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Transposition of Tn*1545*-Δ*3* in the Pathogenic Neisseriae: a Genetic Tool for Mutagenesis

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The ability to study the virulence of pathogenic *Neisseria* spp. has been greatly limited by the absence of genetic tools which allow the construction of defined mutants. We have engineered a transposon system which allows random mutagenesis of the *Neisseria* genome at relatively high frequency. Tn*1545*-Δ*3* is a 3.4-kb derivative of the gram-positive transposon Tn*1545* encoding resistance to kanamycin. Tn*1545*-Δ*3* was subcloned into an erythromycin-resistant derivative of the mobilizable shuttle vector pLES2 to yield the plasmid pMGC20. This latter plasmid was introduced by conjugation from *Escherichia coli* S17-1 into *Neisseria meningitidis* 8013N and *Neisseria gonorrhoeae* 15063G. Kanamycin-resistant 8013N and 15063G transconjugants appeared at frequencies of 10^{-5} and 10^{-6}, respectively. Restriction enzyme analysis and Southern blot hybridization of these transconjugants showed that, in *Neisseria* spp., the transposon excised spontaneously from pMGC20 and integrated into chromosomal DNA. Our studies revealed that (i) transposition of Tn*1545*-Δ*3* was in numerous, apparently distinct sites, (ii) in most cases, for each transconjugant a single copy of Tn*1545*-Δ*3* was integrated into the chromosome, and (iii) several passages on selective media did not induce secondary transposition. The kanamycin resistance marker expressed by the transconjugants was subsequently transformed into a parental background without change in the chromosomal location of the transposon. To assess the role of the general recombination system in the transposition of Tn*1545*-Δ*3*, the *recA* gene of *N. meningitidis* has been cloned and a *rec* derivative of 8013N has been engineered. Similar results were obtained when this latter strain was used as recipient, suggesting that *recA* functions were not required for Tn*1545*-Δ*3* transposition in *N. meningitidis*. Transposition with Tn*1545*-Δ*3* may be an important technique for mutagenesis of the pathogenic neisseriae.

The field of prokaryotic biology has greatly benefited from the technique of transposon mutagenesis. *Neisseria meningitidis* and *Neisseria gonorrhoeae* are two important human pathogens with respect to the morbidity and severity of the diseases they induce. Unfortunately, studies of the neisseriae have been hampered by the limited number of genetic tools available (25). Although DNA transformation and conjugation allow the introduction of genetic material into the neisseriae, defined mutations are essentially engineered by means of shuttle mutagenesis (23, 24), the process by which cloned neisserial genes are transposon mutated in *Escherichia coli* and subsequently reintroduced into the neisseriae via DNA transformation and recombination. By its very nature, the generation of such defined mutations is time consuming. Moreover, most of such mutagenesis studies require the efficient expression of neisserial genes in *E. coli*, tolerance of the foreign antigens by the *E. coli* host, and the availability of antibodies to these antigens for identification of recombinant clones. Many such studies are hampered by the lack of one or more of the above requisites. Therefore, a direct and efficient transposon mutagenesis system would greatly aid in studies of the neisseriae.

Transposon Tn*1545*, a 25.3-kb element originally detected in the chromosome of *Streptococcus pneumoniae*, is a member of a closely related family of conjugative transposons which includes Tn*916*, Tn*918*, and Tn*920* from *Enterococcus faecalis* and Tn*919* from *Streptococcus sanguis* (6, 7, 26). These elements are self-transferable to a large variety of gram-positive bacteria where they are able to transpose to various sites. Tn*1545* transposes in *E. coli*, but conjugal transfer does not seem to occur. Deletion studies in Tn*1545* have established that a small derivative of only 3.4 kb, Tn*1545*-Δ*3*, encoding the kanamycin resistance gene *aphA-3* and the *int-Tn* and *xis-Tn* open reading frames, is still able to excise in *E. coli* (19, 20).

Recently, the 16.5-kb transposon Tn*916* has been introduced by transformation into *N. meningitidis* and shown to transpose (10). However, the low frequency of transposition in *N. meningitidis* (10^{-7}) along with the absence of transposition in *N. gonorrhoeae* limit the use of this system as a genetic tool. In this report, we show that Tn*1545*-Δ*3*, introduced by conjugation into *N. meningitidis* and *N. gonorrhoeae*, transposes randomly into the chromosome of these bacteria at a frequency of 10^{-5} and 10^{-6}, respectively. In addition, we demonstrate that in *N. meningitidis* these transposition events are independent of *recA* function.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown in LB broth (15). *Neisseria* strains were grown either on GCB agar containing the supplements described by Kellogg et al. (13) or on brain heart infusion (BHI) agar supplemented with 2.5% calf serum. Only piliated variants of *N. gonorrhoeae* were used in this study. The neisseriae were incubated at 37°C with 5% CO_2. When necessary, the following antibiotics were used with *E. coli*: kanamycin, 20 μg/ml; erythromycin, 300 μg/ml; tetracycline, 6 μg/ml; and chloramphenicol, 20 μg/ml. With neisseriae, the following antibiotic concentrations were

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Neisseria sp.		
8013	*N. meningitidis* serotype C	J.-Y. Riou[b]
8013N	One-step Nal^r mutant of 8013	This work
8013NSp	One-step Spc^r mutant of 8013N	This work
8013N*rec*	Derivative of 8013N *recA*::*tetM* Tet	This work
15063G	*N. gonorrhoeae*	C. Davis[b]
E. coli		
HB101	F$^-$ *hsdS20 recA13 ara-14 proA2 thi lacY1 galK2 leu rpsL20 xyl-5 mtl glnV44*	3
S17-1	*pro thi recA hsdR*; chromosomal RP4-2 (Tn*1*::ISR*1* *tet*::Mu Km::Tn*7*)	27
Plasmids		
pLES2	*bla*$^+$	28
pHSS6	Km	24
pOX38::mTnCm3	Tra$^+$, carries a mini-Tn*3* expressing Cm^r	23
pSC101::Tn*1545*-Δ*3*	Tet *aphA-3*, carries Tn*1545*-Δ*3*	18
pAT110	*bla*$^+$ *ermAM*$^+$	29
pAT182	*bla*$^+$ *tetM*	16
pMGC10	pLES2Δ*bla*::*ermAM*	This work
pMGC20	pMGC10::Tn*1545*-Δ*3*	This work
pMGC30	pHSS6 (6.5-kb insert containing the *N. meningitidis recA* gene)	This work
pMGC31	pMGC30*recA*::mTnCM3	This work
pMGC35	pMGC35Δ (*recA*-mTnCm3)::*tetM*	This work

[a] Cm, Chloramphenicol; Tet, tetracycline; Spc, spectinomycin; Km, kanamycin.
[b] *N. meningitidis* 8013 and *N. gonorrhoeae* 15063G were obtained from J.-Y. Riou (Laboratoire des Neisseriae, Institut Pasteur, Paris) and from C. E. Davis (Department of Pathology, Medical Center, University of California at San Diego), respectively, and both were fresh human isolates.

used: kanamycin, 100 μg/ml; erythromycin, 2 μg/ml; tetracycline, 2 μg/ml; nalidixic acid, 20 μg/ml; spectinomycin, 75 μg/ml; and colistin, 20 μg/ml. The sensitivity of *Neisseria* strains to tobramycin was assessed by placing antibiotic sensitivity disks on plates during subcultures. The growth of *E. coli* in the presence of methyl methanesulfonate (MMS) was assessed by adding 2 μl of this compound per 20 ml of LB agar (1).

Transformation. Neisseriae were grown on plates for 18 h and harvested into prewarmed 37°C GCB broth with 5 mM $MgSO_4$ at about 10^8 CFU/ml. This preparation was diluted 1:10 into the same medium with approximately 20 μg of the appropriate DNA per ml and incubated at 37°C with CO_2 for 30 min without shaking. Plasmids were linearized prior to transformation unless otherwise stated. The bacteria-DNA mixture was then diluted 1:10 into GCB broth with supplements and incubated at 37°C with CO_2 for 3 to 5 h. Dilutions were made in GCB broth and plated on appropriate media. The transformation efficiency was estimated as the number of transformants per parental cell.

Conjugation. Conjugations were performed by filter mating. *E. coli* donor strains were grown overnight in LB broth containing the appropriate antibiotics, diluted 1:100 in GCB broth with supplements, and incubated without shaking at 37°C for 1.5 h. Eighteen-hour-old plates of the recipient *Neisseria* strains were harvested in 5 ml of prewarmed GCB broth at about 10^8 CFU/ml and diluted 1:50 in the same prewarmed media. This subculture was incubated without shaking for 2 to 4 h at 37°C with 5% CO_2 so that the surface of the subculture had a high surface-to-volume ratio in the flasks. A total of 10^7 recipient cells and 10^6 donor cells from these subcultures were mixed and filtered through a GS membrane filter (Millipore Corp.; pore size, 0.22 μm) by use of a syringe. Filters were placed on GCB agar plates, and the bacteria were allowed to mate at 37°C with 5% CO_2 for 3 to 5 h before being eluted in 3 to 5 ml of GCB broth. Transconjugants were then selected on appropriate selective media. *N. meningitidis* and *N. gonorrhoeae* were allowed to grow for 24 and 48 h, respectively. The transfer frequency was estimated as the number of transconjugants per recipient cell present after the matings.

DNA manipulations. Chromosomal DNA was isolated from neisseriae by the following method. Colonies were grown in GCB medium containing the appropriate antibiotics. Cells from a single 100-mm-diameter petri plate were harvested with a sterile Dacron swab into 0.5 ml of TES (50 mM Tris [pH 8.0]–20 mM EDTA–50 mM NaCl). Boiled RNase A (20 μg/ml) and 0.1% sodium dodecyl sulfate were added, and the cells were lysed at 42°C. Two TES-saturated phenol extractions and one chloroform-isoamyl alcohol (24:1) extraction were performed to remove proteins. The DNA was then precipitated with 2 volumes of isopropanol and washed with 70% ethanol. The pellet was dried and dissolved in TE (10 mM Tris [pH 8.0]–1 mM EDTA). Plasmid DNA from both *Neisseria* spp. and *E. coli* was isolated by the technique of Birnboim and Doly (2). Large-scale preparations were further purified by CsCl-ethidium bromide gradient centrifugation.

The methods used for transferring DNA and hybridizing, constructing, and manipulating recombinant DNA were essentially those outlined by Sambrook et al. (22). The probe used to detect Tn*1545*-Δ*3* was obtained by DNA amplification of a 439-bp fragment located in the *int-Tn* gene (see Fig. 1). The following oligonucleotides were chosen based on the previously published sequence: 5′-GATTTCATGTGTCG CAGAATCCAC-3′ and 5′-CTTTTGGAGAAATATACGTC TGGG-3′ (19). The probe used to detect *tetM* sequence was a 605-bp fragment located within the *tetM* gene. This probe was obtained by amplification with the oligonucleotides (16) 5′-GATTTCATGTGTCGCAGAATCCAC-3′ and 5′-CTTT TGGAGAAATATACGTCTGGG-3′ (see Fig. 4). The polymerase chain reaction was carried out as previously described (21). The amplified fragments were further purified by electroelution from agarose gel. Double-stranded DNA

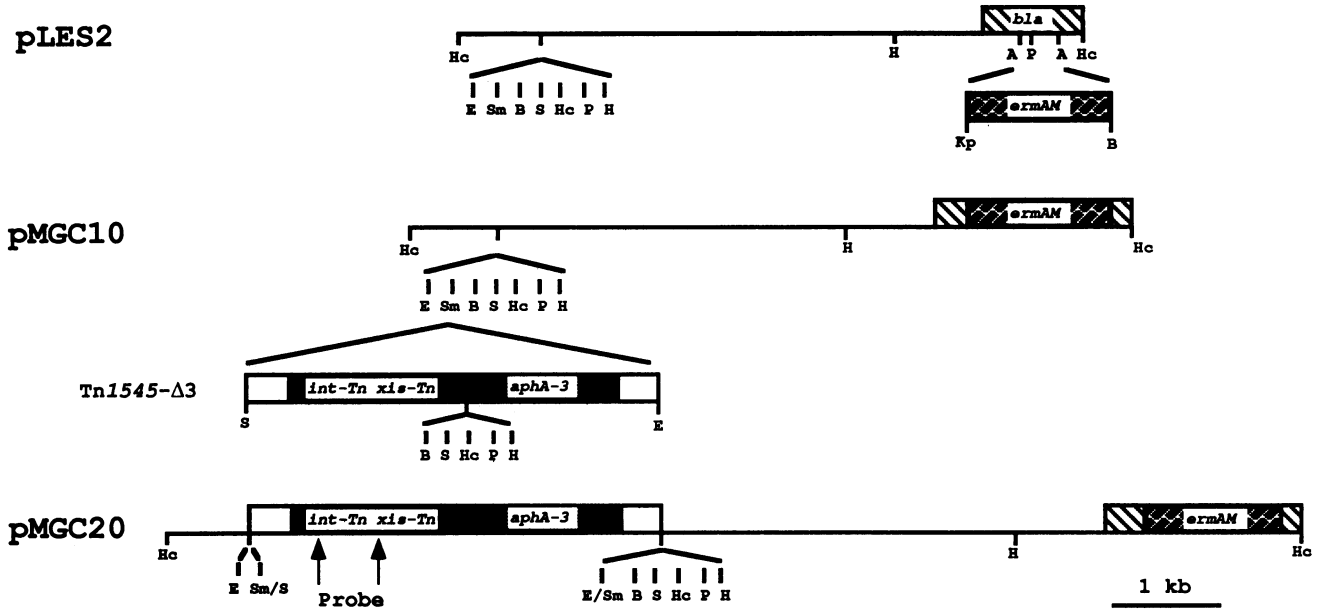

FIG. 1. Restriction map of the transposon-containing vector. White boxes represent sequences corresponding to the target gene in which Tn*1545* was inserted when cloned from *S. pneumoniae*. The 439-bp fragment amplified and used as probe is indicated. Abbreviations for restriction endonuclease sites: A, *Ava*II; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Hc, *Hin*cII; H, *Hin*dIII; Kp, *Kpn*I; N, *Not*I; P, *Pst*I; RV, *Eco*RV; S, *Sal*I; Sc, *Sca*I; Sm, *Sma*I.

fragments were ^{32}P labeled by using a random primer as previously described (8).

RESULTS

Transposition of Tn*1545*-Δ*3* in *N. meningitidis*. pLES2 (Fig. 1) is the only shuttle vector able to replicate in *E. coli* and *Neisseria* spp. (28). It has been shown to be mobilizable from *E. coli* into *N. gonorrhoeae* by derivatives of RP1 (17). Penicillin is one of the major therapeutic options against *N. meningitidis*. To adapt pLES2 for use in *N. meningitidis*, the *bla* gene of pLES2 was deleted and replaced by the *ermAM* gene which encodes resistance to erythromycin (Fig. 1). The *ermAM* gene was purified from pAT110 as a 1.2-kb *Kpn*I-*Bam*HI fragment and blunt-end ligated between the *Ava*II sites of pLES2. This recombinant plasmid was named pMGC10. *E. coli* S17-1, which contains a derivative of RP4 integrated into the chromosome, was able to mobilize pMGC10 into *N. meningitidis* 8013N at a frequency of 10^{-4}. The analysis of the plasmid content of 15 transconjugants showed that the recipient meningococcal cells harbored an incoming plasmid of the same size (data not shown).

Tn*1545*-Δ*3*, a deletion of the gram-positive transposon Tn*1545*, is capable of excision (19). This deleted product contains three open reading frames: one encodes AphA-3, which provides resistance to kanamycin but not to tobramycin (5). The products of the other open reading frames, *xis-Tn* and *int-Tn*, are responsible for excision and integration of the transposon (20). Tn*1545*-Δ*3* was provided to us as pSC101::Tn*1545*-Δ*3* (18); this plasmid was constructed by using an approach similar to that which yielded pUC::Tn*1545*-Δ*3* (19). Briefly, a 1.9-kb *Eco*RI-*Hin*dIII DNA fragment containing the left extremity of Tn*1545* plus the kanamycin resistance gene was cloned between the *Eco*RI and *Hin*dIII sites of pSC101 (4). In a subsequent step, the 2.1-kb *Sau*3A fragment containing the *xis-Tn* and *int-Tn* genes and the right extremity of Tn*1545* was cloned into the multiple cloning sites of pUC1813 (11). This fragment flanked by *Hin*dIII sites was then subcloned into the single *Hin*dIII site of the pSC101 derivative described above to yield pSC101::Tn*1545*-Δ*3*. The proper orientation of the 2.1-kb *Sau*3A fragment was determined by restriction analysis. Tn*1545*-Δ*3* was observed to excise at high frequency from a high-copy-number replicon such as pUC; in contrast, this transposon excised at low frequency from pSC101 (18).

Tn*1545*-Δ*3* was purified from pSC101::Tn*1545*-Δ*3* as a *Sal*I-*Eco*RI fragment and blunt-end ligated into the *Sma*I site of pMGC10 to produce pMGC20 (Fig. 1). pMGC20 was then introduced into *N. meningitidis* 8013N by mobilization from *E. coli* S17-1. *N. meningitidis* 8013N transconjugants were selected by placing the mixture on GCB agar plates containing erythromycin, kanamycin, and nalidixic acid or on BHI agar plates containing kanamycin and nalidixic acid. Since GCB agar appeared to inhibit the effect of kanamycin (unpublished data), BHI agar was utilized instead of GCB agar when kanamycin was the only antibiotic used for selecting the transconjugants. The transconjugants were purified by streaking on the same media. To confirm that the resistance to kanamycin was related to the production of the AphA-3 encoded by the transposon, each transconjugant was assessed for tobramycin sensitivity as described in Materials and Methods. The frequency at which the meningococcal recipients became resistant to kanamycin was approximately 10^{-5} (range, 0.7×10^{-5} to 5×10^{-5}). This frequency was the same regardless of the selection media used. Upon overnight growth on media without kanamycin, the transconjugants retained their resistance to kanamycin (reversion frequency, less than 10^{-2}). To eliminate the possibility that, during the matings, kanamycin-resistant *N. meningitidis* strains arose by transformation of pMGC20 DNA free in the media and not by conjugation, *N. meningitidis* 8013N was transformed with 1 μg of pMGC20 DNA. The experiment was performed by using *Eco*RI-linearized

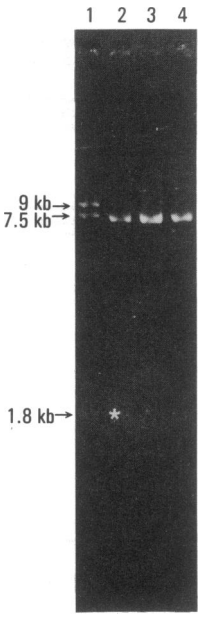

FIG. 2. Plasmid content of *N. meningitidis* transconjugants. Lane 1, *Sal*I digests of the plasmid content of *E. coli* S17-1(pMGC20); lanes 2 through 4, *Sal*I digests of the plasmid content of three *N. meningitidis* transconjugants.

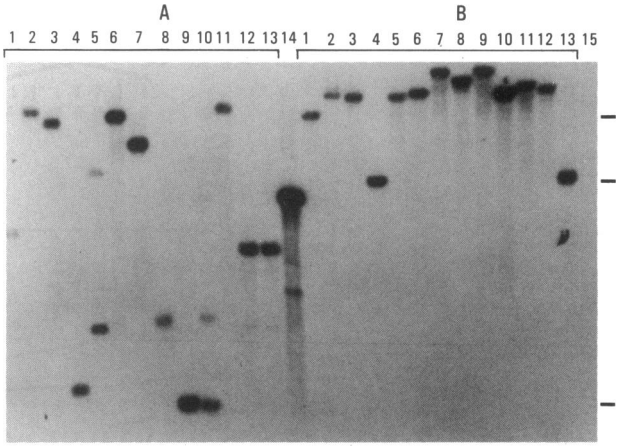

FIG. 3. Southern hybridization analysis with the 439-bp probe described in Materials and Methods of the total DNA of 13 *N. meningitidis* transconjugants (lanes 1 through 13) after digestion with *Cla*I-*Hin*dIII (A) and *Cla*I (B). Lane 14 corresponds to *Hin*dIII-cut pMGC20; lane 15 is loaded with total DNA from the recipient *N. meningitidis* 8013N. Molecular weights are indicated on the side of the figure and are, from top to bottom, 8.5, 5.6, and 1.9 kb. *Cla*I does not cut within the transposon. *Hin*dIII cuts once, this site being outside the fragment used as probe.

DNA and uncut DNA. No kanamycin-resistant transformant arose on kanamycin-containing BHI agar plates (frequency less than 10^{-7}).

Although *N. meningitidis* 8013N apparently does not harbor any cryptic resident plasmid, the plasmid content of *N. meningitidis* transconjugants and *E. coli* S17-1(pMGC20) differs slightly. Plasmid extracts of some transconjugants and S17-1(pMGC20) digested with *Sal*I are shown in Fig. 2. The restriction profile of a *Sal*I digest of pMGC20 prepared from *E. coli* S17-1 contains three bands of 9, 7.5, and 1.8 kb (Fig. 2, lane 1), although the predicted pattern deduced from the restriction map is two fragments of 8.9 and 1.8 kb (Fig. 1). Since Tn*1545* tends to excise spontaneously when carried on a multicopy vector in *E. coli* (7), the most likely explanation for this disparity of results is that the fragment of 7.5 kb corresponds to a transposon-deleted derivative of pMGC20. Such a deleted plasmid would contain pMGC10 and a 500-bp fragment from the transposon within the *Sma*I site (Fig. 1). This 500-bp sequence most likely corresponds to the streptococcal target in which the transposon was inserted and brought along with the transposon when it was cloned. *Sal*I digestion of this transposon-deleted derivative of pMGC20 would generate only one fragment of 7.3 kb, which is in good agreement with the band of 7.5 kb seen in Fig. 2, lane 1. Supporting this hypothesis, an internal fragment of the transposon (Fig. 1) in a Southern blot did not hybridize to the 7.5-kb plasmid fragment (data not shown).

The study of the plasmid content of the *N. meningitidis* transconjugants revealed that the transposon was not present on the shuttle vector. *Sal*I digests of the plasmid content of three *N. meningitidis* transconjugants are shown in Fig. 2, lanes 2 through 4. Only one band of 7.5 kb is present in all cases. Hybridization studies with the 439-bp Tn*1545*-Δ*3* internal fragment as probe confirmed the absence of a signal specific for Tn*1545*-Δ*3* in the plasmid of these transconjugants (data not shown). The same plasmid pattern was obtained with 49 other transconjugants generated from three different matings. These results suggest that, when introduced into *N. meningitidis*, the transposon cannot be maintained stably on pMGC20 and excises spontaneously. Since the transconjugants are resistant to kanamycin and sensitive to tobramycin, Tn*1545*-Δ*3* must be inserted into the chromosome.

Total DNA from 39 of the 49 transconjugants was purified. As previously mentioned, these transconjugants were generated from three different matings, which will be called conjugations 1, 2, and 3. Twelve of the 39 studied transconjugants were from conjugation 1, 12 were from conjugation 2, and 15 were from conjugation 3. The DNA of these 39 transconjugants was digested with *Cla*I and *Cla*I-*Hin*dIII, and Southern blot hybridizations were performed by using the probe described in Fig. 1. *Cla*I does not cut within Tn*1545*-Δ*3*, while *Hin*dIII cuts once within the transposon, this site being outside the fragment used as probe (Fig. 1). Results of these Southern blot hybridizations indicate that (i) the transconjugants have a single copy of the transposon and (ii) 8 of the 12 transconjugants isolated from each of conjugations 1 and 2 carry the transposon in different sites, and 11 of the 15 selected transconjugants from conjugation 3 have the transposon inserted into different places. In addition, 18 of the 19 transconjugants carrying independent transposition events from conjugations 1 and 3 were further studied. The transposon was inserted in 17 different places among these 18 insertions. A Southern blot hybridization of 13 of these 18 chromosomal DNA preparations is shown in Fig. 3. These results suggest that the identical events obtained in each experiment are more likely to be siblings from the matings than to correspond to hot spots of insertions of the transposon.

The hybridization pattern of two transconjugants was determined after seven passages on kanamycin-containing GCB agar. When the same probe was used as described above, the hybridization profile of these transconjugants remained the same, suggesting that once the transposon is

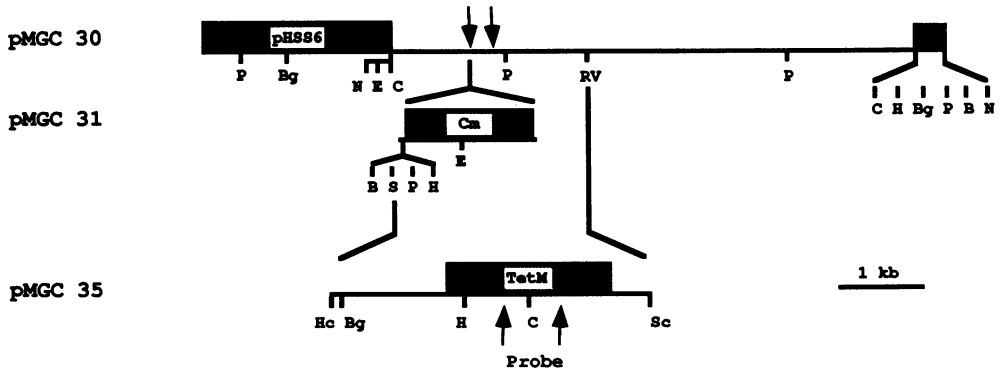

FIG. 4. Restriction map of the sequences encompassing the *N. meningitidis recA* gene. Arrows indicate locations of the mTnCm3 insertions in pMGC30 inactivating the ability of HB101 to grow on MMS plates. The amplified *tetM* internal fragment used as probe is indicated. Abbreviations for restriction endonuclease sites are described in the legend to Fig. 1.

inserted into the chromosome no secondary transposition occurs (data not shown).

Transformation of the chromosomal kanamycin-resistant marker. Chromosomal DNA from 2 of the 39 transconjugants selected above was transformed into the parental strain 8013N, and the transformants were selected on BHI agar plates containing kanamycin. Transformants were obtained at a frequency of approximately 10^{-5}. All of the transformants tested (20 for each transformation) were erythromycin sensitive, indicating that the transposon insertions into the chromosome are free of vector sequences. For each transformation experiment, DNA from four transformants was isolated and subjected to Southern blot analysis with the same Tn*1545*-Δ*3* internal probe. The second-generation transformants had the same hybridization patterns as the donor DNA used to transform them (data not shown). These data suggest that transfer of the kanamycin resistance to the recipient cells was due to homologous recombination between donor and recipient via the sequences flanking the transposon and not to transposition events.

Construction of a *recA* mutant of *N. meningitidis*. To assess the role of the *recA* gene in the transposition of Tn*1545*-Δ*3*, a *recA* derivative of 8013N was engineered. Total DNA from 8013N was digested with *Cla*I and ligated into the *Cla*I site of pHSS6, which encodes resistance to kanamycin. The ligation product was transformed into *recA E. coli* HB101. Transformants were selected by plating on LB plates containing kanamycin and MMS. *rec* strains are deficient in homologous recombination and DNA repair functions and therefore cannot grow in the presence of alkylating agents like MMS unless complemented (1, 9, 12, 14). One recombinant plasmid, pMGC30, allowing interspecific complementation of the RecA phenotype of HB101, was selected for further analysis. The restriction map of this plasmid is shown in Fig. 4. The location of the *recA* gene on the 6.5-kb insert of pMGC30 was further determined by using the shuttle mutagenesis system (23, 24). Two independent insertions of the mTnCm3 inactivated the MMS resistance conferred by pMGC30 to HB101 (Fig. 4). These mapped close to the 0.9-kb *Pst*I-*Eco*RV fragment which corresponds to the 0.8-kb *Pst*I-*Eco*RV fragment located within the *recA* gene of *N. gonorrhoeae* (14).

Since chloramphenicol is one therapeutic option in the treatment of meningococcal disease, the mutation carried by these insertions could not be shuttled back into *N. meningitidis* 8013N. pMGC31, which carries one of the mTnCm3 insertions inactivating the *recA* gene of pMGC30, was selected for further experimentation. The *Sal*I-*Eco*RV fragment of pMGC31, containing the chloramphenicol resistance gene, was replaced with a 4-kb *Hin*cII-*Sca*I fragment carrying the *tetM* gene from pAT182 to yield pMGC35 (Fig. 4). pMGC35 was digested with *Not*I and used to transform *N. meningitidis* 8013N. Transformants were selected on GCB agar plates containing tetracycline. One transformant, named 8013N*rec*, was chosen for further studies. Southern hybridizations of 8013N*rec* DNA, using as probes the 3-kb *Sal*I-*Eco*RV fragment of pMGC31 and the amplified 605-bp *tetM* internal fragment generated with the oligonucleotides described in Material and Methods (Fig. 4), showed that the wild-type *recA* gene had been replaced by the mutated one (Fig. 5). The effects of this mutated allele in 8013N*rec* on

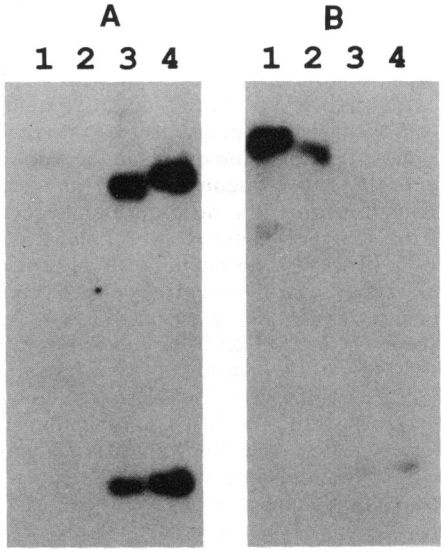

FIG. 5. Southern hybridizations showing the replacement of the wild-type *recA* gene by the mutated one in 8013N*rec*. Lane 1, *Cla*I digest of pMGC 30; lane 2, *Cla*I digest of total DNA of 8013N; lane 3, *Cla*I digest of 8013N*rec*; lane 4, *Cla*I digest of pMGC35. A, Hybridization with the 605-bp *tetM* internal fragment which carries a *Cla*I site; B, hybridization with the 3-kb *Sal*I-*Eco*RV fragment of pMGC31.

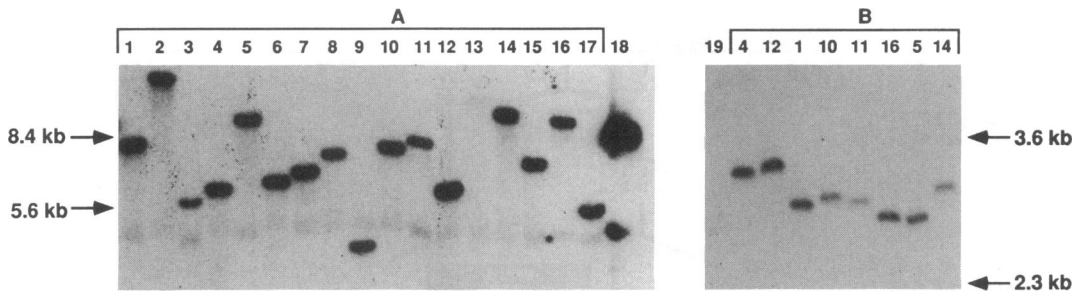

FIG. 6. Southern hybridization analysis with the 439-bp probe described in Materials and Methods of the total DNA of 17 random *N. gonorrhoeae* transconjugants (lanes 1 through 17) after digestion with *Cla*I (A) and after digestion with *Cla*I-*Hin*dIII (B). Lane 18 is loaded with uncut pMGC20; lane 19 is loaded with total DNA from the recipient *N. gonorrhoeae* 15063G.

properties associated with RecA functions were assessed by the ability of the strain to be transformed with chromosomal markers, since this step involves homologous recombination. Total DNA of *N. meningitidis* 8013NSp, a spectinomycin-resistant derivative of 8013N, was used to transform 8013N and 8013N*rec*. The transformants were selected on GCB spectinomycin plates. Transformation frequency of 8013N was approximately 10^{-3}, while 8013N*rec* was not transformable (frequency, less than 10^{-7}). These results strongly suggest that the lesion in 8013N*rec* affects a major recombination function.

Transposition of Tn*1545*-Δ*3* in a *recA* recipient. *N. meningitidis* 8013N*rec* was then used as the recipient in conjugation-transposition experiments. Mobilization of pMGC20 into 8013N*rec* from S17-1 yielded kanamycin-resistant derivatives at a frequency of 2×10^{-5}. Thirty-four randomly selected transconjugants were further purified, and their sensitivity to tobramycin was confirmed. Analysis of plasmid DNA of these 34 transconjugants showed that they all contained a plasmid of 7.5 kb, the transposon-deleted form of pMGC20 discussed above. Again our results suggest that the transposon cannot be stably maintained in *N. meningitidis* on a multicopy plasmid. The chromosomal location of the 34 Tn*1545*-Δ*3* insertions were detected by Southern blot hybridizations of total DNA digested with *Cla*I and *Cla*I-*Hin*dIII, with the 439-bp Tn*1545*-Δ*3* internal fragment as probe. Again the transposon has inserted into different sites, although double transposition events in the same strain have been observed in two transconjugants. This result indicates that, in *N. meningitidis*, the *recA*-dependent recombination system of the host bacterium is not involved in Tn*1545*-Δ*3* transposition. This is consistent with a previous report which did not show any involvement of the RecA function in the transposition of Tn*1545* in *E. coli* (7).

Transposition of Tn*1545*-Δ*3* in *N. gonorrhoeae*. The shuttle vector pMGC10 can be transferred by conjugation from *E. coli* S17-1 to *N. gonorrhoeae* 15063G at a frequency of 10^{-5}. pMGC20 was then introduced by conjugation into this *N. gonorrhoeae* strain as described in Materials and Methods. Selection of transconjugants was performed on GCB agar plates containing kanamycin, erythromycin, and colistin or on BHI agar plates containing kanamycin and colistin. Since all pathogenic *Neisseria* spp. are naturally resistant to colistin, this antibiotic was used to inhibit the growth of *E. coli*. Kanamycin-resistant transconjugants occurred at a frequency of approximately 3×10^{-7} on GCB agar plates (range, 2×10^{-7} to 4×10^{-7}) and at a frequency of approximately 10^{-6} on BHI agar plates (range, 2×10^{-6} to 1×10^{-7}). These numbers are the mean of three different matings. Nine clones selected on plates containing both erythromycin and kanamycin were tobramycin sensitive. Of the 21 clones selected on kanamycin media, three were tobramycin resistant. These latter clones are believed to be kanamycin resistant by a mechanism other than the insertion of Tn*1545*-Δ*3* and were discarded.

Analysis of the plasmid content of the 27 kanamycin-resistant, tobramycin-sensitive transconjugants revealed, in addition to the *N. gonorrhoeae* cryptic plasmid, a plasmid whose size was compatible with the transposon-deleted derivative of pMGC20 (data not shown). Southern hybridization of the plasmid DNA with the Tn*1545*-Δ*3* internal fragment as probe confirmed that Tn*1545*-Δ*3* was not present on a plasmid in these strains. In addition, total DNA of 20 of the kanamycin-resistant, tobramycin-sensitive *N. gonorrhoeae* transconjugants was used to transform the parental strain 15063G. Transformants were selected on kanamycin-containing BHI agar plates. For each transformation, three kanamycin-resistant transformants were assessed for erythromycin sensitivity. All of the kanamycin-resistant transformants tested were found to be sensitive to erythromycin. This result confirms that, in the kanamycin-resistant, tobramycin-sensitive *N. gonorrhoeae* transconjugants, Tn*1545*-Δ*3* has been excised from pMGC20.

A Southern blot of *Cla*I-digested DNA from 17 transconjugants is shown in Fig. 6A. Only one signal is observed per strain. When the signal appeared to be on the same *Cla*I fragment in more than one strain, a Southern blot of a *Cla*I-*Hin*dIII digest of the corresponding DNA was performed to further analyze the sites of insertion (Fig. 6B). Our results show that two pairs of transconjugants (lanes 1 and 11; lanes 5 and 16) have an insertion of Tn*1545*-Δ*3* in the same restriction fragment. These strains could correspond to sibling selection.

DISCUSSION

The genetic analysis of the pathogenic *Neisseria* spp. has been hampered by the lack of an efficient direct transposon mutagenesis system. In this article, we have reported the transposition of Tn*1545*-Δ*3*, a derivative of the gram-positive transposon Tn*1545*, into *N. meningitidis* and *N. gonorrhoeae*. The transposition of Tn*1545*-Δ*3* into the chromosome is in numerous sites and is *recA* independent. In most cases, there is only one copy of the transposon per cell, and after several passages of the recipient in vitro no secondary transposition occurred. Back-crosses by DNA transformation showed that the transposon-mutated DNA had recombined with its wild-type homolog in the new recipient.

In our system, the kanamycin-resistant Tn*1545*-Δ*3* was cloned into pMGC10, a penicillin-sensitive derivative of the shuttle vector pLES2, and introduced into the *Neisseria* spp. by conjugation with *E. coli*. pLES2 had been reported previously to be mobilizable by RP4 derivatives from *E. coli* into *N. gonorrhoeae* but not into *N. meningitidis* (17). In *E. coli*, Tn*1545* is observed to be stable on a low-copy replicon but to excise spontaneously from high-copy-number plasmids (7). When cloned into the shuttle vector used in this study, Tn*1545*-Δ*3* was partially stable in *E. coli* and could not be maintained on the vector in the neisseriae. Therefore, by growing the transconjugants in the presence of kanamycin, recipients containing the transposon in its chromosome could be selected.

Two lines of evidence argue in favor of the excision of the entire transposon from the shuttle vector: (i) Tn*1545*-Δ*3* was not detected in the plasmid content of the transconjugants either by restriction analysis or by Southern blotting, and (ii) back-crossing by DNA transformation of the kanamycin resistance marker showed that the gene encoding resistance to kanamycin is not linked to the plasmid-encoded erythromycin gene. We do not know why the transposon is unstable in *Neisseria* spp. but partially stable in *E. coli*. It is possible that the neisseriae may be more susceptible than *E. coli* to the Int-Tn and Xis-Tn proteins expressed by Tn*1545*-Δ*3*. This explanation could account for the difference in the frequency of conjugal transfer between the shuttle vector pMGC10 and pMGC20. From *E. coli* to *N. meningitidis*, the transfer frequencies of these two plasmids are 10^{-4} and 10^{-5}, respectively; for *N. gonorrhoeae*, they are 10^{-5} and 10^{-6}, respectively. If high concentrations of Int-Tn and Xis-Tn proteins are toxic for *Neisseria* spp., then these frequencies suggest that the transposon excises from the vector in only 1 of 10 bacteria it enters. In the other cells, the transposon would be lethal if it stayed on the shuttle vector, thus explaining why every kanamycin-resistant transconjugant has the transposon in its chromosome.

Another striking result is the difference between the transposition frequency of Tn*1545*-Δ*3* in *N. meningitidis* and *N. gonorrhoeae*. This difference is most likely due to the difference in transfer frequency of the shuttle vector between *E. coli* and the two *Neisseria* species (10^{-4} for *N. meningitidis* and 10^{-5} for *N. gonorrhoeae*). A vector with a higher frequency of transfer between *E. coli* and the *Neisseria* spp. may increase the frequency of Tn*1545*-Δ*3* transposition in these bacteria.

Recently, Tn*916*, another conjugative transposon from gram-positive bacteria, has been shown to transpose in *N. meningitidis* but not in *N. gonorrhoeae* (10). This transposon was introduced by transformation on a suicide vector, and the low frequency of transposition in *N. meningitidis* (10^{-7}) is likely due to the large size of this element (16.5 kb), which may reduce its introduction by transformation into the *Neisseria* spp. The Tn*1545*-Δ*3* transposon system has several advantages over Tn*916*. (i) Transposition events are detected in *N. gonorrhoeae*. (ii) Tn*1545*-Δ*3* transposes at a frequency approximately 2 logs higher than Tn*916* in *N. meningitidis*, and this frequency could conceivably be increased further by increasing the frequency of conjugative transfer of the vector. (iii) Because of its small size, Tn*1545*-Δ*3* allows easy cloning of the gene into which it has inserted. (iv) Incorporation of suitable restriction sites within Tn*1545*-Δ*3* would allow this transposon to be used to deliver genes into *N. meningitidis* and *N. gonorrhoeae*. For these reasons, we believe that the Tn*1545*-Δ*3* system could be a useful genetic tool for studying the molecular basis of *Neisseria* pathogenesis.

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