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Chromosomal Gentamicin Resistance Transposon Tn*3706* in *Streptococcus agalactiae* B128

THEA HORAUD,† GILDA DE CÉSPÈDES, AND PATRICK TRIEU-CUOT*

Laboratoire des Staphylocoques et des Streptocoques, Institut Pasteur, 75724 Paris Cedex 15, France

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***Streptococcus agalactiae* B128 is the only highly gentamicin-resistant group B streptococcal (GBS) strain described so far. This strain carries a chromosomal gentamicin resistance transposon, designated Tn*3706*, which is similar, if not identical, to the Tn*4001* and Tn*5281* transposons detected in *Staphylococcus aureus* and *Enterococcus faecalis*, respectively. Transposition of Tn*3706* occurred onto the GBS plasmid pIP501 in two different loci of its 7.5-kb *Ava*II fragment carrying the genes for chloramphenicol and erythromycin resistance. Molecular analysis of pIP501 derivatives showed that Tn*3706* is composed of a central fragment containing the *aac6′-aph2″* gene; this fragment is flanked by two tandemly repeated copies of IS*256* at the 5′ extremity of the resistance gene and a single inverted copy of IS*256* at its 3′ extremity. The two tandemly repeated copies of IS*256* were separated by a 6-bp segment identical to that found, in the same orientation, in the IS*256–aac6′-aph2″* junction. The hybrid replicons pIP501::Tn*3706* were found to be structurally unstable following conjugative transfer between GBS strains. Numerous individual copies of IS*256* were detected in B128, but this insertion sequence was not found in the 11 wild-type, gentamicin-susceptible GBS strains studied.**

High-level resistance to gentamicin (Gm^r) in enterococci and streptococci is usually due to the presence of the *aac6′-aph2′′* gene, which directs synthesis of the bifunctional enzyme with 6′-acetyltransferase and 2′′-phosphotransferase activities. The Gm^r determinant is usually plasmid-borne in most *Enterococcus faecalis* (15, 23) and *Enterococcus faecium* (33) strains described so far, where it could be carried by transposable elements (12, 13) structurally related to Tn*4001* (22) or to the hybrid element Tn*4001*-IS*257* (3), both of which were originally detected in strains of *Staphylococcus aureus*. Chromosomally mediated high-level gentamicin resistance has also been reported in several *E. faecalis* strains (5, 26, 31) as well as in one group B *Streptococcus* strain (2) and in several *Streptococcus mitis* clinical isolates (17). In one of the *E. faecalis* strains, the chromosome-borne *aac6′-aph2″* gene is carried by a novel type of transposon designated Tn*924* (20).

Group B streptococcal (GBS) strains are the leading cause of neonatal infections. Severe invasive GBS infections can also occur in pregnant women and nonpregnant adults, in particular, in elderly and immunocompromised patients with chronic diseases. The treatment of choice of GBS infections is penicillin (or ampicillin), often in combination with gentamicin (8). This antibiotic combination, which presents a bactericidal synergism against GBS, is used to treat severe GBS infections since in most cases the number of bacteria per milliliter (10^6 to 10^8) is so high that penicillin (or ampicillin) alone is not bactericidal.

Streptococcus agalactiae B128 is the only highly gentamicin-resistant GBS strain described so far (2). This plasmid-free strain is also resistant to all other available aminoglycosides except spectinomycin and to tetracyclines, including minocycline. The resistance phenotype is due to the presence of the *aac6′-aph2″*, *aph3′*, *aadE*, and *tetM* genes, which encode resistance to gentamicin-kanamycin (Gm^r-Km^r), neomycin-kanamycin (Nm^r-Km^r), streptomycin (Sm^r), and tetracycline-minocycline (Tc^r-Mn^r), respectively. These resistance determinants transfer by conjugation en bloc, singly (Tc^r-Mn^r), or in various combinations (Nm^r-Km^r Gm^r-Km^r Sm^r; Nm^r-Km^r Sm^r; Gm^r-Km^r Tc^r-Mn^r) to only a GBS recipient (2).

In preliminary studies, we have demonstrated that the Gm^r-Km^r determinant of strain B128 is carried by a transposable element designated Tn*3706* and that the Tc^r-Mn^r determinant is carried by a Tn*916*-like element designated Tn*3707* (6). We have also shown that Tn*3706* can transpose from its host chromosome onto the R plasmid pIP501 originally detected in GBS strain B96 (14). To our knowledge, transposition of a chromosomal element carrying antibiotic resistance markers onto an R plasmid has not yet been reported in streptococci. The aim of the study described here was to further characterize the molecular structure of Tn*3706*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *S. agalactiae* strains and plasmids used in the study are listed in Table 1. Bacteria were grown in brain heart infusion broth or agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% (vol/vol) horse blood (Diagnostics Pasteur, Marnes-la-Coquette, France). All incubations were done at 37°C.

Mating experiments. Matings were performed on membrane filters (HAEP; 0.45-μm pore diameter; 47 mm; Millipore Corp., Bedford, Mass.) by mixing 0.1 ml each of 18-h broth cultures of donor and recipient strains plus 0.1 ml of broth. After 18 h of incubation at 37°C, the cells were suspended in 0.5 ml of broth and dilutions were spread onto appropriate selective media. Antibiotics were used at the following concentrations: fusidic acid, 25 μg/ml; gentamicin, 1,000 μg/ml; kanamycin, 2,000 μg/ml; rifampin, 100 μg/ml; streptomycin, 2,000 μg/ml; and tetracycline, 8 μg/ml.

DNA manipulations, PCR amplification, and DNA sequencing. Bacterial and plasmid DNAs were extracted from streptococci and enterococci as described previously (18). Primers O1 (5′-GGACCTACATGATGAATGGA-3′) plus O2 (5′-CCTTTACAGAATATTCAATAATGC-3′) and O1 plus O3 (5′-GTATAG CAATATGCAAATCC-3′) were used to amplify the 285-bp IS*256*-IS*256* and 250-bp IS*256–aac6′-aph2″* junction fragments, respectively. Amplification was performed in a final volume of 100 μl containing 50 ng of genomic or plasmid DNA, 0.1 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate, and 2 U of *Taq* DNA polymerase (Amersham International, Buckinghamshire, United Kingdom) in a 1× amplification buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$). The PCR mixture was submitted to a denaturation step (3 min at 95°C); this was followed by 35 cycles of amplification (2 min of annealing at 55°C, 90 s of elongation at 72°C, and 30 s of denaturation at 95°C) and by final

* Corresponding author. Mailing address: Laboratoire de Microbiologie, Inserm U-411, Faculté de Médecine Necker-Enfants Malades, 156 rue de Vaugirard, 75730 Paris Cedex 15, France. Phone: (33) (1) 40 61 56 79. Fax: (33) (1) 40 61 55 92.

† Present address: Gestion des Colloques, Centre d'Information Scientifique, Institut Pasteur, 75724 Paris Cedex 15, France.

TABLE 1. Bacterial strains and plasmids used in the study

S. agalactiae strain or plasmid	Relevant markers[a]	Comment (reference)
B128	Gm^r-Km^r Sm^r Km^r-Nm^r Tc^r-Mn^r, Tra^+	Wild-type strain (2)
BM132[b]	Fus^r Rif^r	Recipient strain (16)
BM134[b]	Str^r	Recipient strain (16)
BM5237	Fus^r Rif^r, Gm^r-Km^r Tc^r-Mn^r, Tra^+	B128 × BM132 (2)
BM5803	Str^r, Gm^r-Km^r, Tra^-	BM5237 × BM134 (the present study)
B96[c]	Cm^r Em^r Tc^r-Mn^r	Wild-type strain (16)
B97[c]	Cm^r Em^r Tc^r-Mn^r	Wild-type strain (16)
B109	Cm^r Em^r Tc^r-Mn^r	Wild-type strain (16)
B117	Cm^r Tc^r-Mn^r	Wild-type strain (16)
B121	Em^r Tc^r-Mn^r	Wild-type strain (16)
B125	Cm^r Tc^r-Mn^r	Wild-type strain (19)
B126	Cm^r	Wild-type strain (19)
B127	Cm^r Em^r Tc^r-Mn^r	Wild-type strain (19)
NEM318	Susceptible to antibiotics	Wild-type strain (the present study)
NEM623	Km^r-Nm^r Tc^r-Mn^r	Wild-type strain (the present study)
pIP501	Cm^r Em^r, Tra^+	(14)
pIP1693	Cm^r Em^r Gm^r-Km^r, Tra^+	pIP501::Tn*3706* (I1) (the present study)
pIP1694	Em^r Gm^r-Km^r, Tra^+	pIP501::Tn*3706* (I2) (the present study)

[a] Abbreviations: Cm^r, chloramphenicol resistance (*cat* gene); Em^r, erythromycin resistance (*ermB* gene); Gm^r-Km^r, high-level gentamicin-kanamycin resistance (*aac6′-aph2″* gene); Km^r-Nm^r, high-level kanamycin-neomycin resistance (*aph3′* gene); Sm^r, high-level streptomycin resistance (*aadE* gene); Tc^r-Mn^r, tetracycline-minocycline resistance (*tetM* gene); Fus^r, Rif^r, and Str^r, resistance to fusidic acid, rifampin, and streptomycin, respectively; Tra, conjugative transfer.
[b] Isogenic strains derived from B19 by spontaneous mutations (16).
[c] In B96 and B97, Cm^r and Em^r markers are carried by plasmids pIP501 and pIP612, respectively (14, 16).

annealing (4 min at 55°C) and elongation (12 min of elongation at 72°C) steps. The amplified DNA fragments were purified from agarose gels by using the Geneclean kit (Bio 101, Inc.), treated with T4 DNA polymerase and DNA polymerase I (Amersham International) to convert ragged ends to blunt ends, and cloned into *Sma*I-linearized and dephosphorylated pUC18. Double-stranded plasmid DNA was sequenced (34) by using the 24-mer sequencing (primer −47) and reverse sequencing (primer −48) primers (New England Biolabs, Inc.). The entire nucleotide sequences of two cloned amplicons obtained from independent PCRs were determined on both strands.

Southern blotting and hybridization. DNA was digested with appropriate restriction endonucleases, and the resulting fragments were separated by electrophoresis on 0.8% agarose gels and were transferred to nitrocellulose filters by a bidirectional method (30). Prehybridization and hybridization under stringent conditions were carried out as described previously (18). The DNA probes were labeled with [α-^{32}P]dCTP (Amersham International) by the use of the BRL nick translation system (Bethesda Research Laboratories, Inc.). Plasmids pSF815A, containing the *aac6′-aph2″* gene (9); pIP1151, carrying a 452-bp fragment internal to IS*256* (7); and pIP1184 (pTCV1::Tn*916*), a pGB2 derivative carrying Tn*916* (10) in its entirety (24), were used as probes. Amplicons corresponding to DNA fragments internal to the *cat* gene of pIP501 (32) and to the *ermB* gene of Tn*917* (29), which is almost identical to *ermB* of pIP501 (1), were labeled and used as probes. They were obtained by using in PCRs the oligonucleotides homologous to base pairs 66 to 84 (5′-TCAGCAAACTACTTATAGC-3′) and 569 to 550 (5′-GAATGATGAAGGTGCAGAGC-3′) of the *cat* gene and to base pairs 37 to 58 (5′-CGAGTGAAAAAGTACTCAACC-3′) and 560 to 540 (5′-CTGGAACATCTGTGGTATGGC-3′) of the *ermB* gene.

RESULTS

Mating experiments. BM5237 was obtained following the mating B128 × BM132 (2). Strain BM5237 carrying the Gm^r-Km^r and Tc^r-Mn^r markers of strain B128 was subsequently mated with strain BM134 (Table 1). When selection was done with gentamicin or kanamycin, only one transconjugant, designated BM5803, was obtained (transfer frequency, 2×10^{-9} transconjugants per donor). This clone carried only the Gm^r-Km^r marker, and no detectable transconjugants were obtained when BM5803 was mated with BM132 (transfer frequency, $<2 \times 10^{-9}$ per donor). When selection was done with tetracycline, for the mating BM5237 × BM134, transconjugants were obtained at a frequency of 8×10^{-8} per donor cell. Analysis for the unselected markers of the 12 clones obtained revealed that 4 of them carried both Tc^r-Mn^r and Gm^r-Km^r determinants, whereas the remaining 8 clones carried only the Tc^r-Mn^r marker. The same frequency of retransfer (8×10^{-8}) was obtained when transconjugant clones carrying either Tc^r-Mn^r Gm^r-Km^r markers or only Tc^r-Mn^r markers were mated with BM132.

Hybridization experiments. Chromosomal DNAs of B128, BM5237, and BM5803, digested with the restriction endonucleases *Ava*II, *Hae*III, *Hin*cII, *Hin*dIII, and *Sca*I, known to have at least two restriction sites within the staphylococcal transposon Tn*4001* (22), were probed with the *aac6′-aph2″* gene. The results of these experiments, summarized in Table 2, revealed that the internal structure of Tn*3706*, carried by the wild-type strain B128 and the transconjugants BM5237 and

TABLE 2. Hybridization between different streptococcal genomic DNA and the probe *aac6′-aph2″*

Strain or transposon	Size (kb) of the hybridizing fragments								
	*Ava*II	*Hae*III	*Hin*dIII	*Hin*cII	*Sca*I	*Hin*dIII + *Sca*I	*Eco*RI	*Eco*RV	*Xba*I
B128	2.1	3.9	2.5	2.2, 1.2	1.8, 1.5	1.4, 1.1	~19.8	5.6	~19.5
BM5237	2.1	3.9	2.5	2.2, 1.2	1.8, 1.5	1.4, 1.1	~20.7	10.7	~19.5
BM5803	2.1	3.9	2.5	2.2, 1.2	1.8, 1.5	1.4, 1.1	~18.9	5.5	~19.5
Tn*4001*[a]	2.1	3.9	2.5	2.2, 1.2	1.8, 1.5	1.4, 1.1	NT[b]	NT	NT

[a] The sizes of the hybridizing fragments are deduced from the nucleotide sequence of Tn*4001* (4, 28). No recognition sites for *Eco*RI, *Eco*Rv, and *Xba*I have been reported in Tn*4001* (4, 28).
[b] NT, not tested.

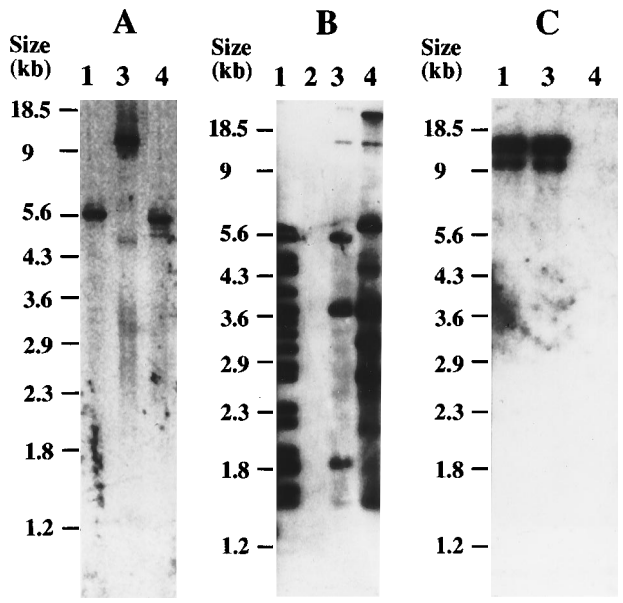

FIG. 1. Southern analysis of digested chromosomal DNA. (A) Hybridization of *Eco*RV digestion with the probe *aac6′-aph2″*; (B) hybridization of *Hae*III digestion with the probe containing IS*256* sequences; (C) hybridization of *Hin*dIII digestion with the probe containing Tn*916*. Lanes: 1, B128; 2, BM132; 3, BM5237; and 4, BM5803. The Raoul I ladder (Appligène, Strasbourg, France) was used as a molecular size marker.

BM5803, is similar, if not identical, to that of Tn*4001* (4, 28). Genomic DNAs of B128, BM5237, and BM5803 were further digested with *Eco*RI, *Eco*RV, and *Xba*I, which do not cleave Tn*4001*, and probed with the *aac6′-aph2″* gene. The single hybridization signal obtained in all cases (Table 2) indicates that these strains harbor only one copy of Tn*3706*. The fact that the isogenic strains BM5237 and BM5803 display a different *Eco*RV hybridization pattern (Table 2 and Fig. 1A) also indicates that Tn*3706* had inserted at different loci in the two chromosomes.

DNA-DNA hybridizations were carried out between the *Hae*III-digested chromosomal DNAs of B128, BM132, BM5237, and BM5803 and the probe pIP1151 containing DNA sequences of IS*256* (Fig. 1B). This probe hybridized with a single *Hae*III IS*256*-chromosome junction fragment of each individual copy of IS*256* and with the internal 3.9-kb *Hae*III fragment of Tn*4001*. This analysis revealed that B128 carries a minimum of 17 copies of IS*256*, which corresponded to the number of *Hae*III fragments hybridizing with probe pIP1151. This is likely an underestimation of the exact number of copies of IS*256* present in B128, since all bands giving a strong hybridization signal may correspond to two or several restriction fragments of similar sizes. A minimum of three and eight copies of IS*256* were found in BM5237 and BM5803, respectively. No sequences related to IS*256* were detected in isogenic GBS strains BM132 (Fig. 1B, lane 2) and BM134. The presence of sequences homologous to IS*256* was similarly studied in 11 *S. agalactiae* wild-type strains susceptible to gentamicin and kanamycin (Table 1) which were from clinical specimens collected from various French hospitals by our laboratory. No DNA-DNA homology with IS*256* was found in any of these strains.

*Hin*dIII-digested chromosomal DNAs of B128, BM5237, and BM5803 were also probed with pIP1184 containing Tn*916*. Whereas two hybridizing bands of 15.0 and 13.2 kb were found in B128 and BM5237, no sequences homologous to Tn*916* were detected in BM5803 (Fig. 1C).

Transposition of Tn*3706* onto plasmid pIP501. Plasmid pIP501 (Table 1) was introduced by conjugation into BM5803, and one of the resulting transconjugant clones, BM5803 (pIP501), was mated with the recipient, strain BM132. When selection was done with gentamicin, eight transconjugants were obtained (transfer frequency, 2×10^{-8} per donor). Seven of these transconjugants carried Cm^r Em^r and Gm^r-Km^r markers, whereas the remaining clone carried only Em^r and Gm^r-Km^r. The retransfer of the resistance markers into the BM134 recipient occurred en bloc at the same frequency as that of pIP501 (2×10^{-2} per donor), whatever drug was used for selection (chloramphenicol, erythromycin, gentamicin, or kanamycin). One plasmid was isolated in each of the eight transconjugants.

Molecular characterization of Tn*3706*. The eight plasmid DNAs were further analyzed after digestion with various restriction enzymes (*Ava*II, *Bst*EII, *Hae*III, *Hin*dIII, *Hpa*I, *Kpn*I, and *Sph*I) by agarose gel electrophoresis and DNA-DNA hybridization with probes specific for IS*256* and the *aac6′-aph2″*, *cat*, and *ermB* genes (Fig. 2 shows the results of part of this work). This analysis revealed that Tn*3706* transposed as a 5.8-kb DNA fragment from the chromosome of BM5803 in the 7.5-kb *Ava*II fragment of pIP501 (Fig. 2, lane 1) to give pIP501::Tn*3706* derivatives. The insertion (I) of Tn*3706* occurred in two different loci, I1 and I2, and the resulting pIP501::Tn*3706* derivatives were designated pIP1693 (Fig. 2, lane 2) (seven plasmids) and pIP1694 (Fig. 2, lane 5) (one plasmid), respectively. Insertion of Tn*3706* at locus I2 of pIP501 inactivated the expression of the *cat* gene of pIP501. Since in all crosses performed in the present study, including the cross BM5803(pIP501) × BM132, the mating time was 18

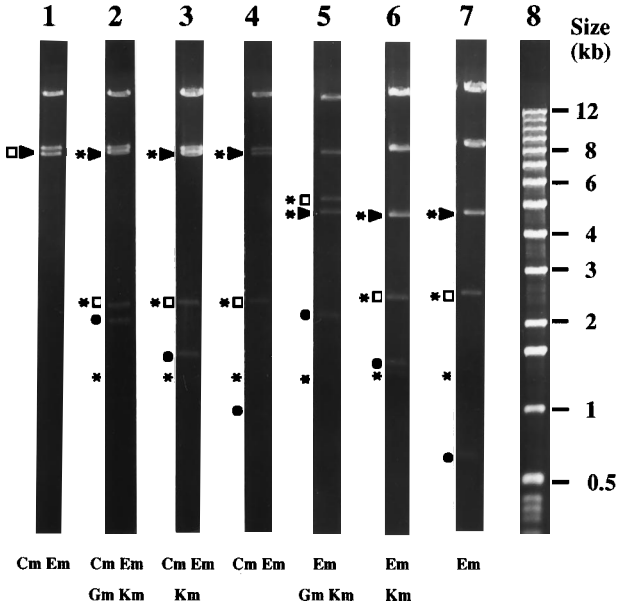

FIG. 2. *Ava*II-digested plasmid DNA. Lanes: 1, pIP501; 2, pIP1693; 3 and 4, pIP1693 deletion derivatives; 5, pIP1694; 6 and 7, pIP1694 deletion derivatives; and 8, 1-kb DNA ladder. Plasmid DNA was separated by electrophoresis (0.8% agarose gel), transferred to a nitrocellulose membrane, and probed with DNA fragments specific for *cat* (▶), *ermB* (□), *aac6′-aph2″* (●), and IS*256* (*). The positions of the DNA band hybridizing with these probes are indicated to the left of each lane. The resistance phenotype conferred to the bacterial host by these plasmids is indicated at the bottom of each lane: Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; Km, kanamycin.

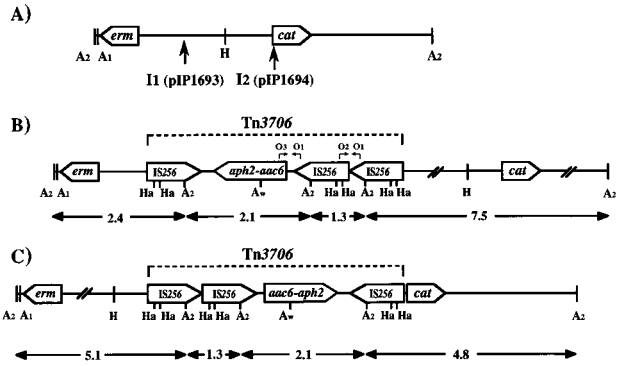

FIG. 3. Molecular analysis of Tn*3706* transposed onto pIP501. Restriction maps are of the 7.5-kb *Ava*II target DNA fragment of pIP501 (A), Tn*3706* in pIP1693 (B), and Tn*3706* in pIP1694 (C). The vertical arrows in panel A indicate the positions of the two insertion sites, I1 and I2, of Tn*3706* on pIP501. The open horizontal arrows in A, B, and C indicate the direction of transcription of the resistance genes (*aac6′-aph2″*, *cat*, and *ermB*) and of the IS-encoded transposase. The positions of the PCR primers O1, O2, and O3 in Tn*3706* are shown in panel B. The molecular sizes (in kilobases) of the *Ava*II fragments are shown below the map in panels B and C. Restriction endonuclease abbreviations: A1, *Ava*I; A2, *Ava*II; Aw, *Alw*I; Ha, *Hae*III; H, *Hpa*II. Only relevant restriction sites are shown.

h, we considered the seven pIP1693-type plasmids to be siblings. The transposition frequency of Tn*3706* onto pIP501, determined by dividing the frequency of the conjugative transfer of the Gm^r-Km^r determinant from BM5803(pIP501) to BM132 (2×10^{-8}) by the transfer frequency of pIP501 (2×10^{-2}) and by the number of generations of the donor cell before selection (about 28 generations), was 4×10^{-8}.

The establishment of the restriction map of Tn*3706* in pIP1693 (Fig. 3B) and pIP1694 (Fig. 3C) revealed that this element was composed of a central fragment containing the *aac6′-aph2″* gene flanked by two tandemly repeated copies of IS*256* at the 5′ extremity of the Gm^r-Km^r determinant and a single inverted copy of IS*256* at its 3′ extremity. The structure of Tn*3706* in pIP1693 was further characterized by determining the nucleotide sequence of the amplified IS*256*-IS*256* (Fig. 4A) and IS*256*–*aac6′-aph2″* (Fig. 4B) junction fragments. For each junction two independently amplified fragments were sequenced. That analysis confirmed the sequence identity between Tn*3706* and Tn*4001* and revealed that the two tandemly repeated copies of IS*256* were separated by a 6-bp segment (Fig. 4A) identical to that found, in the same orientation, in the IS*256*–*aac6′-aph2″* junctions in Tn*4001* (Fig. 4C).

Deletional instability of pIP501::Tn*3706* derivatives. BM132 transconjugants harboring either pIP1693 or pIP1694 were found to spontaneously segregate clones susceptible to gentamicin or to both gentamicin and kanamycin when the transconjugants were grown in the absence of selective pressure. This phenomenom was markedly enhanced when pIP1693 and pIP1694 were serially transferred by conjugation between the isogenic GBS recipients BM132 and BM134. The different plasmids that conferred various antibiotic resistance phenotypes to the bacterial hosts were further characterized on the basis of their *Ava*II restriction profiles and their DNA-DNA hybridization patterns. This analysis revealed that the pIP1693 and pIP1694 deletion derivatives susceptible to only gentamicin (Fig. 2, lanes 3 and 6, respectively) or to both gentamicin and kanamycin (Fig. 2, lanes 4 and 7, respectively) were generated by deletions of various lengths within the 2.1-kb *Ava*II fragment carrying the *aac6′-aph2″* gene in the original plasmids.

DISCUSSION

The results presented in this report demonstrate that high-level resistance to gentamicin in *S. agalactiae* B128 is due to the presence of a chromosome-borne transposable element, designated Tn*3706*, which is similar, if not identical, to Tn*4001* detected in *S. aureus* (22) and to Tn*5281* detected in *E. faecalis* (12). This assumption is based on the demonstration that the *aac6′-aph2″* gene of Tn*3706* is carried by *Ava*II, *Hae*III, *Hin*cII, *Hin*dIII, and *Sca*I DNA fragments (Table 2), which is characteristic, in terms of size and hybridization patterns, of Tn*4001*-like transposons (4, 28). Conjugative transfer of the Gm^r-Km^r determinant carried by Tn*3706* is obtained only between *S. agalactiae* strains (2; the present study) and always depends on the presence in the donor bacteria, B128 or BM5237, of the Tn*916*-like transposon Tn*3707* (6). The possibility that Tn*3706* is carried by Tn*3707* is very unlikely since (i) their resistance determinants can transfer separately into a GBS recipient (2; the present study) and (ii) the transconjugant BM5803 carrying Tn*3706* does not share sequence homology with Tn*916* (Fig. 1C). Therefore, transfer of the nonconjugative transposon Tn*3706* between strains of *S. agalactiae* might be due to its mobilization by Tn*3707*. It could also be conceivable that Tn*3706* and Tn*3707* are both carried by a larger conjugative element within the chromosome of B128.

In contrast to the presence of one copy of the Gm^r-Km^r marker in the chromosomes of B128, BM5237, and BM5803, revealed by single fragments of *Eco*RI, *Eco*RV, and *Xba*I hybridizing with the *aac6′-aph2″* gene, multiple copies of IS*256* were detected in the chromosomes of B128 (≥17 copies), BM5237 (≥3 copies), and BM5803 (≥8 copies). These observations indicate that in these strains, IS*256* could exist as an individual insertion sequence (IS) element which is not always associated with Tn*3706* and that this IS is able to colonize efficiently the genomes of GBS strains. The fact that transconjugant BM5803 carries more copies of IS*256* than donor strain BM5237 is consistent with this notion and suggests that the intracellular dissemination of this element might result from autonomous transposition. Multiple copies of IS*256* are detected in B128 and in its transconjugants BM5237 and BM5803, but this IS is not present in the genomes of the 11

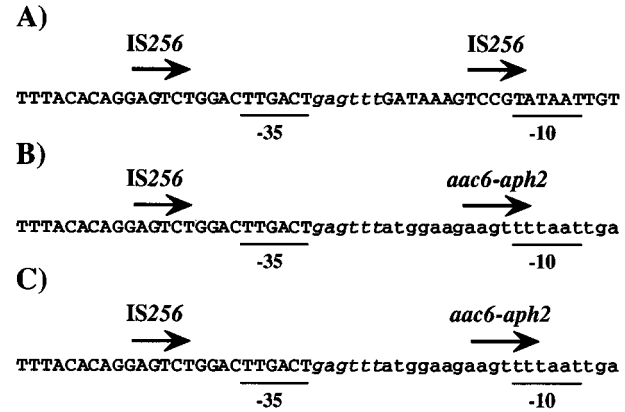

FIG. 4. Nucleotide sequence analysis of Tn*3706*. (A) the IS*256*-IS*256* junction fragment in Tn*3706*; (B) the IS*256*–*aac6′-aph2″* junction fragment in Tn*3706*; (C) the IS*256*–*aac6′-aph2″* junction fragment in Tn*4001*. The sequences of IS*256* and the segment bearing the *aac6′-aph2″* gene are indicated by uppercase and lowercase letters, respectively. The 6-bp directly repeated sequence present in both types of junctions is indicated in lowercase italic letters. The horizontal arrows indicate the direction of transcription of the *aac6′-aph2″* gene and of the IS-encoded transposase. The sequence data for Tn*4001* are from a previous report (28). Presumed −35 and −10 recognition sites are underlined.

gentamicin-susceptible GBS clinical isolates studied here. These results are similar to those reported previously for staphylococci (7) but are in contrast to those reported for enterococci, in which 76% of the gentamicin-susceptible strains studied possessed sequences homologous to IS*256* (27). The data presented here also suggest that the initial event involved in the spread of IS*256* within B128 could be the acquisition of Tn*3706*.

Transposition of Tn*3706* from the chromosome of BM5803 in two different loci of the 7.5-kb *Ava*II fragment of pIP501 was obtained in a mating out assay at a frequency of 4×10^{-8} per generation of the donor cell, a value similar to that already found for other compound transposons (11). Establishment of the molecular structure of Tn*3706* following insertion into pIP501 revealed the presence of two tandemly repeated copies of IS*256* upstream of the *aac6′-aph2″* gene, whereas a single inverted copy of this IS is present downstream of this gene. Thus, Tn*3706* in pIP1693 and pIP1694 might have a structure similar to those of the β forms of Tn*4001* and Tn*5281* identified in plasmids pSK1β in *S. aureus* (22) and pBME10 in *E. faecalis* (12), respectively. The β forms of transposons Tn*4001* and Tn*5281* contain a tandem duplication of IS*256*, but the α forms do not. Tn*3706* is located on 5.6- and 5.5-kb *Eco*RV fragments in B128 and BM5803, respectively (Fig. 1A). The sizes of these restriction fragments indicate that, unlike in pIP1693 and pIP1694, the chromosome-borne Tn*3706* does not possess an additional copy of IS*256* since its β form is approximately 5.8 kb in length. These data suggest that the tandem duplication of IS*256* upstream of the *aac6′-aph2″* gene occurs following insertion of an α form of Tn*3706* into pIP501. Sequence analysis of IS*256*-IS*256* and IS*256*–*aac6′-aph2″* junction fragments of Tn*3706* revealed that the two tandemly repeated copies of IS*256* are separated by a 6-bp segment identical to that found, in the same orientation, in the IS*256*–*aac6′-aph2″* junctions of Tn*4001* (28). The transition from the α to the β form is therefore due to the duplication of an entire copy of IS*256* plus the 6-bp segment present in the IS*256*–*aac6′-aph2″* junction. Transposition of both IS*256* and Tn*4001* has been associated with an 8-bp (three insertions) or 9-bp (one insertion) duplication of the target sequences (4, 7, 25). Thus, the transition from the α to the β form of Tn*3706* does not likely represent a duplication of the target sequence associated with the IS*256* insertion in this location. The promoter of the *aac6′-aph2″* gene is located in the IS*256*–*aac6′-aph2″* junction (28). As a consequence of the duplication event, a hybrid promoter containing a canonical −10 sequence (TATAAT) is generated in the IS*256*-IS*256* junction of the transposon's β form (Fig. 4). This might result in an increase in the level of transcription of both the downstream transposase and the *aac6′-aph2″* gene. The β form of Tn*4001*, when compared with its α form, confers a two- to fourfold increase in the level of gentamicin resistance in an *S. aureus* host (21). However, the β forms of Tn*5281* (12) and Tn*3706* (the present study) do not confer any modification of the initial level of gentamicin resistance in their bacterial hosts. The molecular basis of the rearrangement and the selective pressure, if any, responsible for the transition from the α to the β form of Tn*3706* remain to be characterized.

The ability of the chromosomal Tn*3706* to transpose onto the broad-host-range conjugative plasmid pIP501 prompted us to determine whether this type of plasmid might contribute to the dissemination of the Gm^r-Km^r determinant among streptococci. Surprisingly, both pIP1693 (pIP501::Tn*3706*, I1) and pIP1694 (pIP501::Tn*3706*, I2) exhibit high levels of structural instability following replication in a GBS host or after conjugative transfer between two GBS strains. Deletion derivatives of pIP1693 and pIP1694 conferring resistance to kanamycin, but not to gentamicin, have suffered deletions within the 2.1-kb *Ava*II fragment that contains the *aac6′-aph2″* gene. Subcloning experiments (9) have previously shown that the 5′ moiety of this gene specifies the 6′-acetyltransferase activity responsible for the Km^r phenotype, whereas its 3′ moiety specifies the 2″-phosphotransferase activity responsible for the Gm^r phenotype. These data allow us to postulate that the deletions observed within the 2.1-kb *Ava*II fragment of pIP1693 and pIP1694 derivatives conferring resistance to kanamycin, but not to gentamicin (Fig. 2, lanes 3 and 6), are located at the 3′ extremity of the *aac6′-aph2″* gene. These results constitute the first demonstration that the two enzymatic activities encoded by the *aac6′-aph2″* gene can be dissociated in a bacterial host following a transposition event onto an R plasmid. All of these observations indicate that the presence of Tn*3706* in pIP501 confers a high level of structural instability to this plasmid and suggest that broad-host-range streptococcal pIP501-like plasmids do not constitute appropriate delivery vectors for the dissemination of high-level gentamicin resistance among GBS strains. This property might account for the fact that high-level gentamicin resistance is still rarely encountered in streptococci.

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REFERENCES

1. **Brantl, S., C. Kummer, and D. Behnke.** 1994. Complete nucleotide sequence of plasmid pGB3637, a derivative of the *Streptococcus agalactiae* plasmid pIP501. Gene **142:**155–156.
2. **Buu-Hoï, A., C. Le Bouguenec, and T. Horaud.** 1990. High-level chromosomal gentamicin resistance in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. **34:**985–988.
3. **Byrne, M. E., M. T. Gillespie, and R. A. Skurray.** 1990. Molecular analysis of a gentamicin resistance transposon-like element on plasmids isolated from North American *Staphylococcus aureus* strains. Antimicrob. Agents Chemother. **34:**2106–2113.
4. **Byrne, M. E., D. A. Rouch, and R. A. Skurray.** 1989. Nucleotide sequence analysis of IS*256* from the *Staphylococcus aureus* gentamicin-tobramycin-kanamycin-resistance transposon Tn*4001*. Gene **81:**361–367.
5. **Casetta, A., G. de Cespédès, and T. Horaud.** Unpublished data.
6. **de Cespédès, G., A. Derbyse, P. Trieu-Cuot, and T. Horaud.** 1994. Mobile chromosomal elements (Tn*3706* and Tn*3707*) in *Streptococcus agalactiae* B128 resistant to gentamicin and tetracyclines, p. 272–273. *In* A. Totolian (ed.), Pathogenic streptococci: present and future. Lancer Publication, St. Petersburg, Russia.
7. **Dyke, K. G., S. Aubert, and N. El Solh.** 1992. Multiple copies of IS*256* in staphylococci. Plasmid **28:**235–246.
8. **Edwards, M. S., and C. J. Backer.** 1995. *Streptococcus agalactiae* (group B *Streptococcus*), p. 1835–1845. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Mandell, Douglas and Bennett's principles and practice of infectious diseases. Churchill Livingstone, New York.
9. **Ferretti, J., K. S. Gilmore, and P. Courvalin.** 1986. Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J. Bacteriol. **167:**631–638.
10. **Flannagan, S. E., L. A. Zitzow, Y. A. Su, and D. B. Clewell.** 1994. Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. Plasmid **32:**350–354.
11. **Galas, D. J., and M. Chandler.** 1989. Bacterial insertion sequences, p. 109–162. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
12. **Hodel-Christian, S. L., and B. E. Murray.** 1991. Characterization of the gentamicin resistance transposon Tn*5281* from *Enterococcus faecalis* and comparison to the staphylococcal transposons, Tn*4001* and Tn*4031*. Antimicrob. Agents Chemother. **35:**1147–1152.
13. **Hodel-Christian, S. L., and B. E. Murray.** 1992. Comparison of the gentamicin resistance transposon Tn*5281* with regions encoding gentamicin resistance in *Enterococcus faecalis* isolates from diverse geographic locations. Antimicrob. Agents Chemother. **36:**2259–2264.
14. **Horodniceanu, T., D. H. Bouanchaud, G. Bieth, and Y. A. Chabbert.** 1976. R

plasmids in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. **10:**795–801.
15. **Horodniceanu, T., L. Bougueleret, N. El Solh, G. Bieth, and F. Delbos.** 1979. High-level plasmid-borne resistance to gentamicin in *Streptococcus faecalis* subsp. *zymogenes*. Antimicrob. Agents Chemother. **16:**686–689.
16. **Horodniceanu, T., L. Bougueleret, N. El Solh, D. H. Bouanchaud, and Y. A. Chabbert.** 1979. Conjugative R plasmids in *Streptococcus agalactiae* (group B). Plasmid **2:**197–206.
17. **Kaufhold, A., and E. Potgieter.** 1993. Chromosomally mediated high-level gentamicin resistance in *Streptococcus mitis*. Antimicrob. Agents Chemother. **37:**2740–2742.
18. **Le Bouguénec, C., G. de Cespédès, and T. Horaud.** 1988. Molecular analysis of a composite chromosomal conjugative element (Tn*3701*) of *Streptococcus pyogenes*. J. Bacteriol. **170:**3930–3936.
19. **Le Bouguénec, C., G. de Cespédès, and T. Horaud.** 1990. Presence of chromosomal elements resembling the composite structure Tn*3701* in streptococci. J. Bacteriol. **172:**727–734.
20. **Lee, A. T., J. W. Chow, D. B. Clewell, and M. J. Zervos.** 1994. Tn*924*, a chromosome-borne transposon encoding high-level gentamicin resistance in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **38:**1152–1156.
21. **Lyon, B. R., M. T. Gillespie, and R. A. Skurray.** 1987. Detection and characterization of IS*256*, an insertion sequence in *Staphylococcus aureus*. J. Gen. Microbiol. **133:**3031–3038.
22. **Lyon, B. R., J. W. May, and R. A. Skuray.** 1984. Tn*4001*: a gentamicin and kanamycin resistance transposon in *Staphylococcus aureus*. Mol. Gen. Genet. **193:**554–556.
23. **Patterson, J. E., and M. J. Zervos.** 1990. High-level gentamicin resistance in *Enterococcus*: microbiology, genetic basis, and epidemiology. Rev. Infect. Dis. **12:**644–652.
24. **Poyart, C., J. Celli, and P. Trieu-Cuot.** 1995. Conjugative transposition of Tn*916*-related elements from *Enterococcus faecalis* to *Escherichia coli* and *Pseudomonas fluorescens*. Antimicrob. Agents Chemother. **39:**500–506.
25. **Rice, L. B., L. L. Carias, and S. H. Marshall.** 1995. Tn*5384*, a composite enterococcal mobile element conferring resistance to erythromycin and gentamicin whose ends are directly repeated copies of IS*256*. Antimicrob. Agents Chemother. **39:**1147–1153.
26. **Rice, L. B., G. M. Eliopoulos, C. Wennersten, D. Goldmann, G. A. Jaboby, and R. C. Moellering, Jr.** 1991. Chromosomally mediated β-lactamase production and gentamicin resistance in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **35:**272–276.
27. **Rice, L. B., and A. S. Thorisdottir.** 1994. The prevalence of sequences homologous to IS*256* in clinical enterococcal isolates. Plasmid **32:**344–349.
28. **Rouch, D. A., M. E. Byrne, Y. C. Kong, and R. A. Skurray.** 1987. The *aacA-aphD* gentamicin and kanamycin resistance determinant of Tn*4001* from *Staphylococcus aureus*: expression and nucleotide sequence analysis. J. Gen. Microbiol. **133:**3039–3052.
29. **Shaw, J. H., and D. B. Clewell.** 1985. Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **164:**782–796.
30. **Smith, G. E., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyl-oxymethyl-paper. Anal. Biochem. **109:**123–129.
31. **Thal, L. A., J. W. Chow, J. E. Patterson, M. B. Perri, S. Donabedian, D. B. Clewell, and M. J. Zervos.** 1993. Molecular characterization of highly gentamicin-resistant *Enterococcus faecalis* isolates lacking high-level streptomycin resistance. Antimicrob. Agents Chemother. **37:**134–137.
32. **Trieu-Cuot, P., G. de Cespédès, and T. Horaud.** 1992. Nucleotide sequence of the chloramphenicol resistance determinant of the streptococcal plasmid pIP501. Plasmid **28:**272–276.
33. **Woodford, N., E. McNamara, E. Smyth, and R. C. George.** 1992. High-level resistance to gentamicin in *Enterococcus faecium*. J. Antimicrob. Chemother. **29:**395–403.
34. **Zhang, H., R. Scholl, J. Browse, and C. Somerville.** 1988. Double-stranded DNA sequencing as a choice for DNA sequencing. Nucleic Acids Res. **16:**120.