JOURNAL OF BACTERIOLOGY, Nov. 2000, p. 6161–6168 Vol. 182, No. 21
0021-9193/00/$04.00+0

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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Received 17 May 2000/Returned for modification 22 June 2000/Accepted 1 August 2000

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 insertional 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 Hoehne (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).

L. 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

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TABLE 1. Bacterial strains used in this study

Species and strain	Serotype	Source or reference
L. monocytogenes		
4b1	4b	20
M44 (Tn*916*Δ*E* mutant)	4b	20
4WT	4b	20
LM1320	4b	R. Kanenaka
F4242	1/2b	B. Swaminathan
99-468	1/2a	R. Kanenaka
L. innocua		
F8596		L. Pine
F8596L		This study
1F3 (Tn*916*Δ*E* mutant)		This study
4E6 (Tn*916*Δ*E* mutant)		This study
5F1 (Tn*916*Δ*E* mutant)		This study
12G7 (Tn*916*Δ*E* mutant)		This study
14D9 (Tn*916*Δ*E* mutant)		This study
14D9(pKSV7)		This study
14D9(pNP21)		This study
F7833		L. Pine
F8735		L. Pine
121E9		R. Kanenaka
120A1		R. Kanenaka
SLCC3379 (type strain)	6a	H. Hof
30-1		R. Kanenaka
40-1		R. Kanenaka
44-1		R. Kanenaka
45-1		R. Kanenaka
46-1		R. Kanenaka
K-10		R. Kanenaka
99-248		W. Lin
E. coli DH5α F^- ϕ180d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA relA1*		X. F. Gao

conferring these teichoic acid glycosylations may allow *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. Results described in this communication indicate 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.

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 *Listeria* 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 Tn*916*Δ*E* 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). Teichoic acids from *L. innocua* F8596 and mutant 14D9 were extracted and analyzed as previously described (6, 12).

***Listeria* phage infection assay.** *Listeria* genus-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 *hly* was done as described previously (8). Plasmids were purified with a Wizard Miniprep kit (Promega). Southern blotting was done to determine Tn*916*Δ*E* 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 used as a digoxigenin-labeled probe in Southern blots to confirm its location in the transposon-flanking region and was sequenced.

Primers used to amplify different genomic regions of *L. innocua* F8596 are listed in Table 2. To identify *gtcA* and the flanking genomic sequences of *L. innocua* F8596, we used two primers (RHOST and RR4) from the *gtcA* genomic region of *L. monocytogenes* 4b1, with the annealing temperature set at 46°C. A product of about 1.1 kb was amplified and cloned in pCR2.1, resulting in plasmid pLI2. The cloned fragment was sequenced on both strands.

To construct probes internal to *rho*, *gtcA*, *rpmE*, and locus II of *L. innocua* F8596, we used the primer pairs (M13F and RR8, 1P1ST and 11N, 11R6 and RR4, and 2P3 and CP1, respectively) listed in Table 2. The template was genomic DNA of *L. innocua* F8596, with the exception of the *rho* PCR fragment, which was obtained using pLI2 as the template.

Complementation of the mutant in *trans*. The *gtcA*-containing shuttle plasmid pNP21 (20) was used for genetic complementation of mutant 14D9 in *trans*.

TABLE 2. Oligonucleotide primers used for PCR amplification of genes in the *gtcA* genomic region of *L. monocytogenes* serotype 4b and *L. innocua* F8596

Primer	Sequence	Position	Accession no.
RHOST	5′ GAA TTC AAA GGG ACA GGC AAC AT 3′	3225–3247	AF072894
RR4	5′ GCT GAG TGC GCA AAT TAT TT 3′	4336–4355	AF072894
1P1ST	5′ CAC ATA GAA AGA AGT TAT 3′	3512–3529	AF072894
11N	5′ ACA CGT AGT TCA GTA CAA GC 3′	3916–3935	AF072894
11R6	5′ CGT GTC GAA ATC TCT TCT GA 3′	4210–4229	AF072894
RR8	5′ ATC GCT TTG TTT CGG 3′	3387–3401	AF072894
2P3	5′ GTA ACG TCT CAT ATA TAG GGA G 3′	3434–3454	AF033015
CP1	5′ CAC AGA AGC GAT ACG ATG A 3′	4347–4365	AF033015

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.

Strain typing by REP-PCR and analysis of 16S rDNA. Repetitive extragenic palindrome (REP)-based PCR (REP-PCR) was done using freshly extracted genomic DNA as the template and the REP primers and PCR conditions described by Jeršek et al. (11). PCR products were visualized following electrophoresis in an 18-cm gel. To determine 16S ribosomal DNA (rDNA) sequences of *L. innocua* F8596, we used primers BACT8-27F (5′ AGA GTT TGA TCM TGG CTT AG 3′) and 1510-1492R (5′ RGY TAC CTT GTT ACG ACT T 3′), corresponding to nucleotides 8 to 27 and 1492 to 1510, respectively, in the *E. coli* 16S rDNA sequence. The resulting 1.5-kb PCR product was recovered from the gel, purified with the Geneclean kit and used for nucleotide sequence determinations.

Nucleotide sequence accession numbers. The nucleotide sequence data for the 16S rDNA 5′ region, 16S rDNA 3′ region, and *gtcA* genomic region of *L. innocua* F8596 have been deposited in the GenBank database under accession no. AF201855, AF201854, and AF160251, respectively.

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 *hly*. 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.

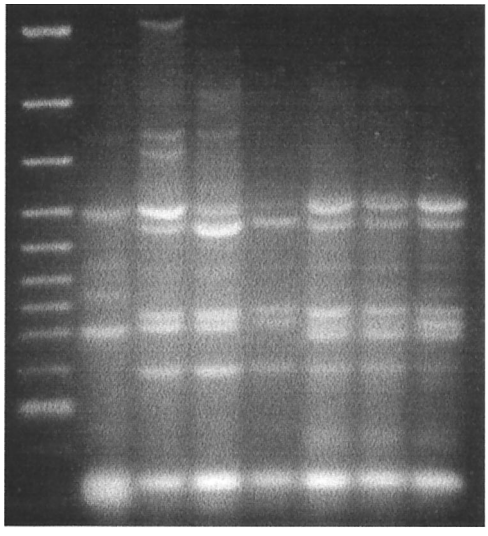

FIG. 1. REP-PCR profiles of *Listeria* spp. Lane M, molecular marker (fragment sizes are, from top to bottom, 3.0, 2.0, 1.5, 1.2, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, and 0.4 kb); lane 1: *L. monocytogenes* 4b1; lane 2, *L. innocua* SLCC3379 (type strain); lane 3, *L. innocua* 121E9; lane 4, *L. innocua* 99-248; lane 5, *L. innocua* F7833; lane 6, *L. innocua* F8596; lane 7, *L. innocua* F8735. REP-PCR was done as described in Materials and Methods.

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* (partial sequence), *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 deducted 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

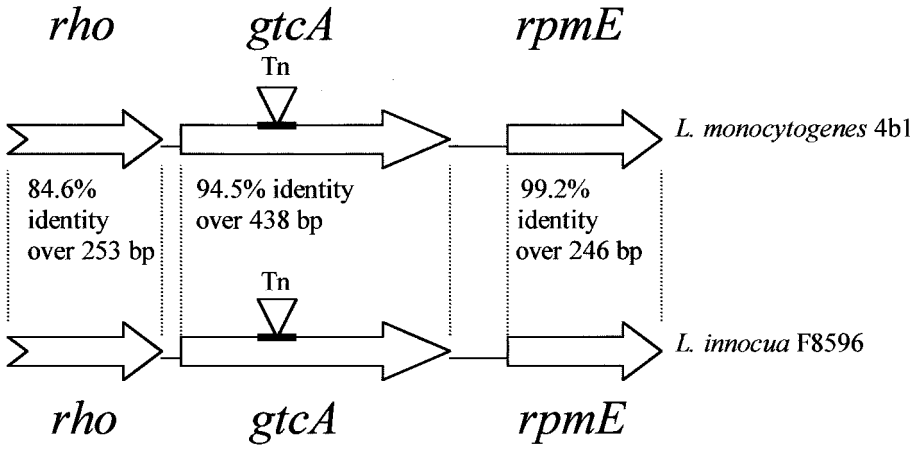

FIG. 2. Characterization of ORFs in the *gtcA* genomic regions of *L. monocytogenes* 4b1 (serotype 4b) (accession no. AF072894) and *L. innocua* F8596 (accession no. AF160251).

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 Tn*916ΔE* 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 Tn*916ΔE* 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 Tn*916* and Tn*916ΔE* mutants of *L. monocytogenes* 4b1 (20) and were similar to the Tn*916* 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 *L. monocytogenes* serotype 4b in regard to teichoic acid composition (Fig. 4). Identical data (not shown) were obtained with F7833 and F8735. GlcNAc was present in the teichoic acid backbone, with galactose and glucose substituents, unlike the case for typical *L. innocua* strains, which have GlcNAc in the teichoic acid backbone but lack the simultaneous presence of both galactose and glucose (6). Inactivation of *gtcA* in mutant 14D9 was accompanied by marked teichoic acid glycosylation defects, identical to those observed in previously characterized *gtcA* mutants of *L. monocytogenes* serotype 4b (20). Galactose could not be detected in the teichoic acid of 14D9, and glucose was markedly reduced in amount. GlcNAc and other teichoic acid components were not affected (Fig. 4). Peptidoglycan appeared to be normal (data not shown).

In strain F8596, *gtcA* is essential for phage A511 sensitivity. Screening of mutant 14D9 with the *Listeria*-specific phage A511 showed that the mutant was resistant to phage infection. The efficiency of A511 plaque formation by 14D9 was less than 1.5×10^{-3} of that obtained with wild-type bacteria. The resistance of 14D9 to A511 may be due to failure of the phage to adsorb, since adsorption efficiency was reduced 28-fold (Table 3). The resistance of 14D9 to phage A511 was unexpected, as this phage appears to utilize peptidoglycan as a receptor (30). In addition, this phage infects listeriae of different species and serotypes (16). Since peptidoglycan was not affected in 14D9, we may conclude that teichoic acid glycosylation is required for proper exposure or conformation of the receptor determinants on the peptidoglycan. Interestingly, *gtcA* mutants of *L. monocytogenes* serotype 4b were also A511 resistant (the efficiency of plaque formation was less than 10^{-3} of that obtained with the wild type), suggesting that glycosylation of serotype 4b-like teichoic acids is essential for infection by the *Listeria* genus-specific phage A511.

***gtcA* of *L. monocytogenes* serotype 4b can complement the mutant phenotypes of the *L. innocua* F8596 *gtcA* mutant.** Plasmid pNP21, which harbors *gtcA* of *L. monocytogenes* serotype 4b on the shuttle vector pKSV7 (20), and pKSV7 alone were electroporated into mutant 14D9. The resulting strains were grown in the presence of chloramphenicol at 30°C, a temperature which permits both replication of the temperature-sensitive plasmid (25) and optimal expression of the serotype 4b-specific surface antigens (14). Reactivity of the mutant with

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L.inno_L31      1 MKTGIHPEYRPVVFVDTSTDFKFLSGSTKSSSETIKWEDGNEYPLLRVEISSDSHPFYTG
L.mono_L31      1 MKTGIHPEYRPVVFVDTSTDFKFLSGSTKSSSETIKWEDGNEYPLLRVEISSDSHPFYTG
B.burg_L31      1 MRKDIHPKNNLVVFKDGSNGAMFLTKSTLNSKETIKYIDGKEYPLVTVEITSKSHPFYTG
B.sub_L31       1 MKAGIHP-----------N----FKKATVKCACGNEFETGSVKEEVRVEICSECHPFYTG

L.inno_L31     61 KQKHATADGRVDRFNKKYGLK
L.mono_L31     61 KQKHATQTAVWTAFNKKYGLK
B.burg_L31     61 QQKFVDAAGRIDKFNKRYKKS
B.sub_L31      46 RQKFASADGRVDRFNKKYGLK
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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).

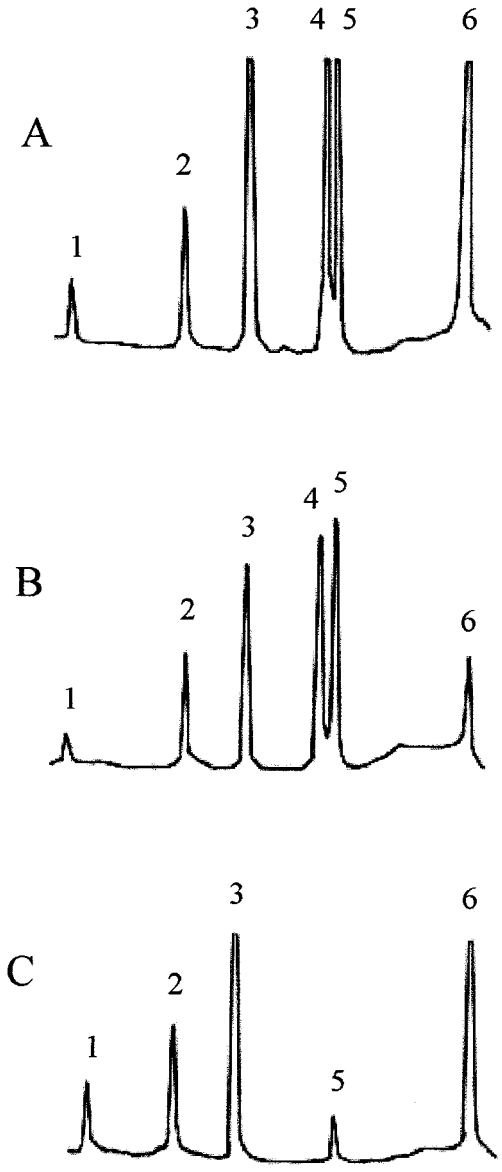

FIG. 4. Teichoic acid compositions of *L. monocytogenes* serotype 4b strain 4WT (A), *L. innocua* F8596 (B), and mutant 14D9 (C). Peaks: 1, glycerol; 2, anhydroribitol; 3, ribitol; 4, galactose; 5, glucose; 6, glucosamine. Teichoic acids were prepared and analyzed as described previously (6, 12, 27). The teichoic acid composition of strains F7833 and F8735 was identical to that of F8596.

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 were still phage resistant (Table 3). These findings suggest that the *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

FIG. 5. Southern blot of *Eco*RI-digested genomic DNAs from different *L. innocua* and *L. monocytogenes* strains with a *gtcA* probe from *L. innocua* F8596. Lane 1, λ molecular size markers (fragment sizes are, from the top to bottom, 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb); lanes 2 to 4, *L. monocytogenes* strains F4242 (serotype 1/2b), 4b1 (serotype 4b), and LM1320 (serotype 4b), respectively; lanes 5 to 17, *L. innocua* strains F8596, F8735, F7833, 121E9, 120A1, SLCC3379, 99-248, 30-1, 40-1, 44-1, 45-1, 46-1, and K-10, respectively. With the exception of F8596, F8735, and F7833 (lanes 5 to 7), the only other *L. innocua* strains 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.

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.

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* alone is not sufficient for expression of the serotype-specific surface antigen and reactivity with these antibodies. In earlier studies we identified another genomic region (locus II) of *L. monocytogenes* serotype 4b, which was found to be specific to serotype 4b-4d-4e as well as to F8596, F8735, and F7833 (15). Probing the same panel of *L. innocua* strains used for the Southern blot shown in Fig. 5

TABLE 3. Phage A511 adsorption deficiency of mutant 14D9

Organism	Phage A511 adsorption (PFU ml^{-1})[a]
L. monocytogenes 4b1	3.7×10^4
L. innocua	
F8596	3.0×10^5
14D9	8.4×10^6
14D9(pKSV7)	7.9×10^6
14D9(pNP21)	1.5×10^5

[a] Adsorption of phage A511 was measured by determining the number of PFU remaining in the supernatant of a mixture containing the phage A511 (8.6×10^6 PFU) and the indicated strain (ca. 1×10^8 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.

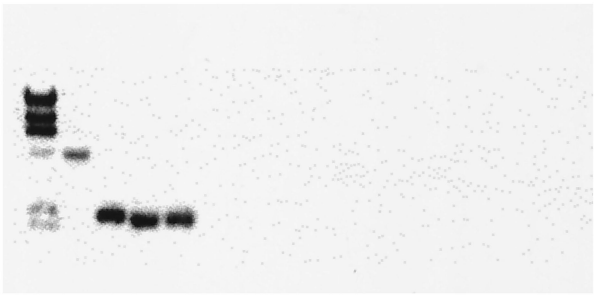

FIG. 6. Southern blot of *Eco*RI-digested genomic DNAs from different *L. innocua* and *L. monocytogenes* strains with a probe derived from locus II of *L. innocua* F8596. Lane 1, λ molecular size markers, as described in the legend for Fig. 4; lane 2, *L. monocytogenes* LM1320 (serotype 4b); lanes 3 to 15, *L. innocua* strain F8596, F8735, F7833, 121E9, 120A1, 44-1, SLCC3379, 99-248, 30-1, 40-1, 45-1, 46-1, and K-10, respectively. Of the *L. innocua* strains, only F8596, F8735, and F7833 (lanes 3 to 5) produced signals. The hybridizing band of *L. monocytogenes* serotype 4b is larger than those of F8596, F8735, and F7833. The probe was derived from PCR using F8596 genomic DNA as the template and primers 2P3 and CP1, as shown in Table 2 (accession no. AF033015).

with a probe derived from locus II revealed that only F8596, F8735, and F7833 harbored homologous sequences (Fig. 6). All other strains, including the c74.22-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

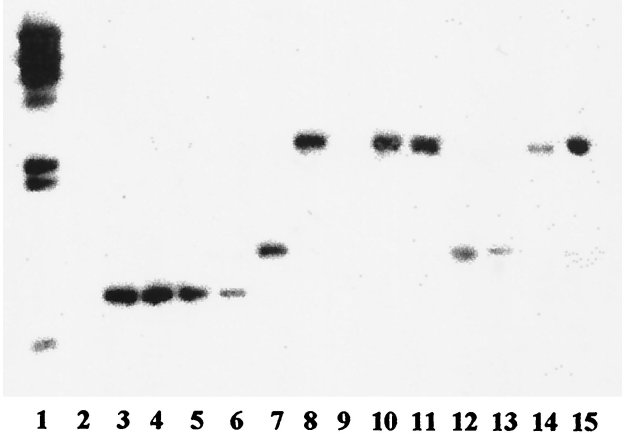

FIG. 7. Southern blot of *Eco*RI-digested genomic DNAs from different *L. innocua* and *L. monocytogenes* strains with a *rho* probe from *L. innocua* F8596. Lane 1, λ molecular size markers, as described for Fig. 4; Lane 2, *L. monocytogenes* 4b1 (serotype 4b); lanes 3 to 8, *L. innocua* strain F8596, F8735, F7833, 121E9, 120A1, and SLCC3379, respectively; lane 9, *L. monocytogenes* 99-468 (serotype 1/2a), lanes 10 to 15, *L. innocua* strains 30-1, 40-1, 44-1, 45-1, 46-1, and K-10, respectively. The *L. innocua* F8596 *rho* probe hybridizes with all other *L. innocua* strains but not with *L. monocytogenes*. Three distinct *Eco*RI restriction fragment length polymorphisms are seen within *L. innocua* with this probe.

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 adherence, 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 moiety, 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 encountered 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 in *Listeria*. Genetic and bacteriologic studies of relevant model systems, such as the *L. innocua* lineage described here, are expected to complement such evolutionary investigations.

ACKNOWLEDGMENTS

This work was partially supported by U.S. Department of Agriculture Competitive Research Initiative AAFS grant 99-35201-8183 and by ILSI-North America.

We thank M. Loessner (Technical University of Munich, Munich, Germany) for providing phage A511 and all of the investigators for providing bacterial strains as indicated in Table 1. We thank Ella Meleshkevitch for REP-PCR. We thank Nattawan Promadej, Xiang-He Lei, Edward Lanwermeyer, and all other members of our laboratories for valuable feedback and support throughout the course of this work.

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