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Molecular Analysis of a Composite Chromosomal Conjugative Element (Tn*3701*) of *Streptococcus pyogenes*

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The plasmid-free *Streptococcus pyogenes* A454 contains a conjugative element, Tn*3701*, encoding resistance to erythromycin (Em^r), tetracycline (Tc^r), and minocycline (Mn^r). We have mapped a 50-kilobase (kb) chromosomal region of A454 corresponding to the internal part of Tn*3701*. Tn*3701* includes a 19.7-kb structure, designated Tn*3703*, on which the Em^r Tc^r Mn^r determinants were localized. Tn*3703* was very similar in structure to Tn*916*. Translocation of the Em^r Tc^r Mn^r markers from A454 onto pIP964, an *Enterococcus faecalis* hemolysin plasmid, yielded different pIP964 derivatives. When the inserts of four of these derivatives were aligned with the 50-kb region of Tn*3701*, three of them were found to result from the transposition of Tn*3703* and one resulted from the insertion of a 44.0-kb portion of Tn*3701*, including Tn*3703*. Tn*3701* inserted, apparently without changing its structure, in the chromosomes of various streptococcal transconjugants, as well as in one of the 12 *E. faecalis* transconjugants studied. Tn*3703* inserted at different chromosomal sites in four *E. faecalis* transconjugants, and one copy of Tn*3701* plus an additional copy of Tn*3703* were detected in the chromosomes of seven transconjugants.

Streptococcus pyogenes (Lancefield group A) is an important pathogen that is implicated in some of the most severe human infections. Antibiotics are the main therapeutic agents known to be effective for treating the diseases caused by this species. The last 25 years have witnessed the appearance in this species of resistance to chloramphenicol, erythromycin (as well as resistance to other macrolides, lincosamides, and streptogramin B) (Em^r), and tetracyclines (Tc^r), as well as high levels of resistance to kanamycin-neomycin and streptomycin. Strains resistant to tetracyclines are generally also resistant to minocycline (Mn^r) (T. Horaud and F. Delbos, unpublished data).

The genetic basis of antibiotic resistance in group A streptococci has been intensively studied for the past 13 years. Antibiotic resistance determinants in *S. pyogenes* strains can either be carried by plasmids (6, 15) or be located on the bacterial chromosome (17, 19, 21). Conjugative transfer of chromosomal antibiotic resistance determinants in streptococci and enterococci has been reported to be mediated by conjugative transposons (7). The prototype of this new class of transposons is Tn*916* (11), which carries the *tetM* gene, encoding resistance to both tetracycline and minocycline (2).

Ten antibiotic-resistant clinical isolates of *S. pyogenes* studied in our laboratory were plasmid free. Three of them transferred their antibiotic resistance markers by conjugation into different streptococcal and enterococcal recipients (3). One of these strains, A454 (17), harbors a mobile chromosomal element encoding Em^r Tc^r Mn^r which has been designated Tn*3701* (24; C. Le Bouguénec, G. de Cespédès, and T. Horaud, Abstr. Xth Lancefield Int. Symp. Streptococci and Streptococcal Dis. 1987, abstr. no. P76, p. 96). Tn*3701* is transferred in filter matings without a vehicle plasmid to the chromosome of streptococcal and of both Rec$^+$ and Rec$^-$ *Enterococcus faecalis* recipients and translocates from the chromosome onto *E. faecalis* hemolysin plasmids and vice versa; translocation onto the hemolysin plasmid pIP964 gives rise to several derivatives which carry inserts of different sizes in various fragments of pIP964 (23).

The purpose of this study was to provide information on the molecular structure of Tn*3701*. We report here (i) the restriction map of the A454 chromosomal region comprising 50 kilobases (kb) of Tn*3701*, (ii) the degree of homology between Tn*3701* and Tn*916*, (iii) the structure of inserts of four pIP964 derivative plasmids, and (iv) the characterization of the chromosomal inserts present in 13 streptococcal and 12 *E. faecalis* transconjugants.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

Media and antibiotics. Media and growth conditions for streptococci and *E. faecalis* strains have been previously described (16). *Escherichia coli* strains were grown in LB medium (9). Ampicillin (100 μg/ml) and tetracycline (4 μg/ml) were used for the maintenance of *E. coli* plasmids.

Mating conditions. Filter matings were performed as described earlier (23).

DNA isolation. DNA was extracted from *E. faecalis* strains and from streptococci of groups A, B, C, and G as follows. An overnight broth culture of 100 ml was diluted with an equal volume of warmed broth (37°C) and incubated for 45 min at 37°C in the presence of 5% (wt/vol) glycine. Sedimented cells were suspended in 50 ml of buffer consisting of 50 mM Tris, 1 mM EDTA, and 7% sucrose (pH 8) and incubated for 10 min at 37°C in 3 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml. For group A, C, and G streptococci, sedimented cells were treated with trypsin (17) prior to lysozyme treatment. Protoplasts were lysed by adding 1% sodium dodecyl sulfate and EDTA (final concentration, 0.04 M) and incubating the mixture for 15 min at 37°C. Lysates from *S. pneumoniae* strains were obtained as described previously (23).

For plasmid DNA isolation, lysates were brought to a final concentration of 1 M NaCl, stored at 4°C for at least 90 min, and centrifuged for 10 min at 8,000 × *g*. The DNA was

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TABLE 1. Bacterial strains used in this study

Strain[a]	Relevant markers[b]	Comment (reference)
Streptococci (species or group)		
A454 (*S. pyogenes*)	Em^r Tc^r Mn^{rc}	Wild-type strain carrying Tn*3701* (17, 23)
A467 (*S. pyogenes*)	Em^s Tc^s Mn^s	Spontaneous loss of Em^r Tc^r Mn^r from A454 (23)
BM137 (*S. pyogenes*)	Fus^r Rif^r	Recipient strain (20)
BM5121 (*S. pyogenes*)	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × BM137 (23)
BM5155[d] (*S. pyogenes*)	Fus^r Rif^r	Spontaneous loss of Em^r Tc^r Mn^r from BM5121 (23)
BM132 (*S. agalactiae*)	Fus^r Rif^r	Recipient strain (18)
BM5122 (*S. agalactiae*)	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × BM132 (23)
BM138 (*S. equisimilis*)	Fus^r Rif^r	Recipient strain (20)
BM5123 (*S. equisimilis*)	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × BM138 (23)
BM140 (G)	Fus^r Rif^r	Recipient strain (20)
BM5127 (G)	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × BM140 (23)
BM120 (*S. sanguis*, Challis)	Sm^r	Recipient strain (20)
BM5128 (*S. sanguis*, Challis)	Sm^r Em^r Tc^r Mn^r	A454 × BM120 (23)
BM121 (*S. pneumoniae*, type 1)	Sm^r	Recipient strain (4)
BM5129 (*S. pneumoniae*, type 1)	Sm^r Em^r Tc^r Mn^r	A454 × BM121 (23)
E. faecalis[e]		
JH2-2	Fus^r Rif^r	Recipient strain (22)
BM133	Sm^r	Recipient strain (18)
BM5156	Sm^r Em^r Tc^r Mn^r	A454 × BM133 (this study)
BM5157	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × JH2-2 (this study)
BM5158	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × JH2-2 (this study)
BM5159	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × JH2-2 (this study)
BM5160	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × JH2-2 (this study)
BM5161	Fus^r Rif^r Em^r Tc^r Mn^r	A454 × JH2-2 (this study)
E. coli JM83	*ara* Δ(*lac-pro*) *strA thi* φ80d*lacZ*ΔM15	29

[a] All the strains are plasmid free.
[b] Fus^r, Fusidic acid resistance; Rif^r, rifampin resistance; Sm^r, high-level streptomycin resistance.
[c] Antibiotic resistance markers carried by Tn*3701*.
[d] A total of four independant clones were tested.
[e] A total of 12 *E. faecalis* transconjugants were studied.

TABLE 2. Plasmids used in this study

Plasmid	Relevant markers[a]	Comment (reference)
E. faecalis host (JH2-2)		
pIP964	Hly-Bcn	65.0 kb[b], *Eco*RI fragments A to K (23)
pIP1037	Em^r	6.2 kb[b] of Tn*3701* inserted into pIP964-*Eco*RI fragment E (23)
pIP1038	Tc^r Mn^r	13.5 kb[b] of Tn*3701* inserted into pIP964-*Eco*RI fragment G (23)
pIP1077	Em^r Tc^r Mn^r H-Hly-Bcn	19.7 kb[b] of Tn*3701* inserted into pIP964-*Eco*RI fragment E (23)
pIP1116	Em^r Tc^r Mn^r Hly-Bcn	44.0 kb[b] of Tn*3701* inserted into pIP964-*Eco*RI fragment A (23)
pAD2	Em^r Km^r Sm^r	Plasmid carrying Tn*917* (8)
E. coli host (DH1)		
pAM170	Ap^r Tc^r Mn^r	*Eco*RI-D::Tn*916* fragment of pAD1 cloned into pGL101 (12)
pAM170LT	Ap^r	pGL101 carrying regenerated *Eco*RI fragment D of pAD1 (12)
E. coli host (JM83)		
pUC8	Ap^r Lac$^+$	29
pIP1145	Ap^r Lac$^-$	pUC8 carrying 9.6-kb *Eco*RI fragment of Tn*3701* (this study)
pIP1146	Ap^r Lac$^-$	pUC8 carrying 4.4-kb *Eco*RI fragment of pIP1077 insert (this study)
pIP1147	Ap^r Lac$^-$	pUC8 carrying 4.9-kb *Eco*RI fragment of Tn*3701* (this study)
pIP1148	Ap^r Lac$^-$	pUC8 carrying 4.4-kb *Eco*RI fragment of Tn*3701* (this study)
pIP1149	Ap^r Lac$^-$	pUC8 carrying 4.0-kb *Eco*RI fragment of Tn*3701* (this study)
pIP1150[c]	Ap^r Lac$^-$	pUC8 carrying 7.5-kb *Eco*RI fragment[d] of pIP1037 insert (this study)

[a] Ap^r, Ampicillin resistance; Km^r, high-level kanamycin resistance; Hly-Bcn, hemolysin-bacteriocin production; H-Hly, hyper-beta-hemolysis; Lac$^+$, β-galactosidase production by JM83(pUC8); Lac$^-$, no production of β-galactosidase by JM83 harboring pUC8 recombinants. See Table 1, footnote *b*, for definitions of the other abbreviations.
[b] All these sizes are slightly different from those previously published (23).
[c] *E. coli* DB11 transformants carrying pIP1150 expressed Em^r (DB11 is an *E. coli* mutant sensitive to erythromycin [obtained from J. Davies]).
[d] This fragment bears the Em^r determinant (14).

recovered from the aqueous phase by ethanol precipitation and dissolved in distilled water. The plasmid DNA was further purified by ultracentrifugation in a cesium chloride-ethidium bromide gradient. Plasmid DNA from *E. coli* strains was isolated as described by Birnboim and Doly (1).

For chromosomal DNA isolation, all lysates were incubated with 100 μg of proteinase K per ml for 15 min at 37°C and then extracted at least twice with phenol-chloroform and twice with chloroform alone. After ethanol precipitation, the DNA was collected by spooling on a glass rod, washed with 70% ethanol and then with 90% ethanol, and dissolved in distilled water.

DNA analysis. The following restriction endonucleases were used: *Ava*I, *Bam*HI, *Eco*RI, *Hin*cII, *Hin*dIII, *Hpa*I, *Kpn*I, *Sal*I, and *Xba*I (Amersham International, Little Chalfont, England). Agarose gel electrophoresis of digested DNA was done as described earlier (23). The sizes of fragments smaller than 2.0 kb were measured on 1.2 to 2.0% agarose gels, and the sizes of larger fragments were measured on 0.6 to 0.8% agarose gels. Bacteriophage λ DNA, double-digested by *Eco*RI and *Hin*dIII, and a 1-kb DNA ladder (Bethesda Research Laboratories, Inc., Cockeysville, Md.) were used as molecular size markers. DNA restriction fragments were isolated from low-melting-point agarose gels by the technique described in the Multiprime DNA-labeling kit (Amersham International).

DNA-DNA hybridization. DNA was transferred from agarose gels to nitrocellulose filters by the method of Southern (27) with previously described modifications (23). DNA was labeled with [α-^{32}P]dCTP (Amersham International) by the method of Feinberg and Vogelstein (10) with the Amersham Multiprime DNA-labeling system. DNA-DNA hybridizations were performed at 65°C under stringent conditions (23). The radioactivity of the probe (specific activity, about 10^9 cpm/μg) loaded onto each filter corresponded to approximately 10^7 cpm.

Cloning. Conditions for alkaline phosphatase (Boehringer GmbH, Mannheim, Federal Republic of Germany) treatment of plasmid vector and ligation with T4 DNA ligase (Amersham International) were as described by Maniatis et al. (25). Ligation mixtures containing 0.2 to 0.4 μg of plasmid DNA and 2 μg of chromosomal DNA were used to transform *E. coli* JM83 as described by Davis et al. (9). The transformed cells were allowed to grow for 90 min at 37°C in LB medium before being plated on selective agar medium containing ampicillin, the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer), and the inducer isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer); 50 μl of 2% X-Gal and 100 μl of 10 mM IPTG were spread on the surface of selective agar medium just before the transformed cells were. Screening was performed by colony hybridization as described by Cami and Kourilsky (5).

RESULTS

Characterization of pIP1116. pIP1116 was the largest derivative plasmid obtained by translocation of Tn*3701* from the A454 chromosome onto the *E. faecalis* hemolysin plasmid pIP964 (23). There was no homology between pIP964 and the A454 chromosome. pIP1116 consisted of pIP964 with a 44.0-kb insert in its *Eco*RI A fragment. pIP1116 had five *Eco*RI fragments not present in pIP964, with sizes of 27.0, 16.5, 6.5, 4.6, and 4.0 kb (Fig. 1A). Since the 16.5- and 6.5-kb fragments were the only fragments of the pIP1116 insert to hybridize with pIP964, these fragments were considered junctions between the latter plasmid and the insert.

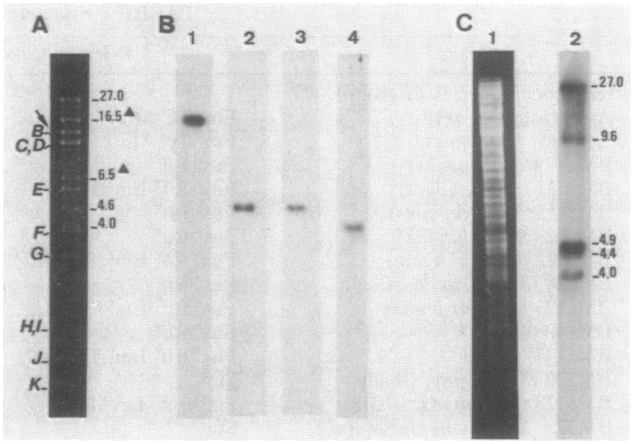

FIG. 1. Homology between A454 DNA and pIP1116. (A) Electrophoretic pattern of *Eco*RI-digested pIP1116. *B* to *K* indicate pIP964 *Eco*RI fragments of 13, 10.7 (×2), 5.7, 3.7, 2.8, 1.3 (×2), 0.8, and 0.6 kb, respectively. The arrow shows the position of the *Eco*RI A fragment (14.4 kb) of pIP964 in which the insertion had occurred that gave rise to pIP1116. The sizes (in kilobases) of the five fragments of the pIP1116 insert are indicated on the right. Symbol: ▲, junctions between the insert and pIP964. A 21.0-kb *Eco*RI fragment, poorly visible on this gel, was occasionally observed in the profile of pIP1116. Hybridization experiments (results not shown) suggest that it may result from molecular rearrangements involving the 27.0- and the 6.5-kb fragments of the insert in a subpopulation of pIP1116 molecules. (B) Autoradiograms of the same DNA after blotting and hybridization with ^{32}P-labeled plasmids as follows: lane 1, pIP1145; lane 2, pIP1147; lane 3, pIP1148; lane 4; pIP1149. (C) Hybridization between A454 DNA and [^{32}P]pIP1116. Lane 1, *Eco*RI-digested A454 DNA; lane 2, the same DNA after blotting and hybridization. The sizes (in kilobases) of the fragments showing homology are indicated on the right.

By hybridization experiments, sequences homologous to pIP1116 were localized in the A454 chromosome. Five *Eco*RI fragments (27.0, 9.6, 4.9, 4.4, and 4.0 kb) of the A454 chromosome showed homology to the insert of pIP1116. Two of these fragments (27.0 and 4.0 kb) were the same size as two of the internal fragments of the pIP1116 insert (Fig. 1C). No homology was detected between [^{32}P]pIP1116 and the chromosomal DNA of antibiotic-sensitive strains BM137, A467, or BM5155 (four clones tested) (Table 1).

Cloning and mapping of the A454 chromosomal region containing Tn*3701*. Ligation was performed between *Eco*RI-digested pUC8 and A454 DNA. Of the 2,500 Ap^r Lac$^-$ JM83 transformants obtained, 75 hybridized with pIP1116. No homology was detected between pUC8 and plasmid or chromosomal DNA of the streptococci and enterococci used in this study. Four recombinant plasmids, designated pIP1145, pIP1147, pIP1148, and pIP1149, were isolated from the 75 transformants. These carried inserts of 9.6, 4.9, 4.4, and 4.0 kb, respectively (Table 2). The cloned fragments shared homology neither among themselves nor with the chromosome of BM137 or A467. pIP1145 was homologous to the 16.5-kb junction fragment of the pIP1116 insert, both pIP1147 and pIP1148 were homologous to the 4.6-kb internal fragment of the pIP1116 insert, and pIP1149 was homologous to the 4.0-kb internal fragment of the pIP1116 insert (Fig. 1A and B). No clones were obtained that contained portions of the A454 chromosome which hybridized with either the 27.0-kb or the 6.5-kb fragment of the insert of pIP1116.

Hybridization experiments were carried out between A454 DNA digested by different restriction endonucleases and

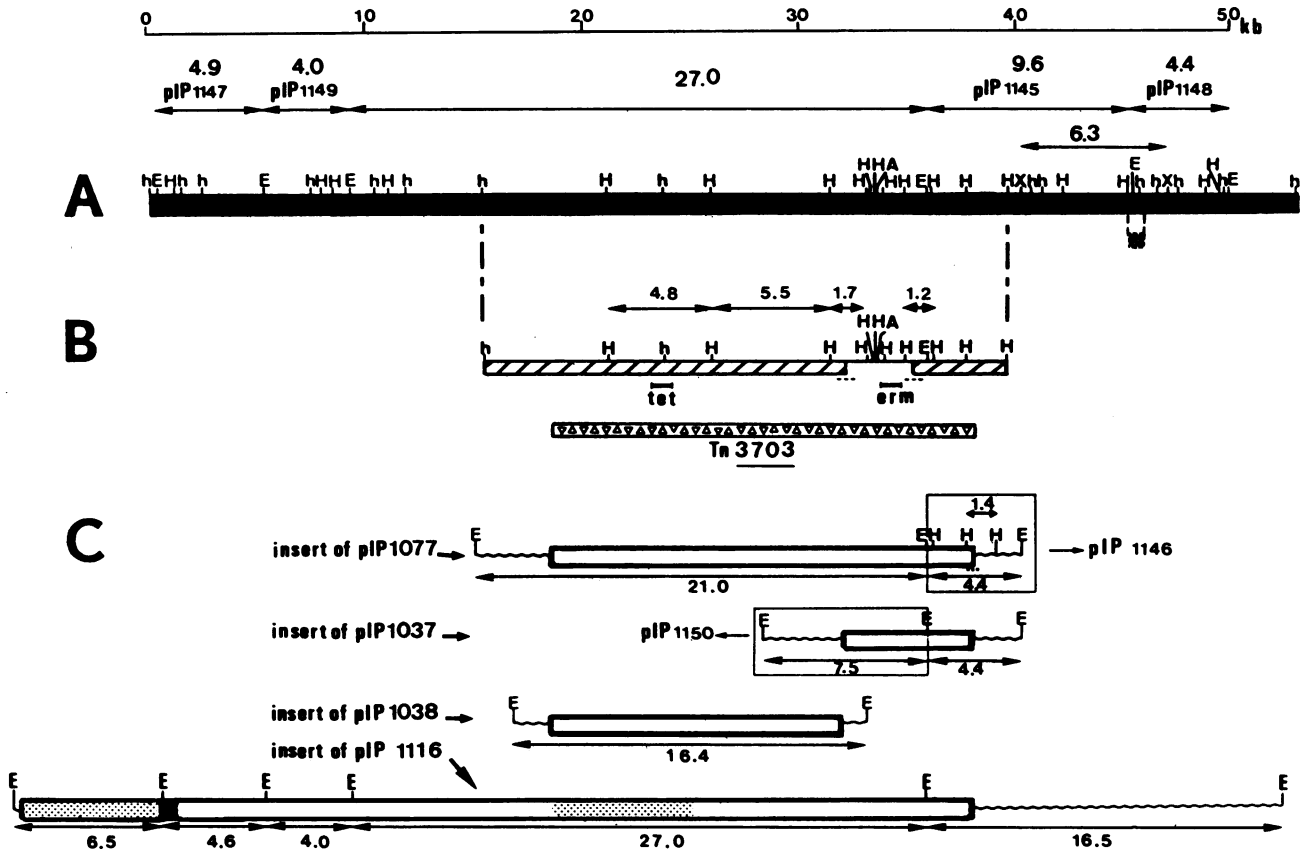

FIG. 2. Map of the A454 chromosomal region containing 50 kb of Tn*3701*. (A) Restriction sites for *Ava*I (A), *Eco*RI (E), *Hin*cII (H), and *Hin*dIII (h). The sizes (in kilobases) and positions of the five *Eco*RI chromosomal fragments which hybridized with pIP1116 are indicated, as well as the position of one *Xba*I (X) fragment of 6.3 kb. For an explanation of the area shown by ▩, see below. (B) Representation of the 24.0-kb *Hin*dIII-*Hin*cII region of Tn*3701* homologous to Tn*916*. The sizes of some *Hin*cII fragments are indicated. Symbols: ▨, regions homologous to Tn*916*; . . ., positions where homology to Tn*916* is thought to stop; erm, tet, locations of the *erm* and *tet* genes, respectively; ▨, Tn*3703* (represented below the 24.0-kb region). (C) Alignment of the inserts of the four pIP964 derivatives with Tn*3701*. The *Eco*RI fragments corresponding to the insert in each derivative are represented, and their sizes are indicated. Symbols: ▭, Tn*3701* DNA; ∿∿, pIP964 DNA. The 4.4-kb *Eco*RI fragment of pIP1077 and the 7.5-kb *Eco*RI fragment of pIP1037 cloned in pUC8, giving rise to recombinant plasmids pIP1146 and pIP1150, respectively, are framed by boxes. On the 4.4-kb *Eco*RI fragment of pIP1077, the position where pIP964 is thought to stop is marked (. . .). In the pIP1116 insert a region that was present twice is indicated (▨) and a region homologous to a part of the 4.4-kb *Eco*RI chromosomal fragment indicated by the same design in panel A is also shown (▩).

[^{32}P]pIP1145, [^{32}P]pIP1147, [^{32}P]pIP1148, and [^{32}P]pIP1149. The 27.0-kb *Eco*RI fragment of the pIP1116 insert was also used as a probe. Figure 2A shows the map of restriction sites for *Ava*I, *Eco*RI, *Hin*cII, and *Hin*dIII of 50.0 kb of A454 DNA corresponding to the internal part of Tn*3701*. On this portion, Tn*3701* was cleaved at multiple sites by *Bam*HI, *Eco*RI, *Hin*cII, and *Xba*I and at one site by *Ava*I and *Kpn*I. No recognition site was found for *Sal*I.

Sequence homology between Tn*3701* and Tn*916*. The presence of a structure similar to Tn*916* (26) on Tn*3701* was demonstrated by reciprocal hybridization experiments performed between pAM170 or pAM170LT and different restriction fragments of the A454 chromosome. The deduced location of the 24.0-kb region of homology between Tn*3701* and Tn*916* bounded by a *Hin*dIII and a *Hin*cII site is presented in Fig. 2B. Tn*3701* carried sequences homologous to the five internal *Hin*cII fragments, as well as to the two ends of Tn*916* (26). Tn*3701* had two *Hin*cII fragments homologous and equal in size to Tn*916* internal fragments (4.8 and 5.5 kb). The presence of the 4.8-kb *Hin*cII fragment homologous to that of Tn*916*, on which the *tet* gene was localized (7), would suggest that Tc^r Mn^r of Tn*3701* is encoded by the *tetM* gene (2). The 1.7- and 1.2-kb *Hin*cII fragments of Tn*3701* were both homologous to the internal 1.6-kb fragment of Tn*916*, but a region of approximately 3.0 kb bordered by these fragments did not hybridize with Tn*916*. By hybridization between the 1.3-kb *Hpa*I-*Kpn*I fragment of pAD2 (bearing the *erm* gene of Tn*917* [8]) and *Hin*cII-digested A454 DNA, the Em^r determinant of Tn*3701* was localized on the 3.0 kb of DNA situated between the two regions of homology with Tn*916*.

The *Eco*RI restriction fragments of the inserts of the four pIP964 derivatives were used to probe A454 DNA to localize them on the map of Tn*3701*. The results are shown in Fig. 2C. pIP1077 carried the region of Tn*3701* homologous to Tn*916*, including the 3.0-kb portion which carried the Em^r determinant. The precise position of the right-hand end of the insert of pIP1077 was deduced from restriction mapping of pIP1146, a recombinant plasmid comprising pUC8 and a 4.4-kb *Eco*RI fragment of the pIP1077 insert. The 1.4-kb *Hin*cII fragment of pIP1146 (Fig. 2C) contained at least 0.4 kb of Tn*3701* and 0.7 kb of pIP964, strongly suggesting that the right-hand end of the pIP1077 insert was homologous to the right-hand end of Tn*916*. The knowledge of the right-

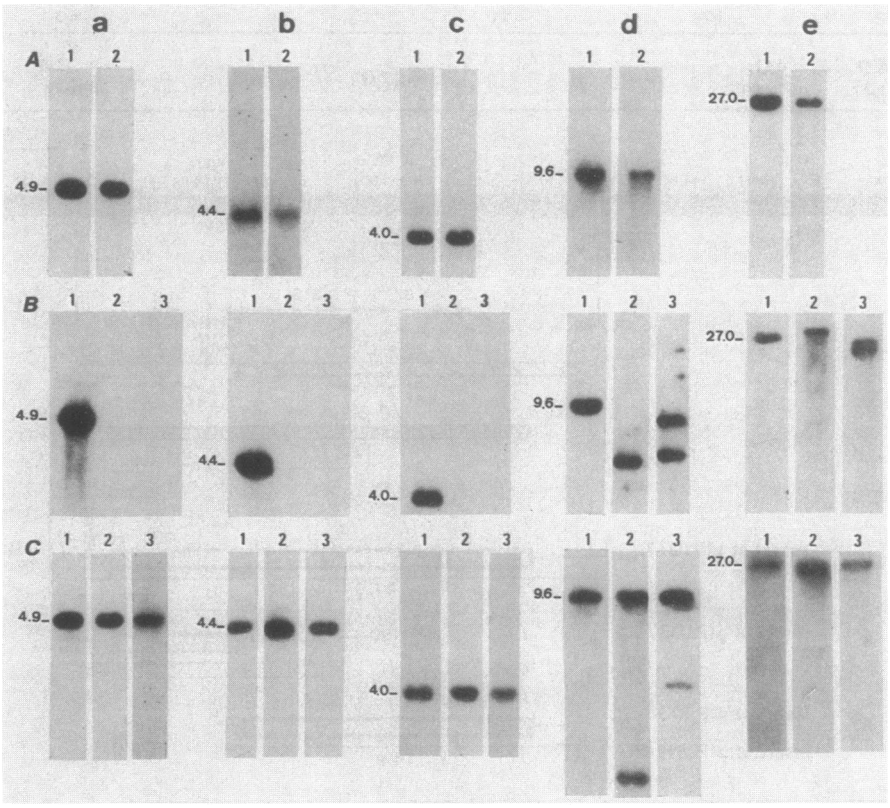

FIG. 3. Composite autoradiogram illustrating the differences between A454 and five *E. faecalis* transconjugants. (A) Category 1 of transconjugants: lane 1, A454; lane 2, BM5156. (B) Category 2 of transconjugants: lane 1, A454; lane 2, BM5157; lane 3, BM5158. (C) Category 3 of transconjugants: lane 1, A454; lane 2, BM5159; lane 3, BM5160. The *Eco*RI restriction fragments, resolved by electrophoresis through a 0.7% agarose gel, were transferred to nitrocellulose filters and hybridized with the following ^{32}P-labeled plasmids: a, pIP1147; b, pIP1148; c, pIP1149; d, pIP1146; e, the E1 probe. The sizes (in kilobases) of the fragments of A454 which hybridized are indicated.

hand end position of the pIP1077 insert allowed us to determine the location of its left-hand end, which was positioned similarly to the left-hand end of Tn*916*, that is, approximately 2.6 kb to the left of the 4.8-kb *Hin*cII fragment. These data suggest that the 19.7-kb insert of pIP1077 corresponded exactly to the structure similar to Tn*916* present in Tn*3701*; this element was designated Tn*3703* (Fig. 2B). The pIP964 derivatives pIP1037 and pIP1038 carried only portions of Tn*3703*. Restriction mapping of pIP1150, containing the cloned 7.5-kb *Eco*RI fragment of pIP1037, showed that the insert of pIP1037 ended next to the left-hand extremity of the 3.0-kb region of Tn*3703* encoding Em^r. The insert of pIP1038 appeared to start next to the site where the insert of pIP1037 stopped. pIP1116 carried 75% of the sequences of Tn*3701* contained in the 50.0-kb region of the A454 chromosome. However, the structural organization of the insert of pIP1116 differed from that of Tn*3701* in the A454 chromosome, as indicated by the following data. The 4.6-kb *Eco*RI fragment of the pIP1116 insert was homologous to parts of the 4.9- and 4.4-kb *Eco*RI chromosomal fragments (4.3 and 0.3 kb, respectively). Moreover, a chromosomal region of 6.5 kb (mapped from 18.0 to 24.5 kb), which corresponded to the left-hand end of Tn*3703*, was present twice in the pIP1116 insert: once in the 27.0-kb *Eco*RI fragment and once in the 6.5-kb junction fragment (Fig. 2C). This result indicated that the left-hand end of the insert of pIP1116 was homologous to that of Tn*3703*. Finally, the right-hand end of the pIP1116 insert corresponded to that of Tn*3703*.

Chromosomal insertion of Tn*3701* in streptococcal and *E. faecalis* transconjugants. *Eco*RI-digested DNA of streptococcal transconjugants belonging to Lancefield groups A, B, C, and G and to *S. sanguis* and *S. pneumoniae*, as well as that of the corresponding recipient strains, used as controls (Table 1), was probed with pIP1116. In each transconjugant tested, including seven other group B transconjugants (14), five *Eco*RI fragments hybridized with pIP1116 which had the same sizes as those in the wild-type strain, A454 (Fig. 1C). The Em^r Tc^r Mn^r markers of these transconjugants transferred by conjugation into appropriate streptococcal or *E. faecalis* recipients (23).

The *Eco*RI-digested DNA from 12 *E. faecalis* transconjugants was probed with pIP1146 and with the 21.0-kb *Eco*RI fragment of the insert of pIP1077 (designated here as the E1 probe) carrying the right-hand and the left-hand *Eco*RI part of Tn*3703*, respectively, as well as with pIP1147, pIP1148, and pIP1149. The 12 transconjugants fell into three categories, which were determined on the basis of the presence of either Tn*3701* or Tn*3703* alone (categories 1 and 2, respectively) or Tn*3701* plus an additional copy of Tn*3703* (category 3). Figure 3 shows the results of hybridizations between the five probes (panels a through e) and the DNA of transconjugants representing each of the three categories (A through C). The single clone of category 1, BM5156, displayed a hybridization pattern identical to that of A454 (Fig. 3A). The Em^r Tc^r Mn^r markers of BM5156 transferred into an *E. faecalis* recipient at a frequency of 2.5×10^{-9} transconjugants per donor cell. Category 2 was represented

by four transconjugants which carried only Tn*3703* (Fig. 3B). No detectable transfer of the Em^r Tc^r Mn^r markers from these clones was obtained (transfer frequency, $<1 \times 10^{-9}$), and homology was not detected with pIP1147, pIP1148, or pIP1149 (Fig. 3B, panels a through c). Moreover, hybridization of *Xba*I-digested DNA of two of these transconjugants with [^{32}P]pIP1145 (data not shown) demonstrated that they lacked the 6.3-kb *Xba*I fragment present in A454 (Fig. 2A). In each of the four clones, the fragments hybridizing with pIP1146 or the E1 probe (Fig. 3B, panels d and e) differed not only from those observed in A454, but also from each other, suggesting that they were junctions between the inserts and the chromosome and that the insertion events had occurred at different sites in the *E. faecalis* chromosome. In the chromosome of BM5158 (Fig. 3B, panels d and e, lanes 3) two fragments hybridized with pIP1146 and the E1 probe, suggesting that two copies of Tn*3703* were present. However, the MIC of tetracycline (64 to 128 μg/ml) for this clone was identical to that for the other 11 *E. faecalis* transconjugants. The seven transconjugants of category 3 carried one copy of Tn*3701* plus an additional copy of Tn*3703* (Fig. 3C). The Em^r Tc^r Mn^r markers of five of the seven clones, including BM5159 and BM5160, transferred into an *E. faecalis* recipient at frequencies of 1×10^{-8} to 1×10^{-9}. The transconjugants of this category had hybridization patterns identical to that of A454 when they were probed with pIP1147, pIP1148, or pIP1149 (Fig. 3C, panels a, b, and c, respectively). Homology to pIP1146 or the E1 probe, however, was detected on two *Eco*RI fragments: one of the hybridizing fragments was identical in size to the single hybridizing fragment in A454, and the second had a different size in each transconjugant (Fig. 3C, panel d, lanes 2 and 3, and panel e, lane 2). Moreover, *Hin*dIII-digested DNA of these transconjugants was hybridized with [^{32}P]pIP1145 or the 27.0-kb *Eco*RI fragment of the pIP1116 insert (data not shown); in each experiment, hybridizing fragments identical in size to all of those detected in A454 were observed, plus one fragment not present in A454. Three of the seven transconjugants carried only portions of either Tn*3701* or the additional copy of Tn*3703*. One of these clones, BM5160, had two *Eco*RI fragments hybridizing with pI 1146 (Fig. 3C, panel d, lane 3) but only one fragment, identical in size to that of A454, homologous to the E1 probe (Fig. 3C, panel e, lane 3), suggesting that it carried Tn*3701* plus only the right-hand region of a second copy of Tn*3703*. Another transconjugant of this category, BM5161, which did not hybridize with pIP1148 (not shown), appeared to carry only a portion of Tn*3701*.

DISCUSSION

S. pyogenes A454 harbors Tn*3701*, a chromosomal conjugative element coding for Em^r Tc^r Mn^r. A restriction map of a linear 50-kb region of the A454 chromosome was constructed (Fig. 2). This region was considered to be an internal part of Tn*3701*, since its two *Eco*RI end fragments were not homologous to the DNA of the antibiotic-sensitive strains *S. pyogenes* BM137 or A467 (Table 1). Tn*3701* is a composite conjugative element carrying a 19.7-kb transposon, designated Tn*3703*, which carries the Em^r Tc^r Mn^r determinants and which is very similar in structure to Tn*916* (26). Tn*3703* may be derived from an element closely similar or identical to Tn*916* by inserting a 3.0-kb sequence carrying the Em^r determinant. All attempts to detect by electron microscopy a stem-loop structure within self-annealed molecules of pIP1077, which carried Tn*3703*, have been unsuccessful (C. Dauguet and C. Le Bouguénec, unpublished data).

Translocation of Em^r Tc^r Mn^r from A454 onto the *E. faecalis* hemolysin plasmids pIP964 and pAD1 gave rise to several derivative plasmids. These plasmids were not considered previously to be the result of a transposition event, since the sizes of the inserts were not constant (23). The data obtained here clearly indicate that the insert of pIP1077 corresponded to Tn*3703*, and additional results (not shown) demonstrated that this was also the case for the inserts of three other pIP964 derivatives, pIP1114, pIP1117, and pIP1118 (23), as well as for the pAD1 derivative, pIP1121 (23). These derivative plasmids could be considered to be the result of a transposition event, since they resulted, in both Rec$^+$ and Rec$^-$ hosts, from insertions of the same size which occurred at different sites in the hemolysin plasmids. The derivative plasmids pIP1038 and pIP1037 carried only a part of Tn*3703*. Thus, it is likely that they resulted from Tn*3703* transposition followed by a deletion in Tn*3703*; in each case, this deletion started at the left end of the 3.0-kb region of Tn*3703*, which encodes Em^r, suggesting that this site is particularly susceptible to molecular rearrangements.

Tn*3703* also appeared to be able to transpose from the derivative plasmids to the host chromosome, as was the case for pIP1117 and pIP1118. These plasmids conferred a nonhemolytic phenotype on their hosts, since their hemolysin genes had been inactivated by the insertion of Tn*3703*. Following the conjugative transfer of pIP1117 and pIP1118, a few Em^r Tc^r Mn^r transconjugants displayed the normal hemolytic phenotype, and the plasmids isolated from these beta-hemolytic clones had restriction profiles identical to that of pIP964 (23). The fact that the hemolysin genes were restored to their normal function suggests the existence of a precise excision step during the transposition of Tn*3703*, as has been reported for Tn*916* (7).

Analysis of Em^r Tc^r Mn^r streptococcal transconjugants, as well as 1 of the 12 *E. faecalis* transconjugants, indicates that Tn*3701* transferred and inserted into the chromosome of the new hosts without changing its structure. However, since the junctions between Tn*3701* and the chromosome of A454 have not yet been identified, it is not possible to know whether the size of Tn*3701* in these transconjugants was exactly the same as in A454. Identification of the ends of Tn*3701* is now in progress in our laboratory. Analysis of the other 11 *E. faecalis* transconjugants shows that Tn*3703* inserted at multiple sites in the *E. faecalis* chromosome, as it did in pIP964. Although Tn*3703*, like Tn*916*, is able to transpose onto hemolysin plasmids, in contrast to Tn*916*, it is not able to transfer by conjugation from one *E. faecalis* chromosome to another. Senghas et al. (26) have reported that the conjugative transfer properties of Tn*916* are modified by insertion of Tn*5* into the right-hand part of the transposon. It is possible that the presence of the 3.0-kb region encoding Em^r inactivated the conjugative transfer capacity of Tn*3703*.

Three of the pIP964 derivatives carried an insert larger than Tn*3703*. Of these, pIP1116 was the largest: it carried a 44.0-kb insert which contained one copy of Tn*3703* at its right-hand end and the left-hand region of another copy of the Tn*3703* at its left-hand end. In addition, sequences of Tn*3701*, outside of Tn*3703*, were located in the internal part of the pIP1116 insert between the two copies of Tn*3703*. These results, as well as those obtained with category 3 of *E. faecalis* transconjugants, suggest that during transposition of Tn*3703*, a duplication of Tn*3703* might have occurred and might have mediated the insertion of other sequences of

Tn*3701*. Moreover, some sequences located in the *Eco*RI end fragments of the A454 chromosomal structure of Tn*3701* were carried by a single internal *Eco*RI fragment of the pIP1116 insert. From all these data, it seems possible that Tn*3701* assumes a circular form before its insertion in a replicon. This form may be similar to the intermediate circular form which is believed to represent an intermediate in cellular or intercellular transposition of Tn*916* (7). Furthermore, the insertion of Tn*3701* into an *E. faecalis* replicon might sometimes resemble the replicon fusion generated by transposons such as Mu (28) or Tn*3* (13).

The results obtained in this study indicate that genetic elements similar to Tn*916* may acquire antibiotic resistance determinants giving rise to elements such as Tn*3703*. Moreover, large mobile elements, such as Tn*3701*, may be composite elements carrying within them structures similar to Tn*3703*. Tn*3701* and Tn*3703* are useful tools for investigating the relationships among the conjugative and nonconjugative elements that frequently occur in plasmid-free antibiotic-resistant streptococci (Le Bouguénec et al., manuscript in preparation).

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