A Sheep in Wolf’s Clothing: *Listeria innocua* Strains with Teichoic Acid-Associated Surface Antigens and Genes Characteristic of *Listeria monocytogenes* Serogroup 4

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*Listeria monocytogenes* serotype 4b has been implicated in numerous food-borne epidemics and in a substantial fraction of sporadic listeriosis. A unique lineage of the nonpathogenic species *Listeria innocua* was found to express teichoic acid-associated surface antigens that were otherwise expressed only by *L. monocytogenes* of serotype 4b and the rare serotypes 4d and 4e. These *L. innocua* strains were also found to harbor sequences homologous to the gene *gtcA*, which has been shown to be essential for teichoic acid glycosylation in *L. monocytogenes* serotype 4b. Transposon mutagenesis and genetic studies revealed that the *gtcA* gene identified in this lineage of *L. innocua* was functional in serotype 4b-like glycosylation of the teichoic acids of these organisms. The genomic organization of the *gtcA* region was conserved between this lineage of *L. innocua* and *L. monocytogenes* serotype 4b. Our data are in agreement with the hypothesis that, in this lineage of *L. innocua*, *gtcA* was acquired by lateral transfer from *L. monocytogenes* serogroup 4. The high degree of nucleotide sequence conservation in the *gtcA* sequences suggests that such transfer was relatively recent. Transfer events of this type may alter the surface antigenic properties of *L. innocua* and may eventually lead to evolution of novel pathogenic lineages through additional acquisition of genes from virulent *listeriae*.

Wall teichoic acids are predominant constituents of the cell envelope of *Listeria monocytogenes* and other gram-positive bacteria. In *Listeria*, pronounced diversity in teichoic acid structure and antigenicity is conferred by glycosidic substitutions of the ribitol phosphate units (6, 7, 12, 27, 28). Such substitutions differ among different listerial serotypes. In the pathogenic species *L. monocytogenes* (the only *Listeria* species pathogenic to humans), serotype 4b strains are unique in bearing both galactose and glucose substituents on the N-acetylglucosamine (GlcNAc) of teichoic acid (6, 27). This is of interest, as serotype 4b accounts for a large fraction of sporadic infections due to *L. monocytogenes* and for almost all confirmed food-borne outbreaks of listeriosis (5, 13, 21).

The genetic basis for teichoic acid glycosylation in *L. monocytogenes* and other species of *Listeria* remains poorly understood. Recently we described the serogroup 4-specific gene *gtcA*, which was essential for decoration of cell wall teichoic acids of *L. monocytogenes* serotype 4b with galactose and glucose. Mutants with insertion mutations in *gtcA* lacked galactose and had only trace levels of glucose in the teichoic acid (20). Several findings suggest that teichoic acid glycosylations may serve important ecological and virulence functions in *L. monocytogenes*: glycosylation-impaired mutants of serotype 1/2a and 4b were found to be resistant to serotype-specific phages (26; N. Promadej, F. Fiedler, and S. Kathariou, unpublished data), and *gtcA* mutants of serotype 4b are impaired in certain aspects of the host cell-pathogen interaction, including invasion of fibroblasts (Promadej et al., unpublished data) and endothelial cell activation (D. A. Drevets and S. Kathariou, unpublished data).

Genetically, the species *L. monocytogenes* appears to be partitioned in two major clonal groups, which are correlated with the flagellin (H antigen) component of the serotypic designations of Seeliger and Hoehe (23). One group includes strains of serotype 1/2a, 1/2c, 3a, and 3c, whereas the other includes serotypes 1/2b, 3b, and 4b (3, 18). The two clonal groups are characterized by nonoverlapping allelic variants in numerous genetic markers, suggesting strong linkage disequilibrium and an apparent lack of gene flow between the groups. Within *L. monocytogenes*, sequences homologous to *gtcA* were found only within serotype 4b and other serogroup 4 strains and were absent from serotypes 1/2b and 3b in the same clonal group (15), suggesting that the distribution of these sequences reflected the presence of serogroup-specific, somatic antigens.

Other *Listeria* species were found to lack sequences homologous to *gtcA*, with the notable exception of certain strains of the nonpathogenic species *Listeria innocua* (15). These unusual *L. innocua* strains were initially identified because they reacted with monoclonal antibodies (MAbs) which otherwise were specific for *L. monocytogenes* of serotypes 4b, 4d, and 4e (14). In *L. monocytogenes* serotype 4b, reactivity with these MAbs requires intact glycosylation of wall teichoic acid, i.e., the presence of galactose and glucose as substituents on the GlcNAc of the teichoic acid backbone (20).

*Listeria innocua* is the species genetically closest to *L. monocytogenes*. Even though these two species differ markedly in pathogenicity, they share the same ecological niches in the environment (including food, vegetation, and soil), and in fact *L. innocua* was not recognized as a species distinct from *L. monocytogenes* until 1981 (22). In spite of the apparent potential for genetic exchange between *L. monocytogenes* and *L. innocua*, such exchanges have not yet been documented. In terms of teichoic acid structure, it is interesting that *L. innocua* shares the teichoic acid backbone (containing integral GlcNAc) with serogroup 4 *L. monocytogenes* but typically lacks the glycosylations that characterize serotype 4b of the latter species (6). It is conceivable, therefore, that lateral transfer of the genes
TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Serotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>L. monocytogenes</td>
<td>4b1</td>
<td>20</td>
</tr>
<tr>
<td>M44 (Tn916ΔE mutant)</td>
<td>4b</td>
<td>20</td>
</tr>
<tr>
<td>4WT</td>
<td>4b</td>
<td>20</td>
</tr>
<tr>
<td>LM1320</td>
<td>4b</td>
<td>R. Kanenaka</td>
</tr>
<tr>
<td>F4242</td>
<td>1/2b</td>
<td>B. Swaminathan</td>
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<tr>
<td>99-468</td>
<td>1/2a</td>
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<td>L. innocua</td>
<td>F8596</td>
<td>L. Pine</td>
</tr>
<tr>
<td>F8596L</td>
<td>This study</td>
<td></td>
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<tr>
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<tr>
<td>14D9(pKSV7)</td>
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<td>14D9(pNP21)</td>
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<tr>
<td>SLCC3379 (type strain)</td>
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<td>46-1</td>
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<tr>
<td>K-10</td>
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<td>99-248</td>
<td>W. Lin</td>
<td></td>
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<tr>
<td>E. coli DH5α F- ΔlacZYA-argF1 lacI2ΔM15 Δ(kmR&amp;catR) supE44 thi-1 gyrA relA1</td>
<td>X. F. Gao</td>
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TABLE 2. Oligonucleotide primers used for PCR amplification of genes in the gtcA genomic region of L. monocytogenes serotype 4b and L. innocua F8596

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
<th>Accession no.</th>
</tr>
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<td>RHOST</td>
<td>5′ GAA TTC AAA GGG ACA GGC AAC AT 3′</td>
<td>3225–3247</td>
<td>AF072894</td>
</tr>
<tr>
<td>RR4</td>
<td>5′ GCT GAG TGC GCA AAT TAT TT 3′</td>
<td>4336–4355</td>
<td>AF072894</td>
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<tr>
<td>1P1ST</td>
<td>5′ CAC ATA GGA AGA AGT TAA 3′</td>
<td>3512–3529</td>
<td>AF072894</td>
</tr>
<tr>
<td>11N</td>
<td>5′ ACA CTG AGT TCA GTA CAA GC 3′</td>
<td>3916–3935</td>
<td>AF072894</td>
</tr>
<tr>
<td>11R6</td>
<td>5′ CGT GTC GGA AAT TCT TCT TCT 3′</td>
<td>4210–4229</td>
<td>AF072894</td>
</tr>
<tr>
<td>RR8</td>
<td>5′ ATC GCT TGT TTT CCG 3′</td>
<td>3387–3401</td>
<td>AF072894</td>
</tr>
<tr>
<td>2P3</td>
<td>5′ GTA ACG TCT CAT ATA TAG GGA G 3′</td>
<td>3437–3454</td>
<td>AF033015</td>
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<tr>
<td>CP1</td>
<td>5′ CAC AGA AGC GAT ACG ATG A 3′</td>
<td>3437–3456</td>
<td>AF033015</td>
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</tbody>
</table>

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used in this study are listed in Table 1. Bacteria were routinely grown in brain heart infusion (Difco) in stationary cultures at 35°C. Growth on agar was on tryptic soy agar (TSA) (Difco) supplemented with yeast extract (0.7%). Hemolytic activity was determined on TSA supplemented with 4% sheep blood (BBL). Escherichia coli was grown in Luria-Bertani broth (Difco) with ampicillin (50 μg/ml). When applicable, antibiotics used for L. innocua were streptomycin (1.200 μg/ml), erythromycin (10 μg/ml), and chloramphenicol (7 μg/ml). Antibiotics were purchased from Sigma.

Transposon mutagenesis. Strain F8596L, a spontaneously derived streptomycin-resistant derivative of L. innocua F8596, was used as a transposon Tn916ΔE recipient in filter membrane matings done as previously described (15), except that conjugations were done at 22°C. Overnight. Antibiotics used for selection and maintenance of transconjugants were streptomycin (1,200 μg/ml) and erythromycin (10 μg/ml). Single transconjugants were inoculated into individual wells of 96-well microtiter plates containing 200 μl of brain heart infusion with the antibiotics, incubated at 35°C overnight, and subsequently kept frozen at −70°C.

Screening of mutants with MAbs. MAbs C74.22, C74.33 and C74.180, which react with serotypes 4b, 4d, and 4e but not with other L. monocytogenes serotypes, have been described before (14). For colony immunoblots with these antibodies, the bacteria were grown at 22°C, transferred to nitrocellulose, and processed as described previously (15).

Biochemical analysis of cell wall composition. The cell wall composition was determined as described by Fiedler et al. (6). Trehalose acids from L. innocua F8596 and mutant 14D9 were extracted and analyzed as previously described (6, 12).

Listeria phage infection assay. Listeria species-specific phage A511 (16) was a gift from M. Loessner. Infections with this phage and determination of adsorption efficiency were done as described previously (26, 31).

DNA manipulation and analysis. Standard molecular procedures (2) were used unless otherwise indicated. Genomic DNA was extracted from L. monocytogenes and L. innocua as described previously (15). Restriction enzymes were purchased from MBI-Fermentas or from Promega. PCR employed Taq polymerase (Promega), and PCR products were purified with a Geneclean kit (Bio101). PCR to detect the listeriolysin gene lrl was done as described previously (8). Plasmids were purified with a Wizard Miniprep kit (Promega). Southern blotting was done to determine Tn916ΔE copy number and for other purposes as indicated, using previously described procedures (15) with the nonradioactive Genius digoxigenin labeling and detection system (Boehringer Mannheim). High-stringency hybridizations were done at 42°C. Nucleotide sequences were determined by automated sequencing at the University of Hawaii Biotechnology Core Facility and analyzed as described previously (20). To isolate transposon-flanking sequences and identify the transposon insertion site in mutant 14D9, we used single-specific-primer PCR (24) as previously described (20). A 0.5-kb product was amplified and cloned in the pCR2.1 vector (Invitrogen), resulting in plasmid pLI1. The cloned fragment was sequenced on both strands.

To construct probes internal to rho, we pursued the characterization of the gtcA genomic region in Listeria. The genomic region of L. innocua strains to express serotype 4b-like teichoic acids.

To better understand the distribution and evolution of teichoic acid glycosylation genes in Listeria, we pursued the characterization of the gtcA genomic region in the unusual L. innocua strains which expressed serotype 4b-like surface antigens. It is known that such L. innocua strains indeed have serotype 4b-like glycosylations in their teichoic acid and support the possibility of a relatively recent transfer of gtcA between serogroup 4 L. monocytogenes and L. innocua, at a genomically equivalent locus. In L. innocua strains which express serotype 4b-like sugar substituents in the teichoic acid, gtcA was found to be functional and essential for teichoic acid glycosylation.
Preparation of Listeria electrocompetent cells and electroporation were done as described previously (20). Transformants were selected on TSA-0.7% yeast extract plates containing chloramphenicol (7 μg/ml) for 2 to 3 days at 30°C.

**RESULTS**

**Bacteriologic and taxonomic characterization of L. innocua strains with serogroup 4-like genes and surface antigens.** Earlier studies suggested that three unusual strains of L. innocua, F8596, F7833, and F8735, reacted with MAbs (c74.22, c74.33, and c74.180) which otherwise reacted only with serotype 4b, 4d, and 4e L. monocytogenes (14). Furthermore, these L. innocua strains also harbored genomic sequences with homology to sequences which otherwise appeared to be unique to L. monocytogenes serogroup 4 (15, 20).

These strains were completely nonhemolytic, as would be expected of L. innocua. To exclude the possibility that they may represent nonhemolytic variants of L. monocytogenes, we used PCR to detect the hemolysin (listeriolysin) gene lly. Such PCRs did not yield any product, suggesting the absence of the corresponding sequences. In addition, Southern blots with an hly probe failed to yield any hybridizing bands using these strains, even under low-stringency conditions (data not shown).

To evaluate the genotypic similarity among these strains, as well as between them and other L. innocua strains, we employed REP-PCR, which is based on the distribution of a class of repetitive elements (REPs) in the genome (29). F8596, F8735, and F7833 were found to have virtually indistinguishable REP-PCR patterns (Fig. 1). The patterns were highly similar to those produced by three other L. innocua strains (including the type strain) and were distinct from the REP-PCR pattern of L. monocytogenes serotype 4b. In particular, PCR fragments of 0.6, 0.75, and 1.1 kb were prominent when the templates were genomic DNAs from F8596, F8735, and F7833, as well as from the other three L. innocua strains, but were absent when DNA from L. monocytogenes 4b was used as the template (Fig. 1). The REP-PCR data suggest that F8596, F8735, and F7833 belong to one genotypic cluster within L. innocua.

We chose L. innocua F8596 as the prototype strain for further molecular studies. 16S rDNA sequence analysis revealed that the sequences (459 and 560 bp at the 5′ and 3′ regions of the 16S rDNA sequence [accession no. AF201855 and AF201854, respectively]) had 99.8 and 100% identity with the L. innocua sequences in the database (accession no. X98527). At nucleotide positions 357, 376, and 390 (accession no. AF201854) in the 3′ portion of 16S rDNA, which appear to differentiate between L. innocua (accession no. X98527) and L. monocytogenes (accession no. X98530), the corresponding nucleotides of F8596 were identical to those of L. innocua (data not shown).

**Identification of the gtcA genomic region of L. innocua F8596.** PCR using primers from the gtcA genomic region of L. monocytogenes serotype 4b and standard conditions (annealing temperature of 48°C) did not produce a product from L. innocua F8596, even though homologous sequences were detected with Southern blots (data not shown). PCR using the same primers at a lower annealing temperature (46°C), however, produced a product of 1,083 bp from F8596, which was subsequently cloned and sequenced.

**ORF analysis.** The 1,083-bp genomic region of L. innocua F8596 had 93.9% identity to the corresponding region in L. monocytogenes 4b1. Three open reading frames (ORFs) were identified, rho, gtcA, and rpmE, in the same order as in L. monocytogenes serotype 4b (Fig. 2). On the basis of sequence similarity to other genes in the database, the partial rho gene and rpmE are expected to encode the putative transcription termination factor Rho and ribosomal protein L31, respectively, similarly to the corresponding sequences in L. monocytogenes serotype 4b (20). The available 3′ portion of rho of L. innocua F8596 had 84.6% identity over 253 bp to its counterpart in L. monocytogenes 4b1 (Fig. 2). The deduced 84-amino-acid portion of the Rho factor, however, was identical in the two strains. The gtcA coding sequence had 94.5% identity over its entire length (438 bp) to its counterpart in L. monocytogenes 4b1, and only one amino acid substitution was detected in the 17.4-kDa deduced gene product (Glu75 instead of Asp75). The rpmE coding sequence had 99.2% identity over 243 bp to its counterpart in L. monocytogenes 4b1. The deduced amino acid sequences (81 amino acids), however, diverged in the C terminus (residues 67 to 73) due to two apparent frameshift mutations which resulted in QTAVWTA in L. innocua F8596 instead of ADGRVDR in L. monocytogenes 4b1 (Fig. 3). The G+C content of gtcA in L. innocua F8596 was 30%, which is noticeably lower than the overall value of 38% for L. innocua. On the other hand, the available sequences of rho and rpmE had G+C values of 37 and 40%, respectively.

**gtcA is functional in L. innocua F8596 and is essential for teichoic acid glycosylation and MAb reactivity.** L. innocua F8596, F8537, and F7833 were first noticed because they reacted with the serotype-specific MAbs c74.22, c74.33, and c74.180 (14). In...
L. monocytogenes serotype 4b, insertional inactivation of gtcA resulted in the c74.22-negative phenotype (20). We pursued, therefore, the generation of c74.22-negative mutants of L. innocua F8596, in order to determine whether such mutants would have insertions in the gtcA gene as well and, if this was the case, in order to evaluate the possible function(s) of gtcA in this strain. Screening of about 2,200 Tn916ΔE mutants with MAb c74.22 identified six which were c74.22 negative. Of these, five (1F3, 4E6, 5F1, 12G7, and 14D9) were found to have insertions in the gtcA region, using a gtcA probe in Southern blotting. Southern blotting using the transposon probe revealed the presence of a single copy number of Tn916ΔE in mutants 4E6, 5F1, and 14D9 (data not shown).

The single-copy mutant 14D9 was chosen for further studies. Sequence analysis of the transposon-flanking region revealed that the insertion was inside the coding region of gtcA, between nucleotides 484 and 485 (accession no. AF160251). The transposon target sequence, TTTTCTAATAAAAA, was the same as that targeted in other Tn916 and Tn916ΔE mutants of L. monocytogenes 4b1 (20) and were similar to the Tn916 preferred target sites reported for other gram-positive bacteria (17).

Biochemical analysis of the teichoic acid composition of 14D9 was pursued to determine whether the insertion affected the teichoic acid components. The wild-type parental strain, L. innocua F8596, was found to be indistinguishable from F8596 (accession no. AF072894), L. monocytogenes (B. burg) (accession no. AE001133), and Bacillus subtilis (B. sub) (accession no. X73124).

L. monocytogenes 4b1 was used as that targeted in other Tn916ΔE mutants of L. monocytogenes 4b1 (20) and were similar to the Tn916 preferred target sites reported for other gram-positive bacteria (17).

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FIG. 2. Characterization of ORFs in the gtcA genomic regions of L. monocytogenes 4b1 (serotype 4b) (accession no. AF072894) and L. innocua F8596 (accession no. AF160251).

FIG. 3. Multiple-sequence alignment (CLUSTAL) of the deduced sequences of ribosomal protein L31 of L. innocua (L. inno) F8596 (accession no. AF160251), L. monocytogenes (L. mono) 4b1 (accession no. AF072894), Borrelia burgdorferi (B. burg) (accession no. AE001133), and Bacillus subtilis (B. sub) (accession no. X73124).
5). Since the latter strains appeared to belong to a REP-PCR were found to harbor strains (121E9 and 99-248), which were negative with c74.22, and F7833) that reacted with MAb c74.22, two additional Interestingly, in addition to the three strains (F8596, F8735, and F7833) that yielded a signal were 121E9 (lane 8) and 99-248 (lane 11). The rather weak signal in lane 6 reflected relative small amounts of DNA.

c74.22 was partially restored in the presence of pNP21, whereas constructs with the vector pKSV7 alone remained negative (data not shown). Furthermore, the complemented strain recovered sensitivity to phage A511, whereas the constructs with the vector pKSV7 alone remained negative with c74.22 (data not shown), suggesting that gtcA alone is not sufficient for expression of the serotype-specific surface antigen and reactivity with these antibodies. In earlier studies we identified an- possible reasons for the c74.22-negative phenotype of strains 121E9 and 99-248 may be that gtcA is not functional in these strains or, alternatively, that additional genes may be required for expression of the teichoic acid-associated surface antigens recognized by c74.22. Introduction of the gtcA gene of L. monocytogenes serotype 4b into these strains in trans on plasmid pKSV7 failed to render them positive with c74.22 (data not shown), suggesting that gtcA gene of L. monocytogenes serotype 4b can complement in trans the mutant phenotypes conferred by inactivation of gtcA in L. innocua F8596.

Identification of gtcA sequences in c74.22-negative L. innocua. A probe derived from gtcA of L. innocua F8596 was used in Southern blots of a panel of 13 L. innocua strains. Interestingly, in addition to the three strains (F8596, F8735, and F7833) that reacted with MAb c74.22, two additional strains (121E9 and 99-248), which were negative with c74.22, were found to harbor gtcA homologues in their genomes (Fig. 5). Since the latter strains appeared to belong to a REP-PCR genotypic cluster separate from that of the c74.22-positive L. innocua strains (Fig. 1), we can conclude that at least two separate L. innocua lineages harbor gtcA homologues, even though only one (comprising F8596, F8735, and F7833) expresses the c74.22-specific surface antigen. In strains such as 121E9 and 99-248, gtcA was cryptic, not being associated with a known phenotype.

TABLE 3. Phage A511 adsorption deficiency of mutant 14D9

<table>
<thead>
<tr>
<th>Organism</th>
<th>Phage A511 adsorption (PFU ml⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>L. monocytogenes 4b1</td>
<td>3.7 × 10⁴</td>
</tr>
<tr>
<td>F8596</td>
<td>3.0 × 10⁵</td>
</tr>
<tr>
<td>14D9</td>
<td>8.4 × 10⁵</td>
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<td>14D9(pKSV7)</td>
<td>7.9 × 10⁵</td>
</tr>
<tr>
<td>14D9(pNP21)</td>
<td>1.5 × 10⁵</td>
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</table>

*a Adsorption of phage A511 was measured by determining the number of PFU remaining in the supernatant of a mixture containing the phage A511 (8.6 × 10⁶ PFU) and the indicated strain (ca. 1 × 10⁴ CFU), as described in the text. 14D9(pKSV7) and 14D9(pNP21) are the mutant 14D9 harboring the cloning vector pKSV7 and a recombinant pKSV7 with the gtcA gene of L. monocytogenes 4b1, respectively. Results are averages from two experiments.
with a probe derived from locus II revealed that only F8596, F8735, and F7833 harbored homologous sequences (Fig. 6). All other strains, including the C47.22-negative strains that harbored gtcA homologues, were negative with the locus II probe (Fig. 6). These results suggest that expression of serotype 4b-like surface antigens is a unique property of a unique L. innocua lineage that harbors gtcA as well as at least one additional serogroup 4-specific locus.

**gtcA of L. innocua** F8596 represents a monocistronic serogroup 4-specific cassette. DNA sequence analysis of the available rho sequence upstream of gtcA in L. innocua F8596 suggested that this sequence had diverged substantially (84.6% identity) from its counterpart in L. monocytogenes serotype 4b, in contrast to the observed conservation of gtcA (94.5% identity). To determine whether the rho sequence of F8596 represented a serotype 4b-like sequence that had undergone divergence or, alternatively, was typical of the rho sequences endemic in L. innocua, Southern blotting of a panel of L. innocua strains was done using the F8596 rho portion as a probe. The probe did not produce detectable signals with L. monocytogenes serotype 4b in high-stringency hybridizations (Fig. 7), as expected on the basis of the sequence divergence between the two rho sequences (84.6% identity). L. monocytogenes serotype 1/2a also failed to yield a signal with the rho probe from L. innocua F8596. In contrast, all screened L. innocua strains harbored homologues to the F8596 rho (Fig. 7), suggesting that in F8596, gtcA is flanked upstream by typical L. innocua sequences.

The gene rpmE, immediately downstream of gtcA, was highly conserved among different L. monocytogenes serotypes (20) as well between L. monocytogenes serotype 4b and L. innocua F8596 (99% identity). A probe specific to rpmE hybridized with all screened L. innocua and L. monocytogenes strains (data not shown). Thus, the combined Southern blot and nucleotide sequence data suggest that in L. innocua F8596, gtcA represents a monocistronic cassette that is serogroup 4 specific and is flanked by sequences not specific or unique to serogroup 4.

**DISCUSSION**

L. innocua is genetically and bacteriologically the species closest to L. monocytogenes, but it is notably nonpathogenic to humans and other animals. Horizontal transfer of genetic determinants between the two species would be expected to take place and could be of special interest in terms of elucidating the evolution of pathogenicity and virulence in *Listeria*. However, to date evidence for such transfer has been lacking. The results described in this work can be best explained as the outcome of horizontal transfer of a serotype-specific gene cassette between one lineage of *L. monocytogenes* (serogroup 4) and a lineage of *L. innocua*, which we designate lineage I and which includes strains F8596, F7833, and F8735. The strong conservation of the gtcA genes in *L. monocytogenes* serotype 4b and *L. innocua* F8596 (94.5% identity) suggests a relatively recent transfer of gtcA sequences between *L. monocytogenes* serotype 4b and *L. innocua* lineage I. Since all screened strains of *L. monocytogenes* serogroup 4 harbor the gene, whereas only a subpopulation of *L. innocua* does so, the direction of transfer would be more likely to have been from *L. monocytogenes* serogroup 4 to *L. innocua* than vice versa.

An alternative hypothesis, that gtcA was present in a common *L. monocytogenes*-L. *innocua* ancestor and was subsequently maintained only in selected lineages, is less likely, as nucleotide sequence divergence would be expected to be substantially higher under such conditions. The same reason renders less likely the hypothesis that gtcA was transferred independently, from a common source, to *L. monocytogenes* serogroup 4 and to *L. innocua* lineage I, unless one also presumes relatively recent transfers to multiple serotypes (4a, 4b, 4c, 4d, and 4e) as well as to *L. innocua* lineage I.

Recent data from our laboratory revealed that within *L. monocytogenes*, strains other than those of serogroup 4 harbor apparent gtcA alleles, although the genetic divergence of these alleles from the serotype 4b sequences is significant (79 to 80% in serotypes 1/2a and 1/2b) (Z. Lan and S. Kathariou, unpublished data). Such data suggest that these gtcA alleles may be of different origin than the sequences detected in serogroup 4 and *L. innocua* lineage I. We presently do not know the ultimate origin(s) of gtcA sequences in *Listeria*. The relatively low G+C content of the sequences (20; Lan and Kathariou, unpublished data), which differs from that characteristic of the overall *Listeria* genome, may be indicative of a
nonlisterial origin(s), as speculated for surface polymer glycosylation genes of other bacteria as well (1).

On the basis of present data it is not clear what the possible advantage(s) of the acquisition of gtcA may be for L. innocua. Sugar substituents on the teichoic acid have been shown to be essential for phage adsorption in L. monocytogenes (26, 30; Promadej et al., unpublished data), and the serotype 4b-like teichoic acid of L. innocua lineage I may confer some yet-unidentified selective advantages to the microorganism in terms of phage infection. Teichoic acid glycosylation may also affect other surface-related attributes of the microorganism (e.g., attachment to surfaces and biofilm formation) and other aspects of the adaptive physiology of the bacteria, especially under conditions of environmental stress.

Although gtcA was found to be essential for glycosylation of the serotype 4b-like teichoic acid of L. innocua F8596, it is likely not acting alone. This is suggested by the identification of strains of L. innocua which harbored cryptic gtcA sequences and lacked the serotype 4b-like glycosylation. Furthermore, unlike all other L. innocua strains which we examined, lineage I harbors additional serotype-specific sequences (locus II) that are otherwise harbored only by L. monocytogenes serotypes 4b, 4d, and 4e (15). It is reasonable to postulate, therefore, that L. innocua strains with serotype 4b-like teichoic acid glycosylation have acquired not only gtcA but additional sequences (locus II and possibly others, yet unidentified) from L. monocytogenes serogroup 4.

Our molecular data indicated that in lineage I of L. innocua and in L. monocytogenes serotype 4b, gtcA is integrated as a monocistronic gene cassette in the rho-rpmE region of the genome. One may speculate that the rpmE locus, which is highly conserved between L. monocytogenes and L. innocua, may have served as a target for a recombination system, perhaps phage mediated, that resulted in the integration of gtcA in this region. The sequence information presently available, however, does not provide evidence for phage involvement in the introduction of gtcA, and such ideas remain speculative.

L. monocytogenes serotype 4b may have special pathogenesis-related features, being responsible for the majority of outbreaks of listeriosis (as well as a large fraction of sporadic cases). In many bacterial pathogens, surface carbohydrates play critical roles in host cell recognition and adhesion, and in fact surface galactose has been shown to serve as a ligand for interactions of L. monocytogenes with certain host cells (4, 9; Promadej et al., unpublished data).

Although the unique L. innocua lineage described here possesses serotype 4b-like sugars on the teichoic acid moietly, it would still be expected to be nonpathogenic, since the virulence-essential hemolysin (listeriolysin) gene hly (19) appears to be absent. Nonetheless, these strains are of special interest in terms of the evolution of listerial pathogenesis. For instance, their serotype 4b-like teichoic acid determinants can serve as receptors for transducing serotype 4b-specific phages, examples of which have been recently described (10). The sugars on the teichoic acid moiety are essential for phage adsorption of serotype-specific phages of L. monocytogenes (26, 30; Promadej et al., unpublished data). Strains such as those of lineage I may represent an early step in the emergence of novel pathogenic lineages of Listeria, as in the course of time and under appropriate selection regimes, virulence genes from L. monocytogenes may be transferred (e.g., by transduction) and stabilized into the genomes of initially nonpathogenic strains.

In the past 15 years, extensive work has been performed on the genetic and cell biologic aspects of listerial pathogenesis (13, 19). In contrast, mechanisms underlying the evolution of virulence in this genus, which contains both pathogenic and nonpathogenic species and is widely encompassed in the environment, have not been investigated. Recently, the European Commission funded the complete sequencing of the genomes of L. monocytogenes (strain EGD of serotype 1/2a) and of L. innocua, and the projects are now complete, although the sequences have not yet been released (http://www.pasteur.fr/recherche/unites/gmp/Gmp_projects.html#lm/). The availability of these genome sequencing data to the international scientific community will promote the establishment of novel approaches to the study of the evolution of virulence genes and biologic studies of relevant model systems, such as the L. innocua lineage described here, are expected to complement such evolutionary investigations.

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