Cell Wall Teichoic Acid Glycosylation in *Listeria monocytogenes* Serotype 4b Requires *gtcA*, a Novel, Serogroup-Specific Gene

N. PROMADEJ,[1]† F. FIEDLER,[2] P. COSSART,[3] S. DRAMSI,[3] AND S. KATHARIOU[2]*

Department of Microbiology, University of Hawaii, Honolulu, Hawaii 96822[1]; Institute for Genetics and Microbiology, University of Munich, Munich, Germany[2]; and Pasteur Institute, Paris, France[3]

We have identified a novel gene, *gtcA*, involved in the decoration of cell wall teichoic acid of *Listeria monocytogenes* serotype 4b with galactose and glucose. Insertional inactivation of *gtcA* brought about loss of reactivity with the serotype 4b-specific monoclonal antibody c74.22 and was accompanied by a complete lack of galactose and a marked reduction in the amounts of glucose on teichoic acid. Interestingly, the composition of membrane-associated lipoteichoic acid was not affected. Complementation of the mutants with the cloned *gtcA* in *trans* restored galactose and glucose on teichoic acid to wild-type levels. The complemented strains also recovered reactivity with c74.22. Within *L. monocytogenes*, sequences homologous to *gtcA* were found in all serogroup 4 isolates but not in strains of any other serotypes. In serotype 4b, *gtcA* appears to be the first member of a bicistronic operon which includes a gene with homology to *Bacillus subtilis rpmE*, encoding ribosomal protein L31. In contrast to *gtcA*, the latter gene appears conserved among all screened serotypes of *L. monocytogenes*.

Listeria monocytogenes is a gram-positive, facultative intracellular pathogen that is widespread in the environment and capable of causing severe infections in humans and animals. Populations at risk include pregnant women, newborns, and immunocompromised individuals, and infections are commonly food-borne. Although several serotypes of *L. monocytogenes* are present in the environment and in foods, the majority of infections are caused by strains of serogroup 1/2 (mostly serotypes 1/2a and 1/2b) and 4 (almost always serotype 4b) (6, 35). Both flagellar and somatic (mostly carbohydrate) surface antigens contribute to the serotypic designation of the organism (36).

As for many other gram-positive bacteria, the cell wall of *L. monocytogenes* contains large amounts of the anionic polymer teichoic acid (TA), covalently linked to peptidoglycan. In *Bacillus subtilis*, TAs have been shown to be essential for viability (22, 28). The TA of *B. subtilis* 168 consists of polyglycerol phosphate, in contrast to polyribitol phosphate in *B. subtilis* W23 (1, 5, 28). In *L. monocytogenes*, a polyribitol phosphate type of TA appears to be the prevalent accessory cell wall polymer (7, 14, 40). Pronounced diversity in TA structure and antigenicity is conferred by glycosidic substitution(s) of the ribitol phosphate units (7, 10, 40). TA of strains of serogroup 1/2 and 3 consists of polyribitol phosphate, with *N*-acetylglucosamine (Glc-NAc) and rhamnose (in the case of serogroup 1/2) substituents on ribitol (Fig. 1). In contrast, in serogroup 4 strains, Glc-NAc is incorporated in the TA chains, and, depending on the serotype (4a, 4b, etc.), bears galactose and/or glucose substituents. Serotype 4b strains are unique in bearing both galactose and glucose substituents on the Glc-NAc of TA (Fig. 1).

Genetic studies of TA biosynthesis in gram-positive bacteria have mostly involved *B. subtilis* (19, 28). Although several structural and immunochemical studies of TA in cell walls of different *L. monocytogenes* serotypes have been performed (7, 10, 14, 39), the molecular genetic mechanisms underlying TA biosynthesis and diversity in *Listeria* remain to be elucidated. In this communication, we describe *gtcA*, a novel, serogroup 4-specific gene, which is essential for the presence of normal amounts of galactose and glucose on the TA of *L. monocytogenes* serotype 4b.

MATERIALS AND METHODS

Bacterial strains and growth media. Bacteria were grown in brain heart infusion broth (BHI; Difco) at the indicated temperature as previously described (16). Growth on agar was on tryptic soy agar (Difco) supplemented with yeast extract (0.7%) (TSA-YE). All *Listeria* strains were preserved in BHI at −70°C. When appropriate, antibiotics used for *Listeria* were streptomycin (1,200 μg/ml), erythromycin (10 μg/ml), tetracycline (10 μg/ml), and chloramphenicol (7 μg/ml). Antibiotics used for *Escherichia coli* were ampicillin (50 μg/ml) and kanamycin (50 μg/ml). Antibiotics were purchased from Sigma.

Generation and screening of mutants. Transposon mutants of the serotype 4b strains 4b1 and 2381L were produced as described previously (15) by using filter-mating conjugations with *Enterococcus faecalis* CG110 or *E. faecalis* RH110, donors of Tn*916* and Tn*916*ΔE, respectively. Strains 4b1 and 2381L were streptomycin-resistant derivatives of NCTC 10527 (sporadic case isolate) and of strain F2381 (implicated in the Jalisco cheese outbreak), respectively (44). The mutants were grown in 96-well microtiter plates with the appropriate antibiotics (streptomycin-tetracycline and streptomycin-erythromycin for Tn*916* and Tn*916*ΔE mutants, respectively). The mutant banks were kept frozen at −70°C.

Monoclonal antibodies (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 (16). For colony immunoblots with these MAbs, the bacteria were grown at 22°C, transferred to nitrocellulose, and processed as described previously (20). An enzyme-linked immunosorbent assay (ELISA) using these MAbs was done as described before (16). Mutant M44 was derived from an earlier Tn*916*ΔE mutant bank of strain 4b1. Its lack of reactivity with c74.22 and reactivity with c74.33 and c74.180 have been described (16).

Biochemical analysis of cell wall composition. Cell wall composition was determined as described by Fiedler et al. (7). Preparation and analysis of TA and lipoteichoic acid (LTA) from *Listeria* were done as previously described (7, 8, 14).

Isolation of transposon-flanking sequences in the mutants. Procedures for extraction of plasmid DNA from *E. coli* and genomic DNA from listeriae were previously described (20). Unless otherwise indicated, restriction enzymes and other molecular biologic reagents were purchased from Promega (Madison, Wis.).

The thermostable polymerase used was Tfl (Epicenter, Madison, Wis.). The

* Corresponding author. Mailing address: Department of Microbiology, University of Hawaii, 2538 The Mall, Snyder 207, Honolulu, HI 96822. Phone: (808) 956-8015. Fax: (808) 956-5339. E-mail: ksophia@hawaii.edu.

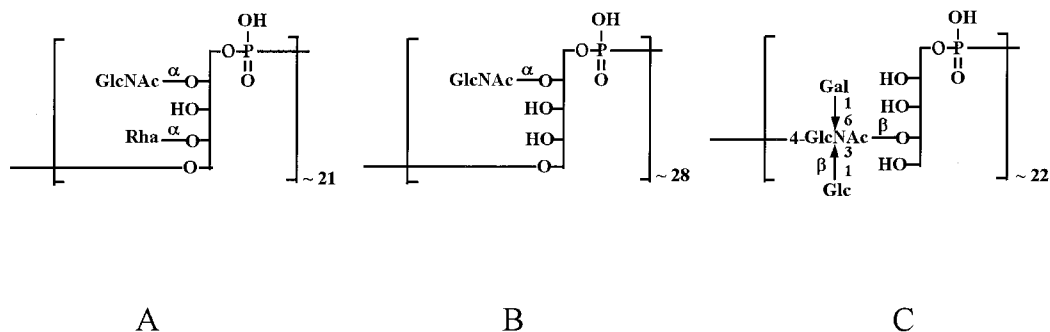† Present address: Centers for Disease Control and Prevention, Atlanta, Ga.

FIG. 1. Diagrammatic representation of TA composition in different *L. monocytogenes* serotypes. (A) Type 1/2 (serotypes 1/2a, 1/2b, and 1/2c); (B) type 3 (serotypes 3a, 3b, and 3c); (C) serotype 4b. Modified from the work of Uchikawa et al. (40).

transposon copy number in the mutants was determined by using Southern blots as previously described (15, 20) with the Genius digoxigenin labeling and detection system (Boehringer-Mannheim). The single-copy transposon mutant M44 was used to amplify a transposon-flanking fragment by means of the single specific primer PCR (SSP-PCR) (37) using *Eco*RI-digested M44 DNA and pUC18, which was digested by *Eco*RI and dephosphorylated (Pharmacia) as described previously (43). A 500-bp product was amplified, cloned in pCR1000 (Invitrogen), and sequenced. Primers internal to this fragment (PALN, 5′ TGG GTT ACT ACA AGA AGA G 3′, and PALT, 5′ AGT ACT GAT GCG ATA AAA GCA 3′) were used to amplify a 300-bp fragment which was cloned in pCR1000, resulting in plasmid pNP1. Transposon-flanking fragments from the other mutants were obtained by PCR using primer OTL (5′-CGG AAT TCC GTG AAG TAT CTT CCT ACA G-3′) derived from one of the Tn*916* terminal sequences and primer PLNEC (5′ CGG AAT TCC TGA GTT ACT ACA AGA AGA G 3′). Both primers contained *Eco*RI sites (underlined) at their 5′ end.

Plasmid constructions (pNP500, pNP21, and pNP95). A 4b1 genomic library (5,000 clones; average insert size, 4 to 10 kb) was constructed as described previously (43). This library was screened with pNP1 as a probe, but no positive clones were identified. Another 4b1 library (2,000 clones) was constructed similarly, except that inserts were in the 2- to 4-kb range. One positive clone, pC11A, was identified in this library following screening with pNP1. Plasmid pNP500 was constructed by digesting pC11A with *Eco*RI (one *Eco*RI site was in the multiple cloning site of the vector, and the other was in the cloned insert) and subcloning the *Eco*RI fragment into *Eco*RI-digested and dephosphorylated pUC18. The locations of the fragments cloned in pNP1, pNP500, and pC11A are indicated in Fig. 3A (see below).

To construct plasmid pNP21, we used primers RhoBam (5′ GCG CGG ATC CAA AGG GAC AGG CAA CA 3′) and SmeBam (5′ GCG CGG ATC CAC TTG AAA ACC TCC T 3′) corresponding to nucleotides (nt) 6 to 22 and 1302 to 1319, respectively, and containing *Bam*HI recognition sites (underlined) at their 5′ ends. The PCR fragment was digested with *Bam*HI and ligated to the *L. monocytogenes-E. coli* shuttle vector pKSV7 (38), which had been digested with *Bam*HI and dephosphorylated. To construct pNP95, pNP21 was digested with *Eco*RI, and the 860-bp *Eco*RI fragment internal to the cloned insert was cloned into *Eco*RI-digested and dephosphorylated pKSV7. The fragments cloned in pNP21 and pNP95 are shown in Fig. 3A.

To amplify the *gtcA* gene from strain 2381L, we used primers RhoBam and SmeBam (see above) and genomic DNA from 2381L as the template. The PCR fragment was purified and sequenced directly.

Preparation of *L. monocytogenes* electrocompetent cells and electroporation. To prepare electrocompetent cells of *L. monocytogenes*, we used the penicillin treatment protocol of Park and Stewart (24). Electrocompetent cells were used immediately or frozen at −70°C. For electroporation, 100 μl of cells (thawed on ice if kept frozen) was mixed gently with 1 μg of plasmid DNA (Wizard minipreps; Promega). The cells were then transferred into 1-mm prechilled electroporation cuvettes (Eppendorf, Madison, Wis.). The electroporator (Eppendorf) was set to 1.5 kV to obtain the field strength of 12.5 kV/cm, as suggested by the vendor. After application of the electrical pulse, 1 ml of BHI with 0.5 M sucrose was added, and the cuvette was incubated at 30°C for 1 h. The cells (100 μl) were then plated on TSA-YE plates containing chloramphenicol (7 μg/ml) and incubated at 30°C for 2 to 3 days.

Northern blotting and reverse transcriptase PCR (RT-PCR). RNA for Northern blots was prepared from mid-logarithmic-phase cultures. Following treatment with lysozyme and pronase as for DNA extraction, a 10× volume of Tri Reagent (Molecular Research Center, Inc.) was added and the mixture was incubated at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes. Extraction and precipitation of RNA were done in accordance with the suggestions of the vendor, and Northern transfer was done as described before (2) by using positively charged membranes (Magnacharge Micron Separations Inc.). Radioactive probe labeling and signal detection were done as described previously (2).

4b1 RNA for RT-PCR was prepared by the procedures of Sato (34) and Roels and Losick (32). RNA was resuspended in 100 μl of diethylpyrocarbonate-treated water and stored at −70°C. Genomic DNA that might be contaminating the RNA preparation was destroyed by DNase I (Promega) (1 U/μl, 37°C, for 2 to 3 h), and the DNase was then inactivated by boiling for 10 min. The absence of contaminating DNA was confirmed by PCR using the DNase-treated RNA as the template.

For RT-PCR, 15 μl of the RNA was used to synthesize cDNA in a 33-μl reaction mixture, including 11 μl of first-strand reaction mix (Pharmacia Biotech), 1 mM dithiothreitol, 1 μl of antisense primer, and 5 μl of RNase-free water at 37°C for 1 h. The samples were then heated (95°C, 5 min) to destroy the RT, and 2 to 3 μl of the cDNA was used as the template in a subsequent 25-μl PCR (94°C, 1 min; 50°C, 1 min; 70°C, 2 min; for a total of 30 cycles in a Perkin-Elmer thermocycler. Antisense primers used for cDNA synthesis were P1 (5′ CTG ATT AAC AGA ATC ATG AC 3′, nt 621 to 640) and P2 (5′ TGT TTA CCA GTA TAG AAC GG 3′, nt 1013 to 1032). The subsequent PCRs included one of these primers as well as one of the following primers, as indicated in Fig. 7: P3 (5′ AAA GGG ACA GGC AAC A 3′, nt 6 to 21), P4 (3′ CGC ATC AGT ACT TTT TGC C 3′, nt 484 to 503), and P5 (5′ GCT TGT TGC CTT CAA CAT TC 3′, nt 751 to 770).

DNA sequencing and sequence analysis. DNA was sequenced on both strands either manually (Silver Sequence; Promega) or by automated sequencing at the Biotechnology Core Facility (University of Hawaii).

For DNA and protein database searches and analyses, we used FASTA (University of Wisconsin GCG package; Genetics Computer Group, Madison, Wis.) and the BLAST protein database (http://www.ncbi.nlm.nih.gov/BLAST/).

Nucleotide sequence accession number. The nucleotide sequence data determined in this study have been deposited in the GenBank database under accession no. AFO 72894.

RESULTS

Mutants negative for serotype-specific MAb c74.22 have impaired TA glycosylation. Within *L. monocytogenes*, MAbs c74.22, c74.33, and c74.180 have been shown to react exclusively with strains of serotypes 4b, 4d, and 4e (16). We have identified three serotype 4b transposon mutants, M44, B6S (also termed XL1), and 27E8 (also termed XL3), which were completely negative for c74.22 but retained reactivity with the other two MAbs (c74.33 and c74.180) on the basis of ELISA and colony immunoblots (16, 20). Mutant M44 has been shown to harbor a single copy of Tn*916*ΔE (20). Western blots of total cellular and extracellular proteins of M44 and its parental strain 4b1 with MAb c74.22 did not identify any specific protein band(s) reactive with this MAb (data not shown). Biochemical analysis of cell wall composition was then undertaken to determine whether the mutant was altered in components of TA, which, on the basis of biochemical and immunological studies, have been shown to vary among different serotypes of *L. monocytogenes* (7, 10, 40, 41). It was found that cell wall TA from M44 lacked galactose and had only trace amounts of glucose (Fig. 2B). In contrast, cell wall TA from a wild-type isogenic strain (4WT) contained both galactose and glucose (Fig. 2A), as is typical of serotype 4b strains. Other cell wall TA components (phosphate, ribitol, Glc-Nac) remained normal (Fig. 2A and
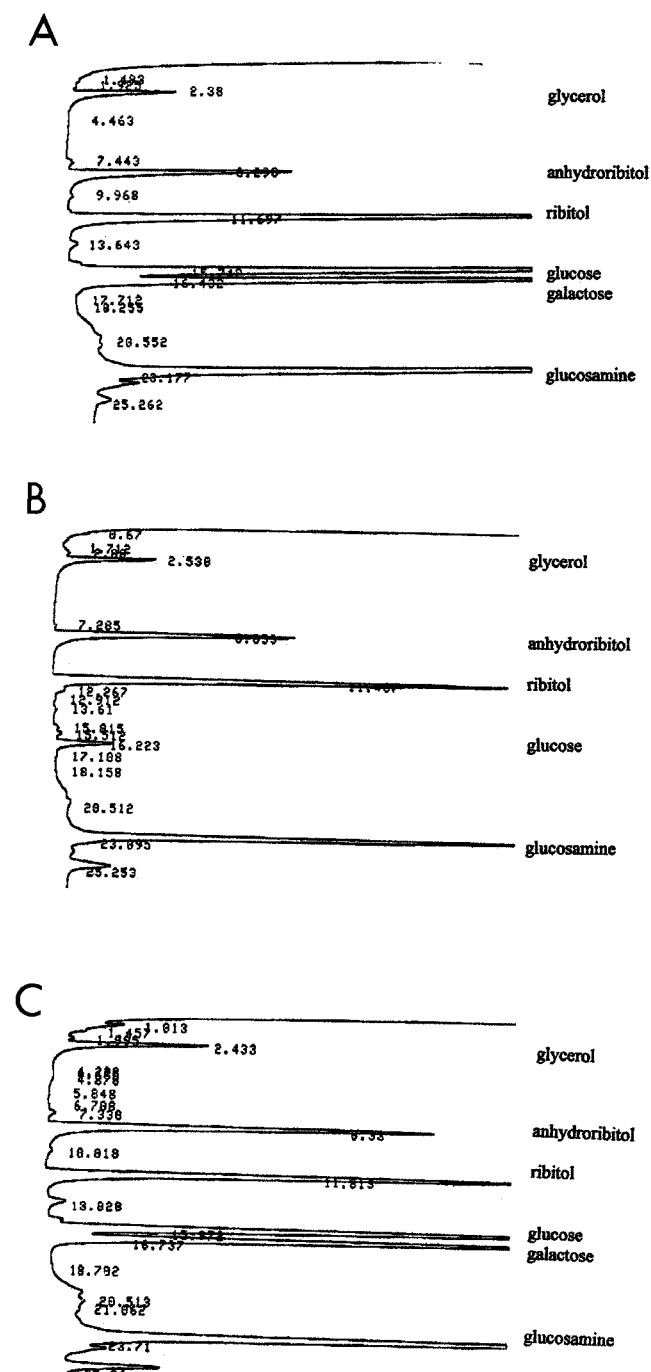
FIG. 2. TA composition in wild-type strain 4WT (A), *gtcA* mutant M44 (B), and M44 complemented with wild-type *gtcA* in *trans* (pNP95). TA preparation and analysis were done as described previously (7, 14).

B), as did the amount and composition of peptidoglycan (data not shown). Interestingly, LTA composition was also completely normal, with both galactose and glucose being present at wild-type levels (data not shown). Results identical to those obtained with M44 were also obtained with the independently derived c74.22-negative mutants B6S and 27E8 (data not shown).

Cloning and molecular characterization of *gtcA*. SSP-PCR was used as described in Materials and Methods to amplify a transposon-adjacent DNA fragment from mutant M44. The amplified flanking fragment (500 bp) was cloned in pCR1000, and the resulting plasmid was used as a probe in Southern blots to determine the sizes of homologous fragments in the wild type (strain 4b1) and in mutant M44. Such blots confirmed that the amplified fragment was indeed from the transposon-flanking region of M44 and localized the transposon insertion in a ca. 0.85-kb *Eco*RI and a 3.5-kb *Hin*dIII genomic DNA fragment. Identical Southern blot results were obtained with mutants B6S and 27E8 (data not shown), suggesting that these independently derived mutants harbored transposon insertions in the same *Eco*RI and *Hin*dIII fragments.

The cloned transposon-flanking fragment was used to screen a genomic library of strain 4b1 (4- to 10-kb fragments) with no success. A single positive clone was identified from the screening of another 4b1 library that contained smaller (2- to 4-kb) inserts. Southern blot analysis suggested that this clone (pC11A) contained 1.2 kb of DNA within the 3.5-kb *Hin*dIII fragment which harbored the transposon in M44 (Fig. 3). DNA sequencing of the original SSP-PCR fragment and of the fragment cloned in pC11A revealed that in M44 the transposon was inserted within an open reading frame (ORF), termed *gtcA* (for galactose-glucose in TA). Nucleotide sequences of transposon-flanking fragments of mutants B6S and 27E8 (amplified as described in Materials and Methods) showed that the transposon had inserted in a common target site within *gtcA*. In M44, Tn*916*ΔE was inserted between nt 512 and 513, whereas Tn*916*ΔE in B6S and Tn*916* in 27E8 were both inserted between nt 514 and 515. The transposon target site in these mutants, TTTTCGAATAAAAA, was in close agreement with the Tn*916* preferred target sites reported for other gram-positive bacteria (21).

ORF analysis. Sequence analysis revealed two complete ORFs (*gtcA* and ORFD) and one incomplete ORF (ORFU) in this region, all transcribed in the same direction (Fig. 3). An inverted repeat followed by a series of T's after the end of ORFD may represent a rho-independent transcription terminator (estimated free energy of formation, −25 kcal/mol). The transposon-harboring ORF (*gtcA*) was preceded by an incomplete ORF (ORFU) with 60 to 74% identity to genes encoding the transcription termination factor rho in *B. subtilis*, *E. coli*, *Salmonella typhimurium*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and other bacteria. The available sequence of this ORF would be expected to correspond to the 275 bp in the 3′ portion of the putative *rho* gene in *L. monocytogenes*, a portion of the coding sequence known to be especially conserved among *rho* sequences (23).

The ORF immediately downstream of *gtcA* (ORFD) showed 55% homology to the *rpmE* gene of *B. subtilis* (31) encoding ribosomal protein L31. Homology was also detected with the *rpmE* gene sequences of *Mycobacterium leprae* (41%), *E. coli* (32%), and other bacteria. BLAST analysis suggested significant similarity between the deduced ORFD product and the L31 proteins from *B. subtilis* and other bacteria (Fig. 4A shows a multiple sequence alignment). It appears therefore likely that ORFD represents the coding sequence of the *L. monocytogenes rpmE* gene. The hydrophobicity plot of the putative RpmE suggested that the protein was hydrophilic (data not shown). A putative ribosomal binding site was identified 5 nt upstream of the start codon of this gene.

The coding sequence of *gtcA* (434 bp) was separated from ORFU and ORFD by intergenic regions of 32 and 101 bp, respectively. A putative ribosomal binding site was identified 5 nt upstream of the *gtcA* start codon, although its similarity with the consensus was noticeably lower than that of the putative ribosomal binding site preceding ORFD. The deduced gene product (17.4 kDa) was calculated to have a pI of 10.17. The
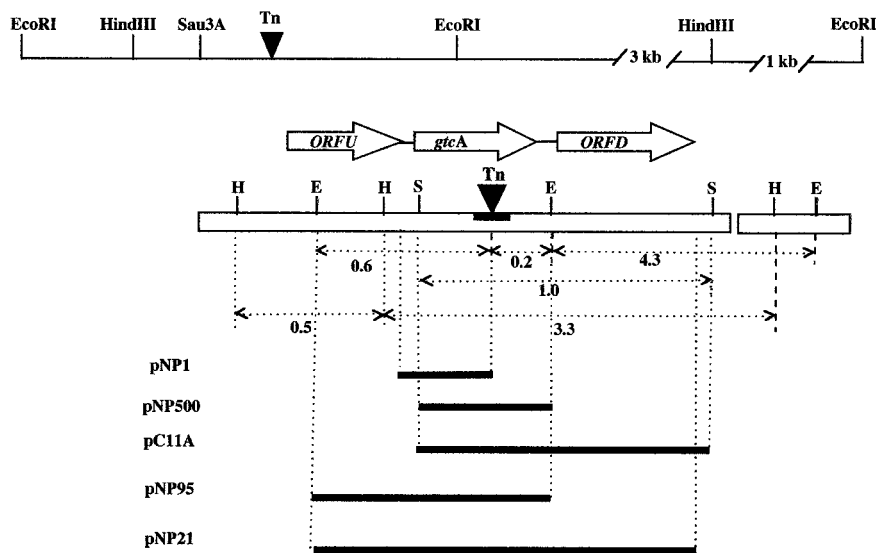
FIG. 3. Organization of the *gtcA* genomic region. Arrows indicate the direction of transcription. The solid bar in *gtcA* indicates the location of the transposon insertion sites (Tn) in M44, B6S, and 27E8. The thick lines indicate the DNA fragments cloned in the indicated plasmids. Numbers indicate sizes of fragments in kilobases. Recognition sites for *Hin*dIII (H), *Sau*3A (S), and *Eco*RI (E) are indicated.

gtcA coding sequence was similar (55% identity) to a gene of *B. subtilis* (*ipa34-d*) which was identified during the *B. subtilis* genome sequencing project and which appears to be a member of an operon involved in galactose metabolism (11). The deduced product of *gtcA* was found to be similar to the deduced product of *ipa34-d* (46% identity over 123 amino acids). The deduced *gtcA* product had lower similarity with several other proteins in the database, i.e., a hypothetical protein of *Actino-*

bacillus actinomycetemcomitans (33% identity over 140 amino acids) and the deduced products of the *E. coli* gene *yeij* (30% identity over 125 amino acids) and of the *Shigella flexneri* gene *rfbI* (27% identity over 122 amino acids). The latter two genes are in genomic regions dedicated to surface antigen biosynthesis in *E. coli* and *S. flexneri*, respectively. Figure 4B shows the alignment between these deduced sequences. It is noticeable that the 22-amino-acid N-terminal region of the deduced *gtcA*

FIG. 4. Multiple sequence alignments (CLUSTAL) of the deduced ORFD (putative L31) sequence (A) and the deduced GtcA sequence (B). Abbreviations: B. subt L31 (accession no. M97678), E. coli L31 (accession no. U00096), and M. lepr L31 (accession no. Z73419), L31 sequences from *B. subtilis*, *E. coli*, and *M. leprae*, respectively; Ipa-34D B. subt (accession no. X73124), o120 E. coli (accession no. U000323), Rfbi S. flex (accession no. X71970), and Unk A. act (accession no. AB002668), sequences with similarity to the deduced *gtcA* product and derived from *B. subtilis*, *E. coli*, *S. flexneri*, and *A. actinomycetemcomitans*, respectively, as described in the text.
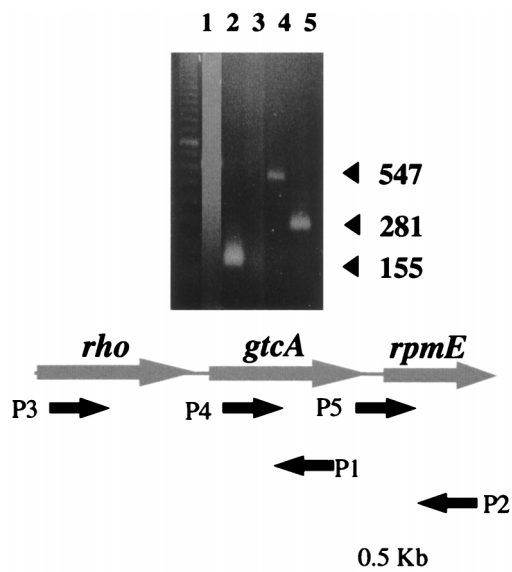
FIG. 5. RT-PCR to determine transcriptional status of ORFU, *gtcA*, and ORFD in *L. monocytogenes* serotype 4b. Primers P1 and P2 were used for cDNA synthesis. Subsequent PCR was done with primers P3 and P1 (lane 1), P4 and P1 (lane 2), P3 and P2 (lane 3), P2 and P4 (lane 4), and P2 and P5 (lane 5). No PCR product was observed in lanes 1 and 3. The leftmost lane contains molecular size markers. The location and orientation of the primers are shown at the bottom of the figure. Precise location and sequence of the primers as well as procedures for RT-PCR were as described in Materials and Methods. Templates for negative control were RNA with the same primer sets. None of the negative control reactions produced detectable PCR products (data not shown).

product does not have a counterpart in the deduced *ipa34-d* sequence of *B. subtilis*. In fact, an alternative start codon (base position 375, residue 23) could be identified 12 nt downstream of a putative ribosomal binding site (AGATCAGGTA). The 23-amino-acid N-terminal region of the deduced *gtcA* product also lacked significant similarity with the hypothetical proteins of *A. actinomycetemcomitans*, *E. coli*, and *S. flexneri*. N-terminal sequencing of the protein will be required for determination of the actual start codon. Hydropathy analysis of the deduced *gtcA* gene product indicated four putative membrane-spanning domains, suggesting that the protein may be membrane associated. The G+C content of *gtcA* was found to be ca. 30%, noticeably lower than the average G+C content of *L. monocytogenes* (38%). In contrast, the G+C contents of the putative *rpmE* and *rho* (partial) sequences were 41 and 39%, respectively.

Interestingly, in *B. subtilis*, *rho* and *rpmE* were located adjacent to each other on the chromosome (31), whereas *ipa34-d* was located in a different genomic region (11). In *L. monocytogenes* serotype 4b, *gtcA* was positioned exactly between the putative *rho* and *rpmE* genes of this organism (ORFU and ORFD, respectively) (Fig. 3).

Determination of *gtcA* sequence in epidemic-associated strain F2381. Strain F2381 is a representative of a clonal lineage implicated in numerous outbreaks of listeriosis and known to be genetically distinct from other serotype 4b strains (3, 25, 44). Sequencing of the *gtcA* region (nt 289 to 1045) showed that the coding sequence of *gtcA* was identical for strains 4b1 and F2381. The two sequence differences identified in this region were both in the *gtcA-rpmE* intergenic region (at nt 756, strain F2381 had an insertion of a T; at nt 819, strain 4b1 had a G that was absent in F2381) (Fig. 3B). The sequenced portion of the coding sequence of *rpmE* (up to and including nt 1045) was identical for strains 4b1 and F2381.

Transcriptional analysis of *gtcA*. Northern blots using the *gtcA*-internal fragment cloned in pNP1 as probe suggested the presence of a ca. 700-nt transcript (data not shown). Signal intensity was quite low in Northern blots, suggesting a low abundance of the message. RT-PCR was therefore used to determine whether *gtcA* was cotranscribed with its flanking ORFs. The results of a typical RT-PCR experiment are shown in Fig. 5. cDNA synthesis was done by using primers P1 (nt 621 to 640, complementary to the expected *gtcA* message) and P2 (nt 1013 to 1032, complementary to the expected *rpmE* message). The RT-PCR results suggest that *gtcA* was cotranscribed with ORFD (Fig. 5, lane 4) but not with ORFU (lanes 1 and 3). Since only 32 bp separated the *gtcA* start codon from the ochre codon of ORFU, the *gtcA* promoter may be located in the 3′-end portion of ORFU. RT-PCR data suggest that the transcript initiation site was within the last 30 to 40 nt of ORFU (16).

TA defects of *gtcA* mutants are complemented by wild-type *gtcA* in *trans*. Plasmids pNP21 and pNP95, harboring *gtcA* with or without ORFD (putative *rpmE*), respectively (Fig. 3), were electroporated into mutant M44. Both plasmids include the ORFU-*gtcA* intergenic region and the 3′-end region of ORFU, since the *gtcA* promoter may be contained within this region. The resulting strains were grown in the presence of chloramphenicol at 30°C, a temperature which permits both replication of the temperature-sensitive plasmid (38) and optimal expression of the serotype 4b-specific surface antigens (16). Reactivity of the mutant with c74.22 was restored by both plasmids. Reactivity was relatively low when pNP21 was used but stronger (albeit still somewhat lower than that of the wild type) with pN95 (Fig. 6). Identical results were obtained with mutant 27E8 (data not shown). Interestingly, introduction of either plasmid completely restored TA composition. The TA of M44 complemented with pNP95 contained both galactose and glucose, at levels indistinguishable from those of the wild type (Fig. 2C); identical results were obtained with M44 complemented with pNP21 (data not shown).

Within *L. monocytogenes*, only serogroup 4 strains harbor *gtcA* sequences whereas ORFD appears to be conserved among *Listeria* spp. Recently, we showed that the region adjacent to the transposon insertion in mutant M44 was present in serogroup 4 (serotypes 4a, 4b, 4c, 4d, and 4e) and in certain strains of *Listeria innocua* (which, in contrast to all other screened *L. innocua* strains, were also positive for MAbs c74.22, c74.33, and c74.180 [16]) but not in other serotypes of *L. monocytogenes* or other *Listeria* species (20). Southern blots using pNP1
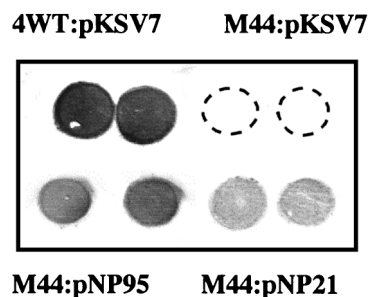
FIG. 6. Complementation of surface antigen expression of M44 with wild-type *gtcA* in *trans*. Colony immunoblotting with MAb c74.22 was done as described in Materials and Methods. Top left (duplicate spots), positive control (wild-type strain 4WT harboring the cloning vector pKSV7); top right (duplicate spots), mutant M44 harboring the cloning vector pKSV7; bottom left and bottom right (duplicate spots in each), M44 harboring pNP95 and pNP21, respectively. No reaction was seen in the top right spots (duplicate spots of M44 harboring pKSV7). Fragments contained in pNP95 and pNP21 were as shown in Fig. 3A.

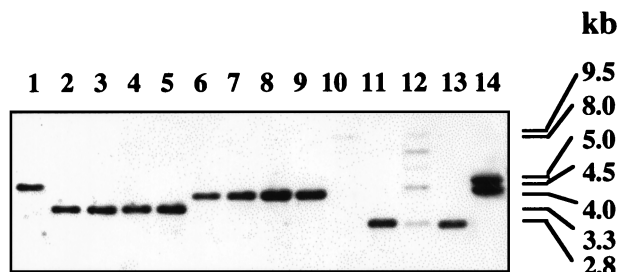FIG. 7. Southern blot of *Hin*dIII-digested genomic DNA from different listerial strains with pC11A as the probe. Lanes 1 to 11, DNA from *L. monocytogenes* serotype 1/2b (strain F4233 [lane 1]), serotype 4b (strains F2381, 33, 15U, and 18 [lanes 2 to 5, respectively]), and serotype 1/2a (strains Mack, G2228, G2295, and G3412 [lanes 6 to 9, respectively]). Lanes 10 to 14 contain DNA from *L. grayi, Listeria welshimeri, L. seeligeri, L. innocua,* and *Listeria ivanovii,* respectively.

as the probe confirmed these findings (data not shown). In addition, Southern blots with pC11A, which includes *gtcA* as well as ORFD, suggested that the latter gene (putative *rpmE*) was present in other serotypes of *L. monocytogenes* as well as in all other *Listeria* species (hybridizing signals were weak in *Listeria grayi* and *Listeria seeligeri*) (Fig. 7). Such Southern blots also revealed *Hin*dIII restriction fragment length polymorphisms (RFLPs) which could differentiate *L. monocytogenes* strains of serotype 4b (Fig. 7, lanes 2 to 5) from those of serotypes 1/2a and 1/2b (lanes 1 and 6 to 9). Similar differentiation could be obtained with *Eco*RI RFLPs (data not shown). Southern blots of *Eco*RI-cut DNA with probe pNP500, which includes *gtcA* and only the *gtcA*-ORFD intergenic region, furthermore indicated that the intergenic region was present in strains of other serotypes. In serogroup 4 (serotypes 4a, 4b, 4c, 4d, and 4e), a hybridizing band of 0.8 kb was observed, whereas a 2-kb hybridizing band was observed with serogroup 1/2 (serotypes 1/2a, 1/2b, and 1/2c), serogroup 3 (serotypes 3a, 3b, and 3c), and serotype 7 (data not shown).

DISCUSSION

The gene described in this report, *gtcA*, appears to be essential for presentation of galactose and glucose substituents on serotype 4b TA. Support for this was provided by the findings that (i) insertional inactivation of *gtcA* in several independently obtained mutants abolished galactose and markedly reduced glucose levels in TA and (ii) the amounts of both sugars were restored to wild-type levels by the wild-type *gtcA* gene in *trans*. These results suggest that the phenotype of *gtcA* insertion mutants was due to the transposon insertion and not to an unrelated (e.g., spontaneous) mutation. In addition, complementation by the wild-type *gtcA* in *trans* strongly suggested that the impact of the *gtcA* mutations on TA composition was indeed due to inactivation of *gtcA* itself and not to a possible polar effect of the transposon insertion on a downstream gene(s).

Cell wall compositional analysis of the *gtcA* mutants led to two unexpected observations: in contrast to its impact on TA, the mutation was found to have no effect on LTA composition, suggesting genetic and enzymatic independence of glycosylation between TA and LTA. Furthermore, TA of *gtcA* mutants was found not only to lack galactose but also to have markedly reduced glucose. A possible explanation for this may be that the enzymatic step(s) mediating incorporation of glucose onto TA may require the prior presence of galactose. Although the precise function of the *gtcA* gene product remains to be determined, our genetic, immunological, and biochemical data suggest that it is involved in glycosylation of TA domains and that such domains serve as serotype-specific surface antigens on bacteria of serotype 4b. Key supporting data include the impaired TA glycosylation of the *gtcA* mutants and their lack of reactivity with MAb c74.22, as well as the finding that normal TA glycosylation and reactivity were restored when *gtcA* was provided in *trans*. The presence of hydrophobic, putative membrane-spanning domains in the deduced *gtcA* product would be in agreement with its involvement in TA biosynthesis. In *B. subtilis*, several TA biosynthesis gene products have been shown to have membrane-spanning and/or membrane-anchoring domains (18, 19), and enzymes involved in TA biosynthesis have been shown to be membrane associated in *Staphylococcus aureus* and *Staphylococcus xylosus* (8, 9). Additional insight into the function of the *gtcA* product will be provided by biochemical characterization of the protein and, perhaps, by characterization of homologous genes or gene products in other bacteria. In this respect, it is of interest that the *gtcA* homologue of *B. subtilis* (*ipa34-d*) appears to be in an operon dedicated to galactose metabolism (11). However, the precise function of *ipa34-d* has not yet been determined, and *B. subtilis* is not known to have galactose in its cell wall TA. In *B. subtilis*, two glucosyl transferases involved in the incorporation of glucose onto TA have been identified, the products of the *gtaB* and *tagE* (*rodD*), respectively (4, 12, 26, 27, 39). *B. subtilis*, however, uses glycerol as the glucose acceptor in its TA, whereas in *L. monocytogenes* serotype 4b, *N*-acetylglucosamine serves as the galactose and glucose acceptor. It is possible that the *gtcA* product may have a glycosyl transferase activity, although no homologies with similar transferases were detected by the protein database searches. Another possibility that cannot be excluded at this point is that *gtcA* may encode a regulatory protein, essential for expression of a gene(s) involved in glycosylation of TA in *L. monocytogenes* serotype 4b. The potential of *gtcA* to confer c74.22 reactivity to heterologous serotypes (e.g., serogroup 1/2 or 3) remains to be determined. It must be kept in mind, however, that these strains (e.g., serogroup 1/2 or 3) differ in their TA backbone structure and in TA glycosylation sites from serotype 4b strains (Fig. 1). A more likely candidate for heterologous expression would be c74.22-negative strains of *L. innocua*, which lack galactose and glucose but have a TA backbone structure identical to that of serotype 4b strains. We were, however, unable to introduce *gtcA* (cloned in pKSV7) into such an *L. innocua* strain (serotype 6a), perhaps because of a restriction barrier (data not shown).

It is of interest that in serogroup 4 strains of *L. monocytogenes*, the *gtcA* gene was positioned between ORFs with homology to *rho* and *rpmE* genes, whereas *rho* and *rpmE* were directly adjacent to each other in *B. subtilis*. Our data suggested that *gtcA* was the first gene of a bicistronic operon which included the putative *rpmE*. This was surprising since no apparent commonality in the function of these two genes was readily evident. Our data also indicated that of the two genes, only *gtcA* was unique to serogroup 4, whereas the putative *rpmE* (as well as the *gtcA-rpmE* intergenic region) was conserved among listeriae. It is tempting to speculate that the *rho-rpmE* genomic arrangement has been preserved in *L. monocytogenes* strains other than those of serogroup 4 (e.g., 1/2 and 3, etc.) which lack *gtcA* and that, in the course of evolution of serogroup 4 strains, *gtcA* was introduced in its present location, conferring serogroup-specific expression of a TA-associated, serotype-specific surface antigen(s). The gene may have been transferred between *L. monocytogenes* serogroup 4 and a lineage of *L. innocua*, a species which is nonpathogenic but otherwise closely related genetically and antigenically to *L. monocytogenes*. The *gtcA* gene may have originated in an organism

other than *Listeria*, and its unusually low G+C content (30%, in contrast to 38% for *L. monocytogenes* and other *Listeria* spp.) may be indicative of this. Introduction of *gtcA* could have been mediated by a phage or transposon, although the currently available sequence data do not reveal any discernible traces or evidence of such elements (inverted repeats, tRNA sequences, or sequences with possible integrase or transposase functions). An alternative possibility is that the *gtcA* sequences were present in an ancestral *L. monocytogenes-L. innocua* lineage but were preserved only in serogroup 4 strains and in certain *L. innocua* strains. We are currently in the process of further characterizing the genomic region that harbors the putative *rho* and *rpmE* genes in strains of *L. monocytogenes* other than those of serogroup 4, in order to gain further insight on this issue.

Extensive molecular studies of virulence determinants have focused on a select group of extracellular and surface-associated proteins of *L. monocytogenes* (reviewed in references 13 and 29). In contrast, the possible involvement of carbohydrate-based surface antigens in pathogenesis remains poorly understood. Earlier studies suggested that glycosylated TA components were important antigenic determinants in *L. monocytogenes* (14, 41). We have obtained data, to be described in a separate presentation, which strongly suggest that galactose on TA of *L. monocytogenes* serotype 4b was essential as a receptor for serotype-specific phages and, in addition, for invasion of several mammalian cell lines by the bacteria (30). Such data are in agreement with findings from other investigations which showed that in serotype 1/2, rhamnose and Glc-NAc on TA served as receptors for serotype-specific phages (42). However, genes involved in the decoration of serotype 1/2 TA with rhamnose and Glc-NAc have not yet been identified, and information regarding their possible serotype-specific distribution in *L. monocytogenes* is lacking. Further investigations are needed to enhance our understanding of the evolution of genetic systems dedicated to TA glycosylation and of the possible function(s) of the different types of TA glycosylation in the adaptive physiology and pathogenesis of the microorganism.

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