

Restriction Fragment Length Polymorphisms Detected with Novel DNA Probes Differentiate among Diverse Lineages of Serogroup 4 *Listeria monocytogenes* and Identify Four Distinct Lineages in Serotype 4b

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***Listeria monocytogenes* of serotype 4b has been implicated in numerous outbreaks of food-borne listeriosis and in ca. 40% of sporadic cases. Strains of this serotype appear to be relatively homogeneous genetically, and molecular markers specific for distinct serotype 4b lineages have not been frequently identified. Here we show that DNA fragments derived from the putative mannitol permease locus of *Listeria monocytogenes* had an unexpectedly high potential to differentiate among different strains of serotype 4b when used as probes in Southern blotting of *Eco*RI-digested genomic DNA, yielding four distinct restriction fragment length polymorphism (RFLP) patterns. Strains of two epidemic-associated lineages, including the major epidemic clone implicated in several outbreaks in Europe and North America, had distinct RFLPs which differed from those of all other serotype 4b strains that we screened but which were encountered among strains of serotypes 1/2b and 3b. In addition, three serogroup 4 lineages were found to have unique RFLPs that were not encountered among any other *L. monocytogenes* strains. One was an unusual lineage of serotype 4b, and the other two were members of the serotype 4a and 4c group. The observed polymorphisms may reflect evolutionary relationships among lineages of *L. monocytogenes* and may facilitate detection and population genetic analysis of specific lineages.**

Invasive illness due to *Listeria monocytogenes* (listeriosis) has severe symptoms (meningitis, septicemia, and abortions) and is a leading cause of death due to food-borne pathogens in the industrialized world, including the United States (11, 13, 22). The pathogen has also been implicated in outbreaks of febrile gastroenteritis (3, 28).

Numerous investigations have revealed that the species is clonally partitioned along serotypic clusters. Two major genomic divisions have been identified: division I includes serotypes 1/2a, 1/2c, 3a, and 3c, whereas strains of serotypes 1/2b, 3b, and 4b are included in division II (1, 5, 6, 8, 14, 24). In addition, genotyping data suggest the existence of a third, less populous division (26, 35) that consists of strains of serotypes 4a and 4c, derived primarily from animal infections (35). Examination of numerous genes has revealed distinct allelic variants in each division, suggesting a strong linkage disequilibrium maintained by lack of gene flow between divisions.

Strains of just three serotypes, 1/2a, 1/2b, and 4b, constitute more than 95% of human clinical isolates (13, 16). Serotype 4b is of special interest, as it has been implicated not only in ca. 40% of sporadic infections but also in numerous outbreaks of food-borne listeriosis. Earlier major outbreaks in Nova Scotia (coleslaw), California (Jalisco cheese), Switzerland (Vacherin Mont d'Or cheese), and France (pork tongue in aspic) and

numerous other outbreaks in Europe and North America (16, 29) implicated strains of this serotype. More recently, a multistate outbreak in the United States, in which contaminated hot dogs were implicated, also involved bacteria of serotype 4b of an apparently novel lineage (2).

Several genotyping data, based on different subtyping tools and approaches, suggest that serotype 4b is genetically rather homogeneous and less diverse than the other serotypes (1/2a and 1/2b) commonly implicated in human illness (5, 14, 21, 23, 27). Furthermore, *L. monocytogenes* strains implicated in outbreaks have been shown to constitute genetically well-defined clonal lineages (epidemic clones) on the basis of multilocus enzyme electrophoresis and pulsed-field gel electrophoresis (PFGE) (7, 9, 24), but the literature contains only a few descriptions of characterized genetic markers that are unique for epidemic-associated strains. Such descriptions primarily concern the strains of a major epidemic lineage (epidemic clone I [ECI]) which has been implicated in numerous outbreaks in Europe and North America, including the above-mentioned epidemics in Nova Scotia, California, Switzerland, and France. Restriction fragment length polymorphism (RFLP) analysis using DNA probes derived from the *ltrB* genomic region, which is essential for cold growth of *L. monocytogenes*, differentiated ECI strains from all other *L. monocytogenes* strains of serotype 4b that were screened (36). In addition, ECI strains appear to possess a unique DNA modification at GATC sites, which renders their DNA resistant to digestion by *Sau*3AI (37). Recently, DNA sequences specific to ECI strains have been identified (15a), and the genome sequencing of one such strain has been undertaken (www.tigr.org). Genetic markers specific

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for other lineages of *L. monocytogenes* serotype 4b, including the strains implicated in the hot dog outbreak in 1998 to 1999 (2), have not yet been described. Such markers would supplement other typing tools (such as PFGE) to facilitate lineage detection and monitoring and would help to elucidate the presently poorly understood evolution of epidemic-associated clonal groups. In this work we describe DNA probes derived from the putative mannitol permease locus of *L. monocytogenes* which can identify four distinct RFLP patterns within serotype 4b, including RFLPs distinct for epidemic-associated strains.

MATERIALS AND METHODS

Bacterial strains and growth media. *Listeria* strains were grown at 35°C in brain heart infusion broth (Difco) or on tryptic soy agar supplemented with 0.7% yeast extract (Difco) and were preserved at -70°C in brain heart infusion broth. *Escherichia coli* strains were grown in Luria-Bertani broth at 35°C. When appropriate, ampicillin was used at 100 µg/ml.

General DNA manipulations and analyses. Unless otherwise indicated, standard molecular procedures (4) were used for extraction, digestion, and other manipulations of DNA. Plasmids were purified with Wizard miniprep columns (Promega). Southern blotting was performed as described previously (20) with DNA probes labeled with digoxigenin (Genius kit; Roche). The probe for the *ltrB* region was pST1, which has been previously described (36). Determination of DNA resistance or sensitivity to *Sau3AI* digestion was as described previously (37). For PCR we used synthetic oligonucleotides (Biosynthesis) and either *Taq* polymerase (Promega) or Red *Taq* (Sigma) under previously described conditions (20). To clone PCR fragments, the desired fragment was purified from agarose gels using GeneClean (Bio101) and cloned into pCR1000 (TA cloning; Invitrogen), following the suggestions of the vendors. Sequences of the cloned fragments were determined and analyzed as described previously (25). Restriction enzymes were purchased from MBI Fermentas or from Promega.

Probe constructions. A genomic region was originally identified in the serotype 1/2a strain 1/2a3 (33) and first used as a probe in Southern blotting in the course of an unrelated research project (33). The region was pursued further in this study because of unusual results obtained with Southern blots of genomic DNAs from different strains. PCR primers HLT28F (5' TTCTTGGTGGTATG ACAGGAAC 3') and HLT28R (5' TCACGACCACATCGCATTCGG 3') derived from the sequence of this region yielded a 464-bp fragment (fragment 28.5) which was cloned in pCR1000, yielding plasmid p28.5. When used as a probe, fragment 28.5 hybridized with a ca. 3.8-kb *EcoRI* fragment of strain 1/2a3. To clone this larger fragment, the ca. 3.8-kb region of an agarose gel (0.8%) of *EcoRI*-digested DNA of strain 1/2a3 was excised, and the DNA was purified with GeneClean. The purified fragments were ligated with *EcoRI*-digested plasmid pGEM-9Zf (Promega) and introduced into *E. coli* DH5α by transformation. White colonies were selected and inoculated in separate wells of two 96-well plates (200 µl of Luria-Bertani broth with ampicillin in each well). Portions of 150 µl from each of 12 wells were combined in one Eppendorf tube, and plasmid extractions from the mixtures were done with Wizard minipreps. The resulting plasmid preparations were screened by PCR using primers HLT28F2 and HLT28R2 (primer sequences are shown above). Three of the mixtures yielded PCR products of the appropriate size. The 12 cultures composing one of the positive mixtures were grown individually, and their plasmids were screened again by PCR using primers HLT28F2 and HLT28R2. The plasmid from one of the cultures (designated p1-4) produced the expected PCR product, and the plasmid was confirmed to harbor the expected ca. 3.8-kb *EcoRI* fragment by Southern blotting using fragment 28.5 as a probe. The cloned ca. 3.8-kb fragment was designated probe 1-4. To prepare probes, p28.5 and p1-4 were digested with *EcoRI* and the cloned fragments were purified from gels using GeneClean and labeled.

Nucleotide sequence analysis. Routine DNA analysis tools (reference 25 and references therein) were used. Sequence databases included the recently released genome sequence data for *L. monocytogenes* strain EGD (serotype 1/2a) and for *Listeria innocua* (<http://bioweb.pasteur.fr/cgi-bin/listeria/blast.cgi>), as well as the genome sequence data for *L. monocytogenes* serotype 4b (www.tigr.org).

Nucleotide sequence accession number. The nucleotide sequence data determined in this study have been deposited in the GenBank database under accession no. AF299095.

RESULTS

The putative mannitol permease gene (*mtlA*) differentiates between ECI and other serotype 4b strains when used as a probe in Southern blots. During our search for putative serotype-specific genes that may be involved in teichoic acid glycosylation of *L. monocytogenes* serotype 1/2a (33), candidate gene fragments were used as probes in Southern blots against a panel of strains representing different known lineages and serotypes. The use of one of these fragments as a probe identified unexpected RFLPs in the panel of strains examined. Although this fragment was subsequently found not to be serotype specific and not to be in a locus involved in teichoic acid glycosylation, we pursued its characterization because of the promising RFLP results. The fragment was sequenced, and a 464-bp internal portion (fragment 28.5) was amplified by PCR and cloned. BLAST analysis of the sequence of this fragment using the recently released (and as of this writing nonannotated) genomic databases of *L. monocytogenes* serotype 4b (www.tigr.org) and *L. monocytogenes* strain EGD (serotype 1/2a) (<http://bioweb.pasteur.fr/cgi-bin/listeria/blast.cgi>) revealed highly similar sequences in both genomic databases, although the identity was higher with strain EGD (97%) than with serotype 4b (94%). This may not be surprising, since the strain which we used, 1/2a3, was of serotype 1/2a, the same as EGD. *L. innocua* also harbored a homologous sequence (92% identity). The corresponding sequences in the databases of serotype 4b and of strain EGD were 93% identical.

Further analysis of the nucleotide sequence of fragment 28.5 identified a partial open reading frame with significant sequence identity to *mtlA*, encoding mannitol permease of *Bacillus stearothermophilus* (15) (60% over 560 nucleotides [nt]), *E. coli* (19) (56% over 557 nt), *Staphylococcus carnosus* (12) (67% over 193 nt), and numerous other bacteria. The deduced gene product was also found to have significant similarity to mannitol permeases of the bacteria named above, with identities of 43% (145 amino acids [aa]), 34% (187 aa), and 37% (149 aa) with the mannitol permeases of *B. stearothermophilus*, *E. coli*, and *S. carnosus*, respectively. TBLASTX analysis of the contig harboring the entire putative *mtlA* gene (contig 22, nt 33967 to 36524 in the *L. monocytogenes* 4b genomic sequence database [www.tigr.org]) showed that the deduced amino acid sequence from fragment 28.5 had 98% identity over 185 aa with an internal fragment of the putative MtlA of serotype 4b.

Use of the cloned fragment 28.5 as a probe (probe 28.5) in Southern blots of *EcoRI*-digested genomic DNA revealed several distinct RFLPs among serotype 4b strains. Interestingly, one hybridizing band of ca. 1.8 kb was identified in strains of the major epidemic clone (ECI) (Fig. 1, lanes 3 and 7), whereas strains from an unrelated epidemic lineage (ECII) had a different hybridizing band, of ca. 5 kb (Fig. 1, lanes 1, 2, and 8). A still different band, of ca. 6.4 kb, was produced with strains which were of apparent sporadic origin, i.e., not associated with any known epidemics (Fig. 1, lanes 5 and 6). One strain (strain 267), which on the basis of other evidence (Z. Lan, R. Y. Kanenaka, and S. Kathariou, unpublished data) represents an unusual genotype, also had a unique hybridizing band, of ca. 9.4 kb (Fig. 1, lane 4).

Four distinct RFLP patterns, designated RFLP patterns I to IV, were also observed in serotype 4b strains when the larger

M 1 2 3 4 5 6 7 8 9

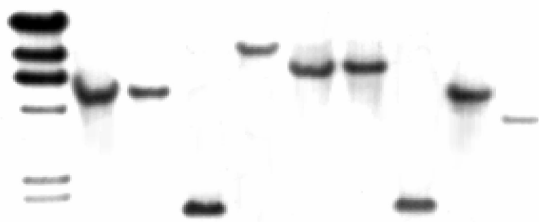


FIG. 1. Southern blot of *Eco*RI-digested genomic DNAs from *L. monocytogenes* strains with probe 28.5. Lane M, λ molecular size markers (fragment sizes [from top to bottom] are 23, 9.4, 6.6, 4.4, 2.3, and 2.0 kb). Lanes: 1, 2, and 8, serotype 4b strains LM20, LM21, and LM50 (clinical isolates from an epidemic unrelated to ECI and referred to here as ECII), respectively; 3 and 7, serotype 4b ECI strains LM265 and F2381 (Jalisco outbreak), respectively; 4, serotype 4b strain LM267; 5 and 6, serotype 4b sporadic strains LM266 and 4b1, respectively; 9, serotype 1/2a strain 1/2a3 (33).

(ca. 3.8-kb) genomic fragment was used as a probe (probe 1-4) with *Eco*RI-digested DNAs (Fig. 2 and Table 1). Pattern I (one band of ca. 6.4 kb) was obtained with sporadic isolates (Fig. 2, lanes 5, 11, 13, and 17), whereas strains of ECI yielded RFLP pattern II, consisting of the previously observed ca. 1.8-kb fragment plus an additional hybridizing fragment of ca. 2.3 kb (Fig. 2, lanes 3, 4, 7 to 10, 12, and 14). The new, ca. 2.3-kb fragment was also observed when the strains of the other epidemic lineage (ECII) were probed, resulting in RFLP pattern III, with two bands of ca. 5 and 2.3 kb for these strains (Fig. 2, lanes 1, 2, and 15). Strain 267, which, as mentioned above, represents an unusual serotype 4b genotype, yielded RFLP pattern IV, consisting of the original ca. 9.4-kb fragment obtained with probe 28.5 and an additional fragment of ca. 2.1 kb (Fig. 2, lane 6). Interestingly, two other isolates from our collection, which on the basis of other genetic studies also appeared to have unusual genotypes similar to those of strain 267, hybridized identically to strain 267 with this probe (Fig. 2, lanes 6 and 16, and Table 1).

Southern blots of *Hind*III-digested DNAs with the pST1 probe derived from the *ltrB* locus, which is essential for cold tolerance of *L. monocytogenes* (36), confirmed that the strains of RFLP pattern II had the epidemic-specific RFLP (data not shown). In addition, the genomic DNAs of these strains were resistant to *Sau*3AI digestion (data not shown), suggesting modification of cytosines at the GATC sites, as described earlier for this epidemic clone (37).

On the basis of PFGE and other genotyping data, strains of the rare serotypes 4d and 4e cannot be distinguished from those of serotype 4b (8, 20, 25). Similar findings were obtained with probes 28.5 and 1-4. The two available strains of serotype 4d and 4e (both type strains, originating at the American Type Culture Collection [ATCC]) hybridized with probe 1-4, yielding RFLP patterns I and II, respectively (Table 1).

TABLE 1. RFLP patterns in different *Listeria* strains determined with probe 1-4

RFLP pattern	Fragments (kb) hybridizing with probe 2-4	Strains (no. screened)		
		<i>L. monocytogenes</i>		Other <i>Listeria</i> species ^a
		Serotype 4b	Other serotypes	
I	6.4	Sporadic ^b (9)	4d (1)	<i>L. innocua</i> (12)
II	2.3, 1.8	ECI (15)	1/2b (4) 4e (1)	
III	5.0, 2.3	ECII (4)	1/2b (6) 3b (1)	<i>L. innocua</i> (3) ^c
IV	9.4, 2.1	B ₂ , A ₂ , 267 (3)		
V	2.0, 1.8		4a (1) 4a or 4c (2)	
VI	2.8, 1.8		4a or 4c (1)	
VII	3.8		1/2a (3) 3a (1) 3c (1) 1/2c (1)	
VIII	8.0, 2.6		"4" ^d (1)	

^a *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* did not hybridize with the probe.

^b Serotype 4b strains not associated with a known epidemic (presumably of sporadic origin).

^c Distinct *L. innocua* lineage (lineage I) with serotype 4b-like teichoic acid composition (18).

^d Unusual clinical isolate of *L. monocytogenes*, with an atypical serotypic designation.

Serotype 1/2b strains have two distinct RFLP patterns, which are also produced by epidemic-associated strains of serotype 4b. Serotypes 1/2b and 3b are the two other constituents of the major genomic division (division II) which includes serotype 4b strains. Two distinct RFLP patterns were observed in strains of serotype 1/2b. Interestingly, the RFLP patterns were identical to patterns II and III, which were found in epidemic-associated strains of serotype 4b but not in other strains of that serotype. In serotype 1/2b, RFLP patterns II and III were obtained in four and six strains, respectively. The single available serotype 3b strain had RFLP pattern III as well. The representative serotype 1/2b RFLPs are shown in Fig. 3 (pattern II in lanes 9 and 10 and pattern III in lanes 8, 11, and 12).

Strains of the other major genetic division of *L. monocytogenes* (division I, consisting of serotypes 1/2a, 1/2c, 3a, and 3c) yielded one hybridizing band of ca. 3.8 kb with either probe (e.g., Fig. 1, lane 9). Thus, a single RFLP pattern (pattern VII) was identified in this division.

The RFLP patterns obtained with the different serotypes of *L. monocytogenes* are summarized in Table 1.

Among other listeriae, only *L. innocua* harbors sequences with detectable homology to probes 28.5 and 1-4 and yields RFLP patterns also encountered within serotype 4b. *L. innocua* strains hybridized with both probes. With probe 1-4, RFLP pattern I (also obtained with non-epidemic *L. monocytogenes* serotype 4b) was produced by the type strain (sero-

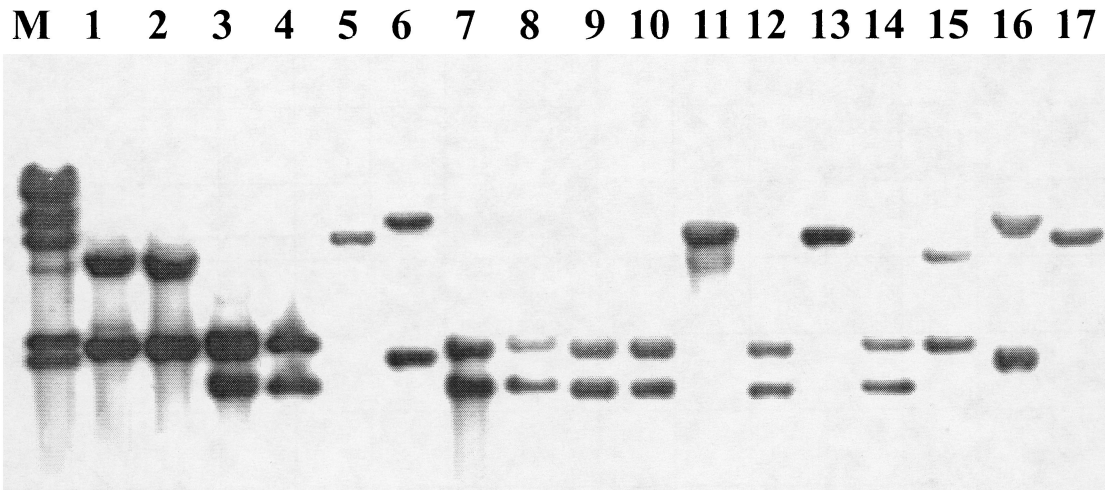


FIG. 2. Southern blot of *Eco*RI-digested genomic DNAs from *L. monocytogenes* serotype 4b strains with probe 1-4. Lane M, λ molecular size markers, as described in the legend for Fig. 1. RFLP pattern I, lanes 5, 11, 13, and 17 (presumably sporadic strains of serotype 4b, not associated with ECI or ECII); RFLP pattern II, ECI strains from Hawaii (lanes 3 and 4), Jalisco (lanes 7, 8, 9, and 12), and Nova Scotia (lanes 10 and 14); RFLP pattern III, ECII strains LM 20 (lane 1), LM 21 (lane 2), and LM 50, (lane 15); RFLP pattern IV, strains 267 (lane 6) and A2 (lane 16) (both serotype 4b and of unusual genotype).

type 6a) and several other strains. Examples of this RFLP pattern are shown in Fig. 3, lanes 5, 6, and 7. Interestingly, pattern III, which was obtained with certain epidemic-associated *L. monocytogenes* serotype 4b (ECII) strains and with some of the serotype 1/2b strains, was also observed with three strains of *L. innocua* when probe 1-4 was used (Fig. 3, lanes 2 to 4). It is noteworthy that these *L. innocua* strains represent a unique lineage, which is indistinguishable from *L. monocytogenes* serotype 4b in terms of teichoic acid composition and the presence of serotype 4b-specific genes (18).

No signals were observed in Southern blots with DNAs from *L. seeligeri* (three strains) or *L. ivanovii*, *L. welshimeri*, and *L. grayi* (one strain each).

M 1 2 3 4 5 6 7 8 9 10 11 12

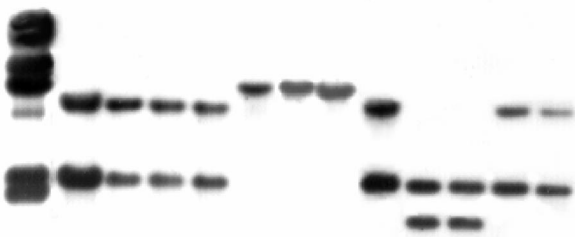


FIG. 3. Southern blot of *Eco*RI-digested genomic DNAs from *L. monocytogenes* serotype 4b strains with probe 1-4. Lane M, λ molecular size markers, as described in the legend for Fig. 1. Lanes: 1, RFLP pattern III of *L. monocytogenes* strain LM 50 (ECII); 2 to 4, RFLP pattern III of *L. innocua* strains F8735, F8596, and F7833 (lineage I, described in reference 18), respectively; 5 to 7, RFLP pattern I of *L. innocua* strains 6a, LM 248, and LM 250, respectively; 8, 11, and 12, RFLP pattern III of *L. monocytogenes* strains LM 249, F4242, and F4236 (serotype 1/2b), respectively; 9 and 10, RFLP pattern II of *L. monocytogenes* strains F4245 and F4260 (also serotype 1/2b), respectively.

The hybridization results with *L. innocua* and other listeriae, using probe 1-4, are summarized in Table 1.

Probe 1-4 can differentiate strains of serotypes 4a and 4c from other strains, including other isolates of serogroup 4 (serotypes 4b, 4d, and 4e). With the small probe 28.5, strains of serotypes 4a and 4c reacted similarly to ECI strains, producing one hybridizing fragment of ca. 1.8 kb (data not shown). When the larger probe 1-4 was used, however, these strains yielded two unique RFLP patterns, V and VI, not seen among any other listeriae (Table 1). Pattern V was produced by the type strain of serotype 4a and two clinical isolates (one animal and one human). The hybridizing bands from the latter strains are shown in Fig. 4 (lanes 1 and 7). The top hybridizing band of these strains was slightly but consistently smaller than the corresponding band of ECI strains (Fig. 4, lanes 2, 4, 5, and 6).

M 1 2 3 4 5 6 7

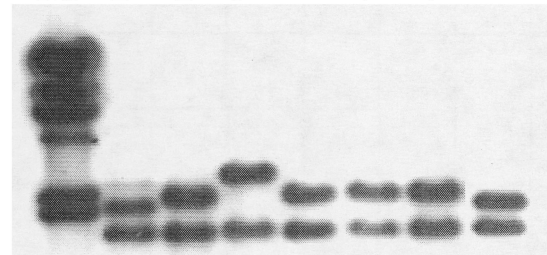


FIG. 4. Southern blot of *Eco*RI-digested genomic DNAs from *L. monocytogenes* serotype 4b strains with probe 1-4. Lane M, λ molecular size markers, as described in the legend for Fig. 1. Lanes 1 and 7, RFLP pattern V of strains OLM 8 and OLM 151 (isolates from animal and human illness, respectively, of serotype 4a or 4c); lanes 2, 4, 5, and 6, RFLP pattern II of ECI strains OLM10, OLM 65, OLM 97, and OLM 116, respectively; lane 3, RFLP pattern VI of strain OLM 59 (human clinical isolate, serotype 4a or 4c).

Pattern VI was encountered in only one strain, a human clinical isolate (Fig. 4, lane 3).

The unique RFLPs of the 4a and 4c strains differentiate them from all other screened strains of *L. monocytogenes*. A similar statement can be made for the three serotype 4b strains (A2, B2, and 267) with RFLP pattern IV, which was not encountered among any of the other strains which we screened (Table 1). An additional unique RFLP pattern (pattern VIII) was encountered in a serogroup 4 strain of *L. monocytogenes* which appears to have an atypical antigenic composition and does not conform to the known serogroup 4 serotypes (Table 1).

DISCUSSION

Numerous genotyping data suggest that *L. monocytogenes* is partitioned in two major divisions that correlate with flagellar (H) antigen types (31) and the corresponding serotypic designations (division I with serotypes 1/2b, 3b, and 4b and division II with serotypes 1/a, 1/c, 3a, and 3c) (1, 5, 6, 8, 14, 24). Strains of serotypes 4a and 4c appear to constitute a third division (26, 35).

Although division-specific genetic variation has been identified in many genes, including those involved in virulence (26, 34, 35), there has been a scarcity of well-defined genetic markers for specific lineages within the major divisions. The lack of lineage-specific genetic markers has been an issue especially for serotype 4b, which appears to be genetically more homogeneous than serogroup 1/2 (5, 14, 21, 23, 27) and which, in addition, is often implicated in food-borne epidemics. The gene probes described here may therefore be of special interest as subtyping tools for this serotype.

The major epidemic clone of *L. monocytogenes*, ECI, yielded a distinct RFLP pattern (pattern II), whereas another epidemic cluster of strains, ECII, had a different RFLP pattern (pattern III). Patterns II and III were not found among any of the other serotype 4b strains which we screened but were obtained for strains of serotypes 1/2b and 3b. This may suggest a common origin of these epidemic serotype 4b lineages and lineages of serotypes 1/2b and 3b, at least in the probed genomic region. The genetic background of ECI and ECII remains poorly understood at this time.

RFLP pattern II was also obtained with the single available serotype 4e strain (the type strain from the ATCC) (Table 1). This was not surprising to us, because on the basis of both *ltrB* RFLP and *Sau3AI* digestion resistance, this strain is indistinguishable from serotype 4b ECI strains (36, 37). In addition, the single available serotype 4d strain (also the type strain from the ATCC) had RFLP pattern I, identical to that of nonepidemic serotype 4b strains (Table 1). None of the genotyping tools that we and others have used, including detection of serotype-specific genes (20, 25) and PFGE (8), has been able to differentiate between serotype 4b and serotype 4d or 4e. Furthermore, serotype 4d and 4e strains also reacted with monoclonal antibodies which reacted with serotype 4b but no other serotypes of *L. monocytogenes* (17).

The nonpathogenic species *L. innocua*, which is considered to be the one most closely related genetically and bacteriologically to *L. monocytogenes* (30), had two RFLPs, both of which were encountered in serotype 4b as well. It is noteworthy that

L. innocua lineage I shared the same RFLP pattern (pattern III) as the epidemic strains of *L. monocytogenes* ECII. This lineage of *L. innocua* is unique in that its teichoic acid-associated surface antigens are identical to those of *L. monocytogenes* 4b and in harboring serotype 4b-specific teichoic acid glycosylation genes (18), facts which may be of relevance in terms of the origin of this lineage. Other *L. innocua* strains had pattern I, which is seen in non-epidemic *L. monocytogenes* serotype 4b strains. The presence of apparently common RFLP patterns between *L. monocytogenes* serotype 4b and *L. innocua* is of potential evolutionary interest.

We identified a surprisingly high number of RFLP patterns in serogroup 4 (e.g., patterns IV, V, VI, and VIII [Table 1]) which were specific and unique to single lineages, suggesting that the RFLPs arose in a specific lineage. Pattern IV was found in only three *L. monocytogenes* serotype 4b strains in our collection which were unusual in terms of PFGE pattern and additional genetic markers (Lan et al., unpublished data). One of these strains was a clinical isolate from 1999, whereas the other two were environmental strains isolated earlier (1995) from a produce storage facility. These strains may represent a unique clone not commonly seen within serotype 4b but apparently capable of causing human illness. The strains of patterns V and VI were of serotypes 4a and 4c (including the 4a type strain). It is worth noting that the use of the large probe was essential in identifying the unique RFLP patterns of these strains, as patterns were identical (1.8-kb band) when the small probe 28.5 was used. Strains of serotypes 4a and 4c are genetically quite distinct from other *L. monocytogenes* strains and represent a distinct lineage (26, 35). In addition, strains of serotypes 4a and 4c had attenuated virulence in mice, although they were still capable of inducing a protective immune response (10, 32). In our laboratory, these strains have been found to have unique RFLPs with probes derived from a serotype-specific DNA region (20). Serotypes 4a and 4c are rarely found in food or environmental samples and are also rare in human infection but constituted 8 to 25% of animal listeriosis isolates in one study (35). This may be a genetically distinct, animal-adapted lineage, although we must note that of the four 4a and 4c strains that we examined, two were of human clinical origin.

The genomic region from which these probes were derived remains to be characterized. Our sequence analysis results suggest that this is a putative mannitol permease locus. Preliminary biochemical characterization of a mutant with an insertion mutation in the putative *mtlA* gene suggests that the mutant was incapable of mannitol uptake (H. L. Tran, S. Kathariou, and R. Hutkins, unpublished findings). In *B. stearo-thermophilus* and *S. carnosus*, *mtlA* is the first gene in an operon that contains additional genes involved in mannitol metabolism (12, 15). Such mannitol metabolism genes are expected to be identified when the annotated *L. monocytogenes* genome sequence data are released.

In conclusion, the probes described here have molecular subtyping potential in identifying several distinct lineages of *L. monocytogenes* of serogroup 4. These and similar probes that may become identified in the future may also be used as tools to elucidate the evolutionary relationships among different lineages (including epidemic-associated lineages) of *L. monocy-*

togenes as well as between *L. monocytogenes* and the closely related nonpathogenic species *L. innocua*.

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