site toward the *Sal*I site (Fig. 3B).

The molecular masses of the fusion proteins created by the Tn*phoA* inserts were determined by immunoblotting (Fig. 3C). Inserts 1, 154, 151, and 172 resulted in the synthesis of polypeptides of approximately 65, 80, 90, and 100 kDa, respectively. Based on the positions of the inserts and the fact that the *phoA* sequence encodes a polypeptide of about 40 kDa, the map position of the DNA sequences encoding the 5′ end of the 60-kDa polypeptide was estimated (Fig. 3B).

Conservation of *msp* among *L. monocytogenes* isolates. A probe specific for *msp* was constructed by cloning the 500-base-pair *Hin*dIII-*Hin*cII fragment from the middle of the gene (Fig. 3B) into *Hin*dIII-*Hin*cII-digested pUC8. The

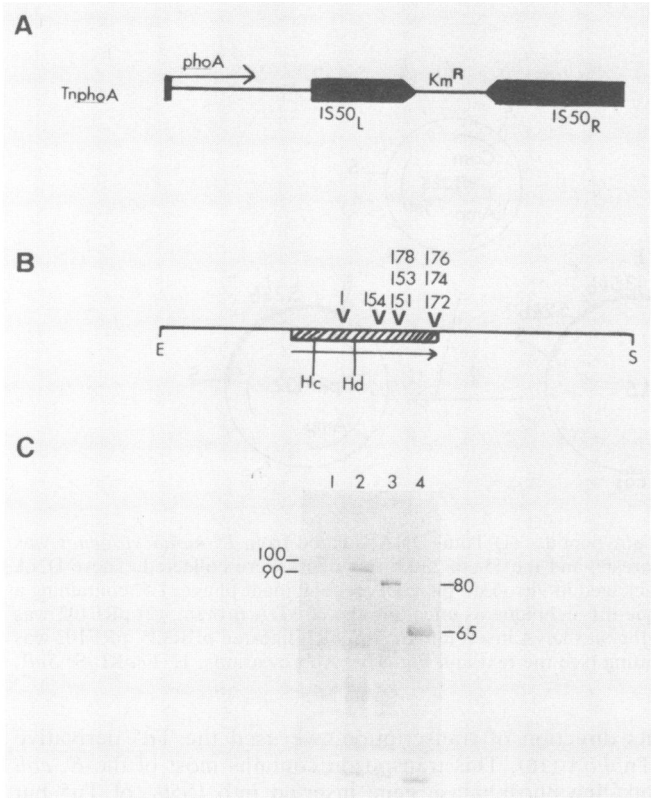

FIG. 3. Mapping of the location and orientation of the *msp* gene. (A) Map of Tn*phoA*. (B) Map positions of eight Tn*phoA* inserts (V) in the 5.2-kb *Eco*RI-*Sal*I fragment of *L. monocytogenes*. The orientation of Tn*phoA* was the same as shown in panel A; the $IS50_L$ sequence was closer to the *Eco*RI site than the $IS50_R$ sequence. All of these inserts resulted in *E. coli* colonies that were blue on XP plates. The coding region for the 60-kDa polypeptide is indicated by the hatched box. The direction of transcription is indicated by the arrow. E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; S, *Sal*I. (C) Detection of fusion proteins by immunoblot analysis. *E. coli* containing Tn*phoA* insert 1 (lane 4), insert 154 (lane 3), insert 151 (lane 2), or insert 172 (lane 1) was grown on LB plates (supplemented with ampicillin at 100 μg/ml, kanamycin at 25 μg/ml, and XP at 20 μg/ml). The cells were harvested, and the proteins were analyzed by immunoblotting, using an antiserum raised against the *L. monocytogenes* extracellular protein preparation. Molecular masses are indicated in kilodaltons.

resulting plasmid, designated pRF106, was then used to determine whether *msp* was conserved among *L. monocytogenes* isolates. Total DNAs from 15 independent strains were isolated and digested with *Eco*RI, and Southern blot transfers were prepared. The filters were then hybridized with ^{32}P-labeled pRF106 under standard stringent conditions. The results were that all of the strains had an *Eco*RI fragment of about 13 kb that hybridized with the probe (Fig. 4, lanes 7 to 11, shows the data for five of the *L. monocytogenes* strains). No hybridization was detected when ^{32}P-labeled pUC8 was used as the probe (data not shown). These data indicated that the *msp* gene was highly conserved among our *L. monocytogenes* isolates. In addition, immunoblot analysis carried out with five of the *L. monocytogenes* isolates (Fig. 5, lanes 7 to 11) indicated that they all produced extracellular polypeptides of about 60 kDa that cross-reacted with the antiserum raised to the gel-purified 60-kDa polypeptide from *L. monocytogenes* 10403.

Screen of additional *Listeria* species and species of other bacterial genera for sequences homologous to *msp*. Six additional *Listeria* species were screened for sequences homologous to *msp*: *L. murrayi*, *L. denitrificans*, *L. grayi*, *L. innocua*, *L. seeligeri*, and *L. ivanovii*. In each case, when standard stringent hybridization conditions were used, no homology was detected (Fig. 4A). With *L. innocua*, *L. seeligeri*, and *L. ivanovii*, however, DNA sequences related to *msp* were observed when nonstringent hybridization conditions were used; with each species, an *Eco*RI fragment of about 7 kb was detected with the *msp* probe (Fig. 4B; the faint band visible with the *L. denitrificans* DNA in lane 2 was due to hybridization with the cloning vehicle). Immunoblot data were consistent with these results. With *L. murrayi*, *L. denitrificans*, and *L. grayi*, we did not detect any secreted proteins that reacted with the antiserum raised to the 60-kDa polypeptide, but with *L. innocua*, *L. seeligeri*, and *L. ivanovii*, cross-reactive polypeptides were observed (Fig. 5).

In addition to the *Listeria* species, four other species of hemolytic gram-positive bacteria were tested for DNA homology with pRF106: two strains of *Bacillus cereus*, two strains of *Bacillus thuringiensis*, one strain of *Streptococcus pyogenes*, and one strain of *Streptococcus pneumoniae*. In no instance was hybridization detected, even under nonstringent conditions (data not shown).

DISCUSSION

Recent outbreaks of listeriosis (4, 7) have stimulated an interest among public health agencies and clinical laboratories in developing a rapid, efficient test for screening human samples and foods for *L. monocytogenes*. One of the possibilities is to design a DNA hybridization test based on the use of a DNA probe specific for *L. monocytogenes*. The data presented here suggest that the cloned *L. monocytogenes* *msp* gene might provide such a probe. Under stringent hybridization conditions, the *msp* gene hybridized with the DNA from all of our clinical *L. monocytogenes* isolates but did not hybridize with DNA from other *Listeria* species or with DNA isolated from *B. cereus*, *B. thuringiensis*, *S. pyogenes*, or *S. pneumoniae*. Datta et al. (5) have shown further that *Rhodococcus equi* and the hemolytic species *Staphylococcus aureus*, *Streptococcus agalactiae*, *Vibrio cholerae*, and *Vibrio parahaemolyticus* do not have DNA sequences homologous with *msp*. It should be noted that the strains of *L. monocytogenes* that we tested were not from one outbreak of listeriosis but were isolated from infected animals and humans over an extended time period in varied geographic locations: e.g., strain EGD was isolated in 1926 from an infected rabbit, strain 78-Li89 was isolated from a patient in New York in 1978, and strain DA3 was isolated from a Dallas patient in 1985. Taken together, these data suggest that the *msp* gene may provide a useful general DNA probe specific for *L. monocytogenes* isolates.

At present, we are uncertain of the function of the *msp* gene product. Its presence in all clinical isolates of *L. monocytogenes* suggests that it may have a role in pathogenicity. Indeed, our data indicate that the *msp* gene product has hemolytic activity. We have constructed plasmids that will direct the synthesis of high levels of the *msp* polypeptide in *E. coli* and have found that such *E. coli* strains are hemolytic on blood agar plates while the parent strains are not (R. Flamm, unpublished results). In addition, Datta et al. (5) have examined 52 *Listeria* isolates and found that only those that tested cyclic AMP positive had DNA sequences homologous with the *msp* probe. However, Portnoy et al.

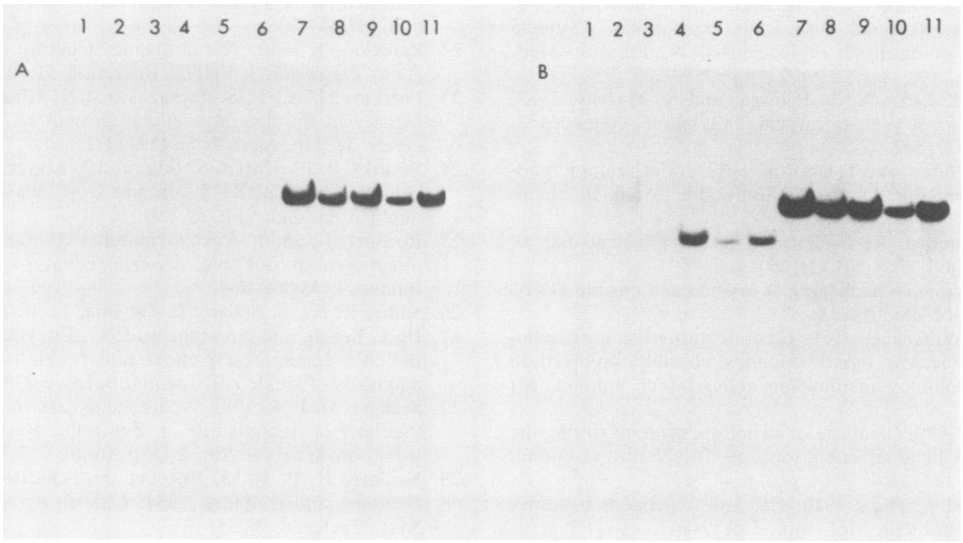

FIG. 4. Conservation of the *msp* gene among *Listeria* species. Total DNAs were isolated from *Listeria* spp. and digested with *Eco*RI. Southern blots were prepared and hybridized with ^{32}P-labeled pRF106. The blots were washed under stringent (A) or nonstringent (B) conditions as described in the text. Total DNAs in lanes 1 to 11 are *L. murrayi* ATCC 25401, *L. denitrificans* ATCC 14870, *L. grayi* ATCC 19120, *L. innocua* ATCC 33090, *L. seeligeri* ATCC 35967, *L. ivanovii* ATCC 19119, *L. monocytogenes* 10403, *L. monocytogenes* DA3, *L. monocytogenes* Scott A, *L. monocytogenes* EGD, and *L. monocytogenes* ATCC 19111, respectively.

(23), using transposon Tn*916*, have isolated a number of mutants of *L. monocytogenes* that are nonhemolytic and have found that all of the mutants are missing a minor secreted protein of about 58 kDa, the molecular mass of listeriolysin O (17). None of the mutants that they obtained had transposon inserts in the *msp* gene (D. Portnoy, unpublished results). Thus, it does not appear that the *msp* gene product is listeriolysin O (also referred to as α-listeriolysin [21]). Whether the *msp* gene encodes β-listeriolysin, a hemolysin that is immunologically distinct from listeriolysin O (21) or possibly a lipase or protease that can lyse erythrocytes (20) remains to be determined. In addition, Kuhn and Goebel (12) have recently reported that a major secreted 60-kDa polypeptide, termed p60, may be involved in the uptake of *L. monocytogenes* by nonprofessional phagocytic cells. Whether *msp* encodes p60 also merits investigation.

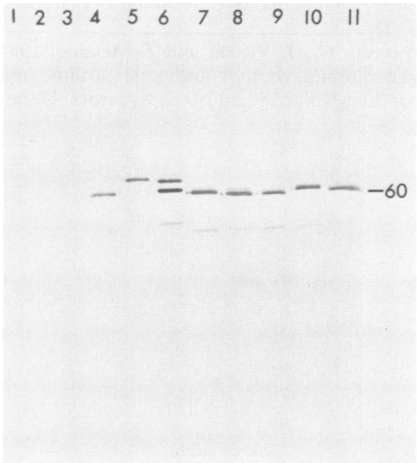

FIG. 5. Synthesis of extracellular polypeptides immunologically related to the 60-kDa extracellular polypeptide of *L. monocytogenes* 10403. Culture supernatants from various *Listeria* species were analyzed for the production of polypeptides that were immunologically related to the *L. monocytogenes* 60-kDa extracellular polypeptide by the immunoblot technique. The antiserum used was that raised to the gel-purified sample of the 60-kDa polypeptide. Culture supernatants in lanes 1 to 11 are *L. murrayi* ATCC 25401, *L. denitrificans* ATCC 14870, *L. grayi* ATCC 19120, *L. innocua* ATCC 33090, *L. seeligeri* ATCC 35967, *L. ivanovii* ATCC 19119, *L. monocytogenes* ATCC 10403, *L. monocytogenes* D3A, *L. monocytogenes* Scott A, *L. monocytogenes* EGD, and *L. monocytogenes* ATCC 19111, respectively.

ACKNOWLEDGMENTS

We thank Dan Portnoy and Ralph Martinez for helpful discussions and critical readings of the manuscript, John Mekalanos and J. Beckwith for Tn*phoA*, H. Lehrach for EMBL3A, and Mitch Cohen, R. Meyer, and D. Shaw for *Listeria* strains.

LITERATURE CITED

1. **Albritton, W. L., G. L. Wiggins, W. E. DeWitt, and J. C. Feeley.** 1980. *Listeria monocytogenes*, p. 139–142. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
4. **Centers for Disease Control.** 1985. Listeriosis outbreak associated with Mexican style cheese—California. Morbid. Mortal. Weekly Rep. **34**:357–359.
5. **Datta, A. R., B. A. Wentz, and W. E. Hill.** 1987. Detection of hemolytic *Listeria monocytogenes* by using DNA colony hybridization. Appl. Environ. Microbiol. **53**:2256–2259.
6. **Flamm, R. K., D. J. Hinrichs, and M. F. Thomashow.** 1984. Introduction of pAMβ1 into *Listeria monocytogenes* by conjugation and homology between native *L. monocytogenes* plasmids. Infect. Immun. **44**:157–161.
7. **Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum,**

P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reingold. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. **312**:404–407.

8. **Frischauf, A., H. Lehrach, A. Poustra, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**:827–842.
9. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
10. **Hirsch, P. R., and J. E. Beringer.** 1984. A physical map of pPH1JI and pJB4JI. Plasmid **12**:139–141.
11. **Hohn, B.** 1979. *In vitro* packaging of lambda and cosmid DNA. Methods Enzymol. **68**:209–218.
12. **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.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Mandel, M., and A. Higa.** 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**: 8129–8133.
17. **Mengaud, J., M. F. Vicente, J. Chenevert, J. M. Pereira, C. Geoffroy, B. Gicquel-Sanzey, F. Baquero, J.-C. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56**:766–772.
18. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Murray, E. G. D., R. A. Webb, and M. B. R. Swann.** 1926. A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n. sp.). J. Pathol. Bacteriol. **29**:407–439.
20. **Njoku-Obi, A. N., E. M. Jenkins, J. C. Njoku-Obi, J. Adams, and V. Covington.** 1963. Production and nature of *Listeria monocytogenes* hemolysins. J. Bacteriol. **86**:1–8.
21. **Parrisius, J., S. Bhakdi, M. Roth, J. Tranum-Jensen, W. Goebel, and H. P. R. Seeliger.** 1986. Production of listeriolysin by beta-hemolytic strains of *Listeria monocytogenes*. Infect. Immun. **51**:314–319.
22. **Paterson, J.** 1940. The antigenic structure of organisms of the genus *Listerella*. J. Pathol. Bacteriol. **51**:427–436.
23. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167**:1459–1471.
24. **Prentki, P., F. Karch, S. Iida, and J. Meyer.** 1981. The plasmid cloning vector pBR325 contains a 482 basepair long inverted duplication. Gene **14**:289–299.
25. **Rocourt, J., and P. A. D. Grimont.** 1983. Description of *Listeria welshimeri* sp. nov. and *Listeria seeligeri* sp. nov. Int. J. Syst. Bacteriol. **33**:866–869.
26. **Saike, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.
27. **Seeliger, H. P. R.** 1981. Apathogene *Listerien*: *L. innocua* sp. n. (Seeliger et Schoofs, 1977). Zentralbl. Bakteriol. Parasitenkd, Infektionskr. Hyg. Abt. 1 Orig. Reihe C **3**:394–398.
28. **Seeliger, H. P. R., J. Rocourt, A. Schrettenbrunner, P. A. D. Grimont, and D. Jones.** 1984. *Listeria ivanovii* sp. nov. Int. J. Syst. Bacteriol. **34**:336–337.
29. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
30. **Skerman, V. B. D., V. McGowan, and P. H. A. Sneath.** 1980. Approved list of bacterial names. Int. J. Syst. Bacteriol. **30**: 225–420.
31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
32. **Thomashow, M. F., R. Nutter, A. L. Montoya, M. P. Gordon, and E. W. Nester.** 1980. Integration and organization of Ti plasmid sequences in crown gall tumors. Cell **19**:729–739.
33. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
34. **Welshimer, H. J., and A. L. Meredith.** 1971. *Listeria murrayi* sp. n.: a nitrate-reducing mannitol-fermenting *Listeria*. Int. J. Syst. Bacteriol. **21**:3–7.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.