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Host-Mediated Modification of *Sau*3AI Restriction in *Listeria monocytogenes*: Prevalence in Epidemic-Associated Strains

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Most major food-related outbreaks of listeriosis have been traced to a cluster of genetically related strains of serovar 4b (epidemic clone). In spite of numerous searches, distinct bacteriologic or virulence-related features unique to these strains have eluded identification, although a restriction fragment length polymorphism (RFLP) characteristic of the epidemic clone has previously been described (W. Zheng and S. Kathariou, Appl. Environ. Microbiol. 61:4310–4314, 1995). We found that DNAs from 75 strains which were derived from three separate outbreaks and which had the epidemic clone-specific RFLP were also invariably resistant to digestion by *Sau*3AI and other restriction endonucleases sensitive to cytosine methylation at 5′ GATC 3′ sites. This modification of *Sau*3AI restriction was host mediated, as it did not persist when DNA was cloned and propagated in *Escherichia coli*, and was uncommon among other *Listeria* strains. Epidemic-associated strains with this modification were resistant to infection by phage propagated in a serotype 4b strain which was not known to be involved in an epidemic and which lacked the epidemic clone-specific RFLP. Screening for susceptibility to *Mbo*I digestion revealed that these epidemic strains lacked methylation of adenines at GATC sites. This type of modification was rare among *Listeria* strains and was found in only three (of eight screened) strains of serovar 1/2b, possibly representing one clonal lineage.

Listeria monocytogenes is a gram-positive bacterium which can cause serious disease (listeriosis) in humans and animals. This bacterium is widespread in the environment and is well-known for its ability to grow at refrigeration temperatures (11). *Listeria* infections can often be traced to contaminated cold-stored foods. In contrast to most other food-borne infections, listeriosis is an invasive disease which often affects the central nervous system (11, 12). During the last 20 years, several high-impact epidemics of food-related listeriosis involving serotype 4b strains, including the Nova Scotia coleslaw-associated outbreak (1981), the Jalisco (Mexican-style cheese) outbreak in Los Angeles (1985), and the Swiss Vacherin Mont d'Or cheese outbreak (1987 to 1989) (11, 25), as well as the latest outbreak of food-borne listeriosis in France (1992) (15), have taken place. Interestingly, phage typing, multilocus gel electrophoresis, and pulsed-field fingerprinting have revealed a high degree of genetic relatedness among the strains implicated in these geographically and temporally unlinked epidemics (15, 24), suggesting that such strains are likely to represent an epidemic clonal lineage (epidemic clone) (23). Recently, we showed that a unique restriction fragment length polymorphism (RFLP) in a DNA region involved in cold growth could differentiate such strains from other strains of this pathogen, including other serotype 4b strains. This RFLP was best detected when Southern blots of *Hin*dIII-cut genomic DNAs were probed with DNA fragments derived from the cold-growth-essential gene *ltrB* (30). During the study of this and other RFLPs, it became apparent that DNAs from strains with the epidemic clone-specific RFLP were not digested by *Sau*3AI (recognition site, GATC), suggesting that GATC sites are modified in these strains. In contrast, *Sau*3AI readily digested genomic DNA of strain 4b1, which was derived from an isolated (sporadic) case of listeriosis and which lacked the epidemic clone-specific RFLP. In fact, partial *Sau*3AI digestion of 4b1 DNA has been used in our laboratory for the construction of genomic libraries (30).

In this report, we show that modification of *Sau*3AI restriction is prevalent in epidemic-associated *L. monocytogenes* strains. Such strains also showed reduced plaque formation after infection by phage propagated in nonmodifying strains, suggesting the involvement of a cognate restriction endonuclease. The observed modification was host mediated, since it did not persist once the DNA was cloned and propagated in *Escherichia coli.*

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used in this study are listed in Table 1. Strains 4b1 and F2381, derived from an isolated (sporadic) case of listeriosis and from the Jalisco cheese outbreak, respectively, were used as prototype sporadic and epidemic clone strains, respectively, for some experiments as indicated. Unless otherwise indicated, *Listeria* strains were grown overnight at 35°C in brain heart infusion broth (Difco).

***Listeria* phage infection assay.** *Listeria* serovar 4b-specific phage 2671 (kindly obtained from J. Rocourt) was purified from the indicated propagating strain and diluted in SM buffer (0.1 M NaCl, 0.015 M $MgSO_4$, 0.01% gelatin, and 0.05 M Tris-HCl [pH 7.5]). Phage (100 μl) was mixed with host bacteria (200 μl of an overnight culture in brain heart infusion broth [Difco]) for 15 min. Five milliliters of melted soft agar (Luria-Bertani [LB] broth with 0.7% agar) was added to the phage-bacteria mixture, briefly mixed, and immediately poured over modified LB agar plates (LB broth with the addition of 0.3% glucose, 0.075 mM $CaCl_2$, 0.004 mM $FeCl_3$, 2 mM $MgSO_4$, and 1.2% agar). Plates were incubated at 35°C overnight.

Molecular procedures. Genomic DNAs from *Listeria* spp. were extracted as described previously (30) from 4.5 ml of overnight cultures grown at 35°C, with the modification that a hexadecyl trimethyl ammonium bromide extraction step was included as previously described (3). Restriction enzyme digestions were conducted according to the suggestions of the vendor (Promega). A nonradioactive digoxigenin-based Genius kit (Boehringer Mannheim) was used in Southern blotting for labeling and detection of DNA according to the suggestions of the vendor.

To clone 5- to 8-kb DNA fragments from the epidemic strain F2381, genomic DNA was partially digested by *Mbo*I and separated on a low-melting-point agarose gel (Amresco). Fragments in the desired size range (2 to 8 kb) were purified as previously described (30) and ligated to *Bam*HI-digested and dephosphorylated pUC18 (Pharmacia).

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TABLE 1. Sensitivities of *Listeria* DNAs to restriction enzymes *Sau*3AI and *Mbo*I

Organism(s)	No. of strains screened	No. of strains that cleave with:	
		*Sau*3AI	*Mbo*I
L. monocytogenes			
Serotype 4b			
Epidemic RFLP[a]	75	0	75
Nonepidemic RFLP	27	27	27
Serotype 1/2a	10	10	10
Serotype 1/2b	8	8	5[b]
Serotype 1/2c	1	1	1
Serotype 3a	1	1	1
Serotype 3b	4	4	4
Serotype 3c	2	2	2
Serotype 4a	4	4	4
Serotype 4c	1	1	1
Serotype 4d	4	4	4
Serotype 4e	1	0[c]	1
Serotype 4ab	1	1	1
Serotype 7	1	1	0
Not determined[d]	26	26	26
L. innocua	24	24	24
L. ivanovii	1	1	1
L. seeligeri	3	3	3
L. welshimeri	1	1	1
L. grayi	1	1	1
Atypical listeriae[e]			
Rhamnose negative			
Serotype 4	2	1	2
Serotype 1/2b	2	0	2
Serotype 6a	2	2	2
Other strains[f]	2	0	2

[a] Includes strains from the Nova Scotia (27 strains), Jalisco (45 strains), Swiss (1 strain), and Philadelphia (2 strains) outbreaks (25).
[b] *Mbo*I-resistant strains were from the Philadelphia outbreak (26).
[c] Strain had the epidemic RFLP (30).
[d] Serotypes were other than 4b, 4d, and 4e on the basis of MAb and DNA probe reactivities (17, 19).
[e] Strains of atypical serotypes and undetermined taxonomic status (17).
[f] Strains Kauai A2 and Kauai B2. The serotype is predicted to be 4b, 4d, or 4e on the basis of MAb and DNA probe reactivities (17, 19); strains had the epidemic RFLP, but the hybridization signals were weak.

RESULTS

Modification of *Sau*3AI, but not *Mbo*I, restriction is prevalent in strains of the epidemic clone lineage. DNAs from numerous *Listeria* strains of different serotypes and epidemiological backgrounds (Table 1) were examined in terms of sensitivity to cleavage by *Sau*3AI and *Mbo*I. Although both of these enzymes recognize GATC sites, methylation of the cytosine at this site inhibits cleavage by *Sau*3AI but has no effect on cleavage by *Mbo*I. In contrast, *Mbo*I does not cut DNA when the adenine residue at the GATC site is methylated (20).

Strains associated with the Nova Scotia and Jalisco cheese outbreaks were independent isolates of both clinical (28 Nova Scotia and 18 Jalisco strains) and food or environmental (2 Nova Scotia and 27 Jalisco strains) origins. Reactivities with monoclonal antibodies (MAbs) (17) and DNA probes (19) confirmed that these strains were of serogroup 4. The nine Massachusetts outbreak strains were independent clinical isolates (the implicated strain was never isolated from food) (11,

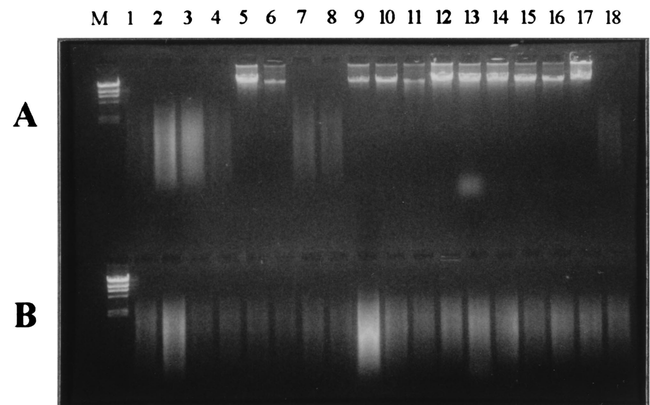

FIG. 1. *Sau*3AI (A) and *Mbo*I (B) digestions of chromosomal DNAs prepared from representative sporadic and epidemic serotype 4b strains of *L. monocytogenes*. Lanes: M, *Hin*dIII-digested λ DNA (fragment sizes are [from top to bottom] 23, 9.4, 6.5, 4.3, 2.3, and 2.0 kb); 1 to 4, sporadic serovar 4b strains 4b1, 1849A, G3622, and G3384, respectively; 5 to 8, serotype 4b strains from the Philadelphia outbreak (F4234, F4243, F4244, and F4258, respectively); 9 to 11, strains F2381, 2115, and 2182, respectively, from the Jalisco outbreak; 12 to 16, strains 15U, 2205, 2208, 2211, and 2212, respectively, from the Nova Scotia outbreak; 17, strain 33 from the Swiss outbreak; and 18, strain 18 (Massachusetts).

25). The Philadelphia outbreak was unusual because it involved strains of multiple serotypes and electrophoretic types (26). We examined eight strains from this outbreak, including four of serotype 4b.

DNA preparations which were not treated with restriction endonucleases contained high-molecular-weight DNA which appeared to be indistinguishable among epidemic-associated and other isolates in terms of mobility on agarose gels (data not shown). Restrictions with *Sau*3AI, however, revealed that DNAs from most (27 of 30) Nova Scotia isolates and all available strains from the Jalisco and Swiss outbreaks resisted cleavage by *Sau*3AI, as did DNAs from 2 serotype 4b strains (of 4 strains of this serotype) implicated in the Philadelphia outbreak. Most other *L. monocytogenes* strains (a total of 93 screened strains, including the remainder of the Philadelphia isolates, the Massachusetts strains, and strains from isolated [sporadic] incidences of listeriosis and from foods or the environment) yielded DNAs which were readily restricted by *Sau*3AI (Table 1). The *Sau*3AI isoschizomer *Mbo*I, however, cleaved *Sau*3AI-resistant DNAs from the epidemic-associated strains listed above (Fig. 1B; Table 1), suggesting that methylation at the GATC sites in the genomes of these strains involved cytosines, not adenines.

To more accurately determine the extent of this modification, a cloned 7-kb DNA fragment derived from the *ltrA* region (29) was used as the probe in Southern blotting of *Eco*RI- and *Sau*3AI-digested genomic DNA of the epidemic strain F2381. To eliminate the possibility that cutting occurred at only a few select sites within the *ltrA* region of F2381 but that the resulting fragments were too large to separate and detect on these blots, we included double digests of DNA with *Sau*3AI and *Eco*RI (Fig. 2). Such blots revealed that none of the *Sau*3AI sites expected to be present in this region were cut, whereas these sites were cut by *Mbo*I. DNA from strain 4b1, in contrast, yielded identical fragments when it was cut with *Eco*RI-*Sau*3AI and *Eco*RI-*Mbo*I.

In addition to being the recognition sequence for *Sau*3AI and its numerous isoschizomers, GATC is a core sequence in the recognition sites of several other restriction endonucleases. Some of these enzymes, such as *Bam*HI and *Bgl*II, which recognize the sequences 5′ GGATCC 3′ and 5′ AGATCT 3′,

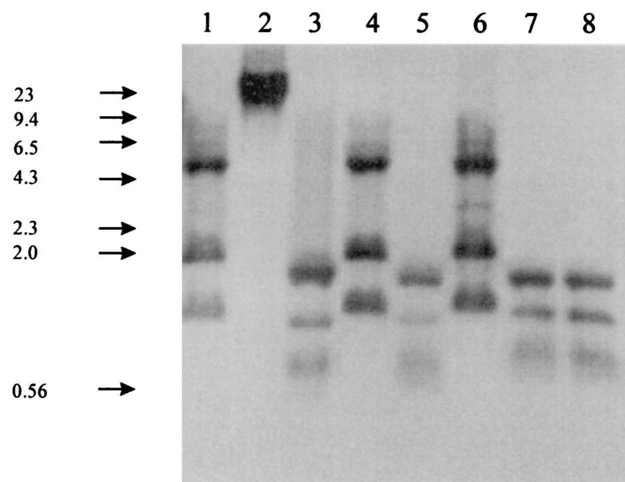

FIG. 2. Evidence for modification of all GATC sites in the *ltrA* region (ca. 7 kb) of strain F2381. The numbers on the left indicate the fragment sizes (in kilobases) of *Hin*dIII-digested λ DNA. Lanes: 1 to 5, F2381 DNA digested with *Eco*RI, *Sau*3AI, *Mbo*I, *Eco*RI plus *Sau*3AI, and *Eco*RI plus *Mbo*I, respectively; and 6 to 8, 4b1 DNA digested with *Eco*RI, *Eco*RI plus *Sau*3AI, and *Eco*RI plus *Mbo*I, respectively. The DNA blot was hybridized with a digoxigenin-labeled DNA fragment from the *ltrA* region cloned in pUC18.

respectively, do not cleave DNA when the cytosine at the internal GATC site is methylated (20). Indeed, we found that *Bam*HI did not cut DNAs from strains of the epidemic clone which resisted *Sau*3AI digestion (Fig. 3). *Bgl*II was also unable to cut the DNAs of these strains (Fig. 3). Strain 4b1 DNA, however, was readily restricted by *Bam*HI and *Bgl*II, yielding numerous large (>20-kb) fragments as well as smaller fragments (Fig. 3). This restriction pattern was typical for these two enzymes. In contrast, when the same DNA preparations were cut with *Hin*dIII or *Eco*RI, a continuous ladder of fragments without a disproportionate amount of high-molecular-weight bands was produced (data not shown).

The putative modification (cytosine methylation) at GATC sites appeared to be quite specific to the strains of the epidemic clone. With very few exceptions (see below), DNAs from other surveyed strains, including other serotype 4b isolates, were

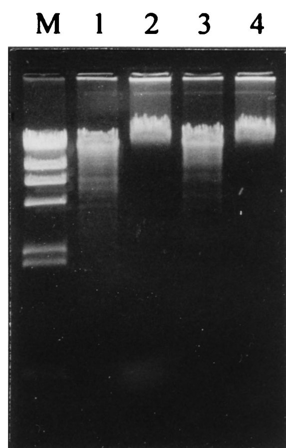

FIG. 3. *Bam*HI and *Bgl*II digestions of DNAs prepared from representative sporadic and epidemic strains of *L. monocytogenes*. Lanes: M, λ DNA digested with *Hin*dIII as described in the legend to Fig. 1; 1 and 3, 4b1 DNA digested with *Bam*HI and *Bgl*II, respectively; and 2 and 4, F2381 DNA digested with *Bam*HI and *Bgl*II, respectively.

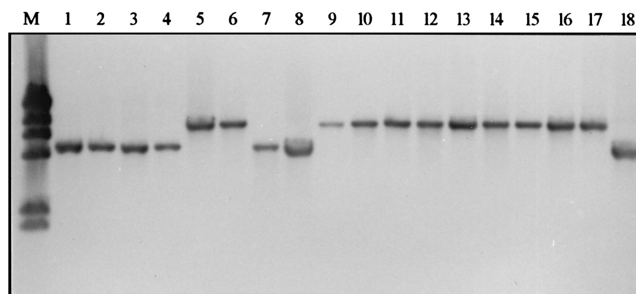

FIG. 4. Southern blot of *Hin*dIII-digested chromosomal DNAs from representative sporadic and epidemic 4b strains of *L. monocytogenes* probed with digoxigenin-labeled probe 1 (pST1), derived from the cold-essential gene *ltrB* (30). Lanes: M, λ DNA digested with *Hin*dIII as described in the legend to Fig. 1; and 1 to 18, the same *L. monocytogenes* strains as those in lanes 1 to 18 of Fig. 1. Southern blotting and probe development were done as previously described (30). The DNA blot was hybridized simultaneously with digoxigenin-labeled *Hin*dIII-digested λ DNA and with digoxigenin-labeled probe 1.

readily cleaved by *Sau*3AI (Table 1). A survey of several strains of other *Listeria* spp., including 1 strain each of *L. ivanovii*, *L. welshimeri*, and *L. grayi*, 3 strains of *L. seeligeri*, and 24 strains of *L. innocua*, suggested the absence of cytosine methylation at GATC sites; DNAs from these strains were readily cleaved by *Sau*3AI (Table 1).

RFLP characteristic of the epidemic clone lineage is present only in strains with the modification of *Sau*3AI restriction. We previously showed that the strains of the epidemic clonal group had a characteristic *Hin*dIII RFLP that was detected on Southern blots with probes derived from the *ltrB* locus, which is essential for low-temperature growth (30). Southern blots of additional strains of this lineage confirmed these results (Fig. 4). The agreement between the epidemic clone-specific RFLP and resistance to *Sau*3AI digestion was demonstrated by examination of the Nova Scotia and Philadelphia outbreak isolates. Of the 30 strains associated with the Nova Scotia outbreak, 27 were found to have the epidemic clone RFLP and yielded DNAs which were resistant to *Sau*3AI digestion; the 3 strains which lacked the epidemic clone RFLP also had DNAs that were readily cut with *Sau*3AI (data not shown). We suspect that these serotype 4b strains were derived from epidemiologically unrelated incidences of listeriosis concurrent with the outbreak. Interestingly, these same three strains were shown earlier to have restriction enzyme patterns different from those characteristic of other strains associated with the Nova Scotia outbreak (27a). In the case of Philadelphia outbreak strains, the two serotype 4b isolates with the epidemic clone-specific RFLP (F4234 and F4243) also had DNAs which resisted *Sau*3AI digestion (Fig. 1A and 4, lanes 5 and 6). Other isolates from this outbreak, including two additional serotype 4b strains (Fig. 1A and 4, lanes 7 and 8), lacked the epidemic clone RFLP and had DNAs which were cut with *Sau*3AI. These results suggest that in this case, the epidemic clone was involved in some, but not most, of the incidences of listeriosis and are in agreement with the previously described involvement of multiple lineages in this unusual outbreak (26).

The agreement between the epidemic clone-specific RFLP and DNA resistance to digestion by *Sau*3AI was also observed for strains ATCC 19118 (serotype 4e) and strains Kauai A2 and Kauai B2. These strains, which were not known to be associated with an epidemic, had the epidemic-type RFLP as well as DNA which resisted *Sau*3AI cleavage (Table 1). In fact, we were unable to identify any strain that had the epidemic RFLP and DNA which was cleaved by *Sau*3AI.

The strains which lacked the epidemic-type RFLP also

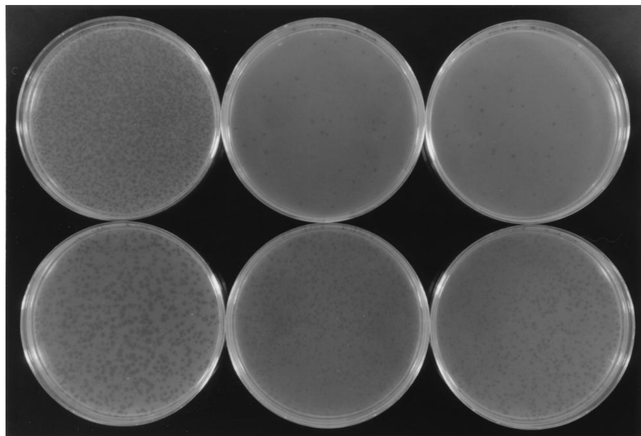

FIG. 5. Phage infection assay that suggests a restriction and modification system in the epidemic clone of *L. monocytogenes*. (top row, left to right) Phage 2671 propagated in the sporadic strain 4b1 was used to infect 4b1, the epidemic-RFLP strain F2381, and F4243, respectively. (bottom row, left to right) Phage 2671 propagated in strain F2381 was used to infect 4b1, F2381, and F4243, respectively. Strains F2381 and F4243 have cytosine methylation at GATC sites.

tended to lack the modification to *Sau*3AI restriction. We identified only 3 exceptions (strains G542, G543, and F9599) from 128 strains screened (Table 1). The actual frequency may be even lower, because two of these *Sau*3AI-resistant strains (G542 and G543) may represent repeated isolations of the same organism. Interestingly, all three were biochemically atypical, rhamnose-negative strains. One of the strains (F9599) was serologically atypical as well, probably representing a novel serotypic designation.

Modification to *Sau*3AI restriction is expressed during growth at a low temperature (4°C). The psychrotrophic nature of *Listeria* spp. prompted us to determine whether the epidemic strains maintained their characteristic DNA modification during growth at low temperatures. Strain F2381, which had the modification to *Sau*3AI restriction, and strain 4b1, the DNA of which was readily cut by *Sau*3AI, were grown for 20 days at 4°C. DNAs from F2381 and 4b1 showed resistance and susceptibility, respectively, to cleavage by *Sau*3AI (data not shown), suggesting that the modification mechanism operates during growth of the epidemic strain at low temperatures as well.

Propagation of epidemic strain DNA in *E. coli* results in loss of modification to *Sau*3AI. Five clones from a genomic library of the epidemic strain F2381 in pUC18 (insert size, 5 to 8 kb) were randomly chosen, purified from the *E. coli* host strain DH5α, and examined for the ability of inserts to resist *Sau*3AI digestion. Plasmid DNA, including the *L. monocytogenes* insert, was completely digested by the enzyme, yielding a number of small (200- to 500-bp) DNA fragments (data not shown). Thus, cytosines at GATC sites were apparently no longer methylated after the replication of F2381 DNA in *E. coli* DH5α.

Strains with the modification of *Sau*3AI restriction resisted infection by phage propagated in nonmodifying strains. Strains F2381 and 4b1 were infected by the serovar 4b-specific phage 2671 propagated in either type of host. 4b1-propagated phage formed many more plaques in 4b1 than in the epidemic strain F2381. In contrast, when the phage was propagated in F2381, equal numbers of plaques were formed in 4b1 and F2381 (Fig. 5). Similarly, numerous plaques were produced when F2381-propagated phage was used to infect strain F4243 (Philadelphia isolate which was *Sau*3AI resistant and had epidemic-specific RFLP). Noticeably fewer plaques were produced when F4243 was infected by 4b1-propagated phage (Fig. 5).

Modification of *Mbo*I restriction in serovar 1/2b strains of *L. monocytogenes*. We found that DNAs from three of eight screened serovar 1/2b strains of *L. monocytogenes* were not cut by *Mbo*I but were readily cleaved by *Sau*3AI (Table 1). These results suggest the presence of adenosine methylation at the GATC sites of these strains. Interestingly, all three *Mbo*I-resistant strains were from the Philadelphia outbreak, suggesting that they represent a serovar 1/2b epidemic-associated clone. This suggests that two distinct epidemic clones (of serovars 4b and 1/2b) accounted for five cases (of a total of eight screened strains) in this unusual outbreak (26). The modification of *Mbo*I restriction was not detected in strains of any serovar other than 1/2b or in other *Listeria* spp. (Table 1).

DISCUSSION

Our data suggest that the modification of cytosine at GATC sites was prevalent in the serovar 4b epidemic clone of *L. monocytogenes*. In contrast, the modification of adenine at GATC sites was observed only among strains of serovar 1/2b, possibly representing one clonal lineage. Although strain-dependent modifications of DNA at GATC sites have previously been described for other bacteria (7, 10, 27), the modification described here is remarkable in that it is associated, almost exclusively, with strains of unique importance in the epidemiology of this pathogen. We found that, without exception, strains with the RFLP which is characteristic of the epidemic clone lineage also appeared to have cytosine modification at GATC sites. The possible impact of this modification in the ecology (e.g., survival in refrigerated foods or a low-temperature environment) or pathogenesis-related attributes of the epidemic clone remains to be determined.

In bacteria, DNA modification systems of the type evidenced here are very often accompanied by restriction counterparts dedicated to the recognition and cleavage of foreign (usually unmodified) DNA (1, 2, 6). Such restriction-modification (R-M) systems are believed to have evolved partly as a defensive strategy against viruses, targeting and cleaving phage DNA injected into the bacterial host cell during phage infections (6). Interestingly, GATC sequences are underrepresented in bacteriophage genomes (16), perhaps as a counterstrategy to minimize the effects of GATC-recognizing endonucleases. In nature, several DNA R-M genes reside on mobile genetic elements (plasmids and prophage) (5, 6, 13). Recent data suggest that R-M systems in fact mediate the stability of the mobile elements which carry them (18, 22, 28). The high association of the *L. monocytogenes* modification system described here with a specific clonal lineage suggests that it was introduced by means of a *Listeria* phage or other mobile genetic element. Outside of this lineage, the resistance of DNA to restriction by *Sau*3AI was seen only rarely in listeriae (3 of 128 strains screened). Interestingly, these strains were not of serovar 4b and showed several unusual bacteriologic characteristics (e.g., they were rhamnose negative), although the possible relevance of this to their modification status is not clear at this time. It is possible that an ancestral epidemic isolate acquired the R-M system by horizontal transfer of the corresponding gene(s) from such an atypical *Listeria* strain or vice versa. Another possibility is that the modification system was introduced independently in the epidemic clone lineage and in the atypical isolates, perhaps from another, currently unidentified gram-positive donor. Genetic characterization of the putative R-M system in the epidemic clone of *L. monocytogenes* will be necessary to further address this issue. The possibility that the modification is transferable from strains of the epi-

demic clonal lineage to other *Listeria* strains (e.g., via conjugation) is being investigated in our laboratory.

Even though the origin of the DNA modification system in epidemic strains remains unknown, it is clear that the presence of a cognate restriction system would be of substantial advantage to bacteria. The phage 2671 infections described here indeed provide evidence for a restriction system in the epidemic strains which exhibit modification to *Sau*3AI restriction, although they do not prove that such restriction is cognate to this modification system. We would expect unmodified DNA (from phage propagated in 4b1) to undergo substantial restriction when it was injected into F2381 host cells. The few plaques that were obtained could be accounted for by phage DNA that managed to become modified and so escaped restriction. Phage propagated in F2381 was indeed shown to form equal numbers of plaques in F2381 and 4b1. These findings support the hypothesis that modified DNA from F2381-propagated phage is expected to persist and replicate equally well in modifying and nonmodifying hosts. A defensive weapon against phage attack may be especially important when growth is slow or compromised, as during long-term exposure to low temperatures or other environmental stresses. The fact that DNA of the epidemic strain F2381 was modified during growth at 4°C suggests that the putative cognate restriction system also operates under such conditions. Phage 2671 infections and lysis of cells appeared to take place quite effectively in the cold (4°C) (31); by controlling phage infections, epidemic strains may increase their ability to persist in refrigerated foods and other low-temperature environments. Furthermore, the presence of methylated bases at frequent sites in the genome may impact on several basic molecular processes, as has been shown in other systems (4, 8, 9, 21). It is known, for instance, that the ability of the *E. coli* Dam methylase to methylate adenine at GATC sites may be reduced in the presence of methylated cytosines at these sites (20). Furthermore, the genomic distribution of certain classes of GATC motifs suggests their involvement in transcriptional regulation during transition periods characterized by decreases in temperature and increases in the availability of oxygen (14). Further work is needed to determine whether the modification observed in the epidemic clonal lineage of *L. monocytogenes* confers some as-yet-unidentified molecular and/or physiological advantages, e.g., in regard to replication and gene expression at low temperatures or perhaps in regard to virulence potential and pathogenesis.

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