

## High-Frequency Mobilization of Broad-Host-Range Plasmids into *Neisseria gonorrhoeae* Requires Methylation in the Donor

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Antibiotic resistance in *Neisseria gonorrhoeae* has been associated with the acquisition of R plasmids from heterologous organisms. The broad-host-range plasmids of incompatibility groups P (IncP) and Q (IncQ) have played a role in this genetic exchange in nature. We have utilized derivatives of RSF1010 (IncQ) and RP1 (IncP) to demonstrate that the plethora of restriction barriers associated with the gonococci markedly reduces mobilization of plasmids from *Escherichia coli* into strains F62 and PGH 3-2. Partially purified restriction endonucleases from these gonococcal strains can digest RSF1010 *in vitro*. Protection of RSF1010-km from digestion by gonococcal enzymes purified from strain F62 is observed when the plasmid is isolated from *E. coli* containing a coresident plasmid, pCAL7. Plasmid pCAL7 produces a 5'-<sup>ME</sup>CG-3' cytosine methylase (M.SssI). The M.SssI methylase only partially protects RSF1010-km from digestion by restriction enzymes from strain PGH 3-2. Total protection of RSF1010-km from PGH 3-2 restriction requires both pCAL7 and a second coresident plasmid, pFnuDI, which produces a 5'-GG<sup>ME</sup>CC-3' cytosine methylase. When both F62 and PGH 3-2 are utilized as recipients in heterospecific matings with *E. coli*, mobilization of RSF1010 from strains containing the appropriate methylases into the gonococci occurs at frequencies 4 orders of magnitude higher than from strains without the methylases. Thus, protection of RSF1010 from gonococcal restriction enzymes *in vitro* correlates with an increase in the conjugal frequency. These data indicate that restriction is a major barrier against efficient conjugal transfer between *N. gonorrhoeae* and heterologous hosts.

In nature, conjugation appears to play an important role in the mobilization of antibiotic resistance plasmids into various species of the genus *Neisseria*. Resistance plasmids can be transferred, for example, from enteric bacteria to *Neisseria gonorrhoeae*, as has been hypothesized by Roberts and Falkow (22). Likewise, other investigators have demonstrated that the broad-host-range incompatibility group P (IncP) plasmid pUB307 can mobilize pLES2 from *Escherichia coli* to *N. gonorrhoeae* (18). Thus, IncP elements may contribute to the establishment of resistance plasmids in *Neisseria* spp., although IncP elements have not been found in *Neisseria* organisms. It is assumed that this group of plasmids cannot be maintained in *N. gonorrhoeae* because of plasmid replication deficiencies (18). In addition to the  $\beta$ -lactamase plasmids, plasmids having homology to the broad-host-range incompatibility group Q (IncQ) plasmids, namely, RSF1010, have been found in a variety of commensal *Neisseria* species and in *Neisseria meningitidis* (5, 19, 23, 24). These RSF1010-like plasmids are the first multiresistant plasmid group found in *Neisseria* spp., having resistance not only to sulfonamide and streptomycin but also to ampicillin (23). The possibility exists for dissemination of these plasmids into *N. gonorrhoeae*.

Naturally occurring IncP elements (as illustrated by RK2, RP1, and R68 [1]) are large (>50 kb), stable, self-transferable plasmids which exist in four to seven copies per chromosome equivalent (7). The IncP plasmid consists of a contiguous block of conjugal transfer functions joined to a block of replication and maintenance functions. The IncQ class of plasmids are characterized by a small size (9 kb), a copy number of 4 to 12 copies per chromosome equivalent, and a host range at least as wide as that of RK2 (7). RSF1010 is not

self-transmissible, but it can be mobilized when *tra* functions are provided in *trans* by derivatives of RK2 (7, 26). Its complete nucleotide sequence as well as the organization of its genome has been determined previously (26).

Our interest in IncP and IncQ broad-host-range plasmids stems not only from their relationship and potential dissemination into the pathogenic *Neisseria* strains but also from their utilization as conjugal tools for transposon delivery and gene replacement in *Neisseria* spp. Initially, we utilized *Neisseria flava* as a model organism to monitor the parameters of the conjugation process (2). These included the number or ratio of donor to recipient bacteria, as well as the type of medium and appropriate antibiotic markers for the selection of transconjugants. The mobilization of IncP and IncQ plasmids were reasonable tools for monitoring this process because of their broad host range, the ease with which they are mobilized, and the variety of antibiotic markers available for selection (2).

We have reported that RSF1010 could be efficiently mobilized into *N. flava*, at frequencies of  $10^{-4}$  (2). Functions provided in *trans* by IncP elements, such as pUB307, were essential for the conjugal mobilization of RSF1010 into *N. flava*. This suggested that efficient mating-pair formation could be established between *Neisseria* spp. and *E. coli* and that IncQ elements could replicate and be stably maintained in *N. flava*.

We have now extended our study to include *N. gonorrhoeae* and report that RSF1010 can also be mobilized into *N. gonorrhoeae* F62-RN at a very low frequency, but this frequency increases by greater than 4 orders of magnitude when the plasmid is methylated *in vivo* by the methylase SssI (M.SssI), a <sup>ME</sup>CG methylase from *Spiroplasma* spp. High-frequency mobilization into *N. gonorrhoeae* PGH 3-2R requires methylation not only by M.SssI but also by M.FnuDI, a GG<sup>ME</sup>CC cytosine methylase. Plasmids isolated

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant description <sup>a</sup>	Source or reference
<b>Strains</b>		
<i>E. coli</i>		
SM10	Rec <sup>-</sup> derivative of C600 with RP4-Tc::Mu integrated in the chromosome	27
LE392	<i>hsdR14</i>	14
ER1821	<i>mcrA5 (mrr-hsdRMS-mcrB)::IS10</i>	E. Raleigh
<i>Neisseria</i> spp.		
<i>N. flava</i> Sm	Sm <sup>r</sup>	This study
<i>N. flava</i> RSp	Rf <sup>r</sup> Sp <sup>r</sup>	This study
<i>N. gonorrhoeae</i> F62-RN	Rf <sup>r</sup> Nf <sup>r</sup> derivative of F62	This study
<i>N. gonorrhoeae</i> PGH 3-2R	Rf <sup>r</sup> derivative of PGH 3-2	This study
<b>Plasmids</b>		
<b>IncQ</b>		
RSF1010	<i>oriT</i> Su <sup>r</sup> Sm <sup>r</sup>	26
RSF1010-km	<i>oriT</i> Su <sup>r</sup> Sm <sup>r</sup> Km <sup>r</sup>	This study
<b>IncP</b>		
pRK2013	RK2 ColEI origin, Ap <sup>r</sup> Km <sup>r</sup>	1
RP1	Ap <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup> <i>tra</i> <sup>+</sup> <i>mob</i> <sup>+</sup>	6
<b>Other</b>		
pFnuDI	P15A origin, Cm <sup>r</sup> <i>FnuDI</i> methylase gene	This study
pCAL7	ColEI origin, Ap <sup>r</sup> , <i>SssI</i> methylase gene	William Jack
pAIT2	P15A origin, Cm <sup>r</sup> Km <sup>r</sup> , <i>SssI</i> methylase gene	William Jack

<sup>a</sup> Antibiotic abbreviations are as follows: Sm<sup>r</sup>, streptomycin resistance; Rf<sup>r</sup>, rifampin resistance; Sp<sup>r</sup>, spectinomycin resistance; Nf<sup>r</sup>, nalidixic acid resistance; Su<sup>r</sup>, sulfamethoxazole resistance; Km<sup>r</sup>, kanamycin resistance; Ap<sup>r</sup>, ampicillin resistance; Tc<sup>r</sup>, tetracycline resistance; Cm<sup>r</sup>, Chloramphenicol resistance.

from *E. coli* donors, containing the appropriate methylases, are protected from digestion by restriction endonucleases purified from gonococcal strains F62 and PGH 3-2. Plasmid protection correlates with an increased frequency of conjugal mobilization. Furthermore, the IncP plasmid RP1 can self-mobilize into *N. flava* and *N. gonorrhoeae* if methylated in vivo by the *SssI* methylase. Although IncP-derivatives can be established in *Neisseria* spp., they are readily lost in the absence of selection.

(A preliminary report of parts of this work has been presented previously [2].)

## MATERIALS AND METHODS

**Organisms.** All bacterial strains and plasmids used are listed in Table 1. The chemicals utilized in this study, unless otherwise indicated, were purchased from either Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific Co. (Fairlawn, N.J.). All media were purchased from Difco Laboratories (Detroit, Mich.), Becton Dickinson (Cockeysville, Md.), or BBL (Baltimore, Md.). Donor *E. coli* strains were grown on Luria broth (LB) or Mueller-Hinton agar medium (14), and the concentrations of antibiotics used to select for appropriate plasmid-encoded resistance were as follows: carbenicillin, 50 µg/ml; sulfamethoxazole, 50 µg/ml; kanamycin, 30 µg/ml; and chloramphenicol, 20 µg/ml. *N.*

*flava* strains were grown on LB agar medium, and transconjugants were selected on LB media containing rifampin (100 µg/ml), spectinomycin (20 µg/ml), streptomycin (100 µg/ml), and kanamycin (30 µg/ml). Gonococcal strains were grown on GC medium supplemented with 1% IsoVitaleX (31), GC medium consists of 3.75 g of Trypticase peptone, 7.5 g of Thiotione-peptone, 4.0 g of dibasic potassium phosphate, 1.0 g of monobasic potassium phosphate, 5.0 g of sodium chloride, 1.0 g of soluble starch, and 10.0 g of agar, each per liter of water. *N. gonorrhoeae* transconjugants were selected on chocolate agar medium. Chocolate agar medium was prepared by mixing equal volumes of bovine hemoglobin with 2× GC agar base as recommended by the manufacturer (Becton Dickinson). The following concentrations of antibiotics were added when necessary: rifampin, 100 µg/ml; nalidixic acid, 10 µg/ml; kanamycin, 25 µg/ml; sulfamethoxazole, 25 µg/ml; and streptomycin, 25 µg/ml.

*N. gonorrhoeae* F62-RN is a spontaneously rifampin-resistant, nalidixic acid-resistant derivative of strain F62. Strain PGH 3-2R is a transformant of the gonococcal strain PGH 3-2 which had been mutagenized with 2% (final concentration) ethyl methanesulfonate (Sigma) for 1 h at 37°C, followed by a 6-h outgrowth and plating on selective chocolate agar plates containing rifampin at 250 µg/ml. DNA from a single rifampin-resistant colony was used to transform an unaltered, pilated strain of PGH 3-2 to rifampin resistance in order to eliminate any additional mutations which may have occurred as a result of the chemical mutagenesis. *N. flava* RSp and *N. flava* Sm are, respectively, spontaneously derived rifampin- and spectinomycin-resistant and streptomycin-resistant derivatives.

**Conjugal matings.** Biparental and triparental matings between *N. gonorrhoeae* and *E. coli* were performed by mixing 4.0 ml (optical density at 600 nm of 1.0 in a Spectronic 20 spectrophotometer with a tube of 17 by 120 mm) of the recipient with 0.4 ml (optical density at 600 nm of 1.0 in a tube of 17 by 120 mm) of the donor(s), centrifuging the mixture, suspending the cell pellet in 300 µl of GC broth (31), and spreading the suspension on a GC plate. The matings were incubated at 37°C for 7 h, swabbed from the plate into GC broth, diluted, and plated on chocolate agar plates containing 100 µg of rifampin per ml (PGH 3-2R) or rifampin and 10 µg of nalidixic acid per ml (F62-RN) for selection against the donors. Matings between *N. flava* RS and *E. coli* or *N. flava* RSp and *N. flava* Sm were performed by mixing 4.0 ml (optical density at 600 nm of 1.0 in a tube of 17 by 120 mm) each of the donor and recipient, centrifuging the mixture, suspending the cell pellet in 300 µl of LB, and spreading the suspension on an LB plate. The matings were incubated at 37°C for 7 h, swabbed from the plate into LB broth, diluted, and plated on media containing rifampin (100 µg/ml) and spectinomycin (20 µg/ml) to select against the donor. In cases in which the donor plasmid was self-mobilizable, such as RP1, biparental matings were performed. Triparental matings were performed when donor plasmids, such as RSF1010-km, required transfer functions in *trans* from pRK2013 in order to be mobilized (4). Some mating experiments were performed in the presence of DNase (100 µg/ml) in order to prevent the acquisition of plasmids by transformation. The frequency of conjugation was determined by dividing the number of transconjugant colonies by the number of recipient colonies at the end of the mating. The frequency represents the mean from at least two experiments.

**DNA manipulations.** Enzymes for restriction or ligation of DNA were purchased from New England Biolabs (Beverly,

Mass.). The purification of plasmid DNA and restriction endonuclease digestion, ligation, and agarose gel electrophoresis were performed according to the method of Maniatis et al. (14). DNA hybridizations were performed on DNA fixed on Hybond membranes (Hybond N+; Amersham Co., Arlington Heights, Ill.) at high stringency (65°C) by following the procedures of Meinkoth and Wahl (16). RSF1010-km was first cut with *R.EcoRI* and then labelled with [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol; Amersham) by following the instructions of the random-priming kit (Boehringer, Mannheim, Germany).

The plasmid RSF1010-km was created by ligating the 1.2-kb *HincII* fragment containing the Tn903 kanamycin resistance gene cassette from pUC4K into RSF1010 devoid of the *PvuII* fragment from bp 1821 to 1948 (26). The resultant plasmid, RSF1010-km, contains the antibiotic markers for streptomycin and sulfamethoxazole resistance as well as kanamycin resistance. RSF1010-km not only provided an additional selectable marker in RSF1010 but also tested whether kanamycin selection in the gonococcus was useful (2).

The plasmid pFnuDI encodes the *M.FnuDI* methylase on a P15A replicon. The gene for *M.FnuDI* was supplied by Elizabeth Raleigh (New England Biolabs) as a *HincII-HindIII* fragment cloned in pUC19. The gene fragment was excised by digestion with *R.BamHI* and *R.HindIII* and transferred into similarly digested pACYC184. The plasmids pCAL7 and pAIT2 contain the *M.SssI* methylase on pBR322 and P15A replicons, respectively, and were provided by the laboratory of William Jack at New England Biolabs. In each case, the ATG codon of the methylase has been fused to the initiation codon of *lacZ*. This assembly was placed downstream of a *tac* promoter. This construction is preceded by a fourfold repeat of the ribosomal transcription terminator. Furthermore, a copy of *lacI<sup>q</sup>* exists on both plasmids.

**Restriction endonuclease purification.** Partial purification of gonococcal restriction endonucleases was performed by a combination of polyethyleneimine precipitation and chromatography on heparin-agarose according to the procedures of Pirrotta and Bickle (20). Approximately 2 g (wet weight) of gonococcal cell paste was washed in Tris-saline (150 mM NaCl, 50 mM Tris hydrochloride, pH 8.0) and then suspended in a mixture containing 2 to 4 ml of 20 mM Tris hydrochloride, pH 8.0, 1 mM EDTA, and 20% sucrose with 10 mg of lysozyme per ml. The cells were broken osmotically by diluting the suspension severalfold in water. The lysate was centrifuged, and polyethyleneimine was added to the supernatant at a final concentration of 1% to remove the nucleic acids. The remaining supernatant was dialyzed against column buffer (20 mM Tris hydrochloride, pH 7.5; 7 mM 2-mercaptoethanol; 0.5 mM EDTA), and the sample was applied to a heparin-agarose column (type II; Sigma Chemical Co.) equilibrated in this buffer. Fractions (1 ml) were eluted from the column with a gradient of 0 to 0.8 M NaCl. Two microliters of each fraction was assayed for the ability to digest 0.4  $\mu$ g of lambda DNA in the presence of assay buffer (6 mM Tris hydrochloride, pH 7.5; 6 mM MgCl<sub>2</sub>; 7.5 mM 2-mercaptoethanol). Fractions which digested lambda DNA were pooled and stored in storage buffer (10 mM Tris hydrochloride, pH 7.5; 10 mM MgCl<sub>2</sub>; 50% glycerol).

For clarity in the text, the name of a gonococcal restriction enzyme or methylase is followed in parenthesis by the commercially available isoschizomer.

TABLE 2. Comparisons of methylation patterns of *N. gonorrhoeae* F62 and PGH 3-2 and *N. flava*<sup>a</sup>

Specificity	Isoschizomer <sup>b</sup>	Digestion or protection <sup>c</sup> of:		
		<i>N. flava</i>	F62	PGH 3-2
<i>R.NgoI</i> (PuGCGCPy)	<i>R.HaeII</i> (38)	+	-	-
<i>R.NgoII</i> (GGCC)	<i>R.HaeIII</i> (102)	+	-	-
<i>R.NgoIII</i> (CCGCGG)	<i>R.SacII</i> (1)	-	-	-
<i>R.NgoIV</i> (GCCGGC)	<i>R.NaeI</i> (0)	+	-	-
<i>R.NgoV</i> (GGNNCC)	<i>R.NlaIV</i> (30)	+	-	-
<i>R.NgoVIII</i> (GGTGA)	<i>R.HphI</i> (19)	+	-	-
Unknown (G <sup>ME</sup> ATC)	<i>R.DpnI</i> (45)	+	-	-
Unknown (GATC)	<i>R.MboI</i>	-	+	+
Unknown (GATC)	<i>R.Sau3A</i>	+	+	+

<sup>a</sup> Chromosomal DNAs from *N. flava* and *N. gonorrhoeae* F62 and PGH 3-2 were digested with the commercially available isoschizomers of the seven characterized gonococcal restriction enzymes and electrophoresed on a 0.7% agarose gel.

<sup>b</sup> The enzymes *R.MboI* and *R.Sau3A* were used as positive controls for the presence or absence of *dam* methylation. The numbers in parentheses indicate the number of predicted sites in plasmid RSF1010 (26).

<sup>c</sup> +, total digestion of DNA; -, protection of DNA from digestion.

## RESULTS

**Can RSF1010 be mobilized into *N. gonorrhoeae*?** Having established the conditions for monitoring successful conjugal events in *N. flava* (2), we attempted to mobilize RSF1010 into *N. gonorrhoeae* F62. With the *E. coli* donor SM10, the highest rate of transfer was 10<sup>-8</sup>; a rate 4 orders of magnitude less than that seen in *N. flava*. It is possible that the low efficiency of conjugation seen in *N. gonorrhoeae* is due to differences in surface components required for stable mating-pair formation. Alternatively, *N. gonorrhoeae* may exclude foreign DNA more efficiently because of its plethora of restriction barriers. Korch et al. (12, 13) as well as Davies (3) have independently noted that any gonococcal strain potentially has seven type-II restriction-modification systems. We initially attempted to determine the differences in restriction-modification systems between the gonococcal strains F62 and PGH 3-2 as well as the commensal *N. flava* by digesting chromosomal DNA from these strains with isoschizomers of the seven characterized gonococcal restriction enzymes. The data in Table 2 confirm that the gonococci have an elaborate methylation system which is not totally shared by *N. flava*. The two gonococcal strains appear to be *dam* mutants in not methylating adenine in the sequence GATC (as shown by the inability to digest with *DpnI*, but the ability to digest with *MboI*), whereas *N. flava* is *dam* methylated. The gonococcal strains and *N. flava* are similar only in their ability to methylate the sequence 5'-CCGCGG-3'. One obvious difference between *N. gonorrhoeae* and *N. flava* which could reduce the rate of transfer of foreign DNA is that *N. gonorrhoeae* has a greater number of potential restriction barriers.

**Do gonococcal restriction enzymes restrict RSF1010?** We analyzed whether purified gonococcal restriction enzymes could digest RSF1010-km by Southern blot utilizing labelled RSF1010-km as a probe. Partially purified gonococcal restriction enzymes from strains F62 and PGH 3-2 were incubated with RSF1010-km DNA for 2 h at 37°C and then electrophoresed on a 0.7% agarose gel. DNA in the gel was transferred to Hybond membranes and probed with <sup>32</sup>P-labelled RSF1010-km. The data in Fig. 1, lanes 10 and 13, indicate that restriction activity from both gonococcal strains can effectively digest RSF1010-km isolated from *E.*

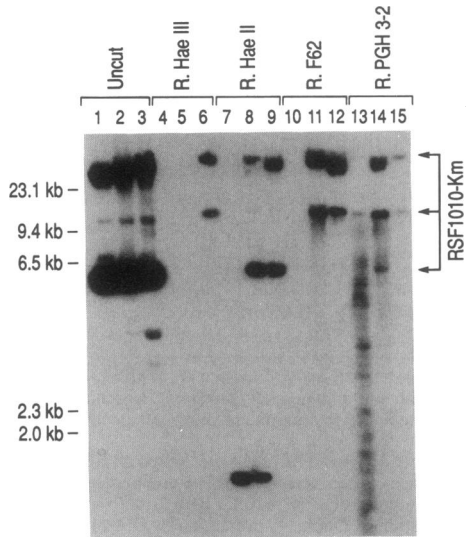


FIG. 1. Autoradiogram of Southern blot analysis of plasmid RSF1010-km digested with various restriction enzymes. Lanes 1, 4, 7, 10, and 13, RSF1010-km isolated from ER1821; lanes 2, 5, 8, 11, and 14, RSF1010-km isolated from ER1821 containing pCAL7; lanes 3, 6, 9, 12, and 15, RSF1010-km isolated from ER1821 containing pCAL7 and pFnuDI. The plasmid DNA was digested with the restriction enzymes as indicated, and the blot was probed with  $^{32}$ P-labelled RSF1010-km.

*coli* ER1821. No hybridization is observed in Fig. 1, lane 10, because the plasmid is digested to small fragments which ran off the gel. Partial digestion is observed in Fig. 1, lane 13, and may represent less activity per microliter of PGH 3-2 restriction extracts than in those of F62. Nevertheless, this result demonstrates that double-stranded DNA is subject to restriction by gonococcal enzymes *in vitro*. We have noted in Table 2 the number of sites in RSF1010 (in parentheses), on the basis of the DNA sequence (26), which are susceptible to gonococcal restriction.

Although the DNA sequence specificity of the partially purified gonococcal endonuclease(s) was not determined, we hypothesized that appropriate methylation of plasmid RSF1010 in *E. coli* would protect against gonococcal restriction. Interestingly, of the seven characterized or inferred gonococcal restriction enzymes, three have internal 5'-CG-3' dinucleotides in their recognition sequences (3). Methylation of the cytosine in a 5'-CG-3' pair would protect against the gonococcal restriction enzymes *R.NgoI* (*R.HaeII*), *R.NgoIII* (*R.SacII*), and *R.NgoIV* (*R.NaeI*).

The *SssI* methylase from *Spiroplasma* spp. (17, 21) is a cytosine methylase which methylates 5'-CG-3' sequences. The *M.SssI* methylase gene has been cloned and expressed from both a pBR replicon (pCAL7) and a P15A replicon (pAIT2) (6). In both constructions, the cloned enzyme in *E. coli* protects not only chromosomal DNA but also coresident plasmid DNA from restriction against type II enzymes having internal 5'-CG-3' sequence specificity (6, 21).

We introduced RSF1010-km into *E. coli* ER1821 coresident with the plasmid pCAL7. RSF1010-km isolated from ER1821 containing pCAL7 was mostly resistant to digestion by *R.HaeII* (the isoschizomer of *R.NgoI*) (Fig. 1, lane 8), whose recognition sequence has internal 5'-CG-3' residues (5'-CCGG-3'), whereas RSF1010-km isolated from ER1821 not containing pCAL7 was sensitive to the enzyme (Fig. 1,

lane 7). A DNA fragment of approximately 1,400 bp, corresponding to the kanamycin resistance gene which lacks *R.HaeII* sites, was observed in these lanes. However, *R.HaeIII* (the isoschizomer of *R.NgoII*), an enzyme whose recognition sequence contains an internal 5'-GC-3' (5'-GGCC-3'), was able to digest RSF1010-km whether or not pCAL7 was coresident (Fig. 1, lanes 4 and 5). This experiment confirms that pCAL7 protects coresident plasmids from digestion by restriction enzymes whose recognition sequences include an internal 5'-CG-3' dinucleotide.

Stein et al. have reported that strain F62 contains a frameshift mutation in the gene encoding *R.NgoII* (*R.HaeIII*), while strain PGH 3-2 has been shown to produce the restriction enzyme (29). In order to combat the *R.NgoII* (*R.HaeIII*) restriction barrier in PGH 3-2, we also introduced coresident with RSF1010-km and pCAL7 a plasmid which produces the methylase *M.FnuDI*, an isoschizomer of the *M.NgoII* methylase. This construction afforded protection of RSF1010-km when *R.HaeIII* was added to DNA purified from this strain (Fig. 1, lane 6).

**Is methylated RSF1010 protected from gonococcal restriction endonucleases?** The addition of partially purified F62 or PGH 3-2 restriction extract to RSF1010-km DNA resulted in total (F62 extract) or partial (PGH 3-2 extract) digestion of the plasmid (Fig. 1, lanes 10 and 13). Protection of RSF1010-km against F62 extract was seen when DNA was isolated from ER1821 containing the methylase plasmid pCAL7 (Fig. 1, lane 11). RSF1010-km DNA was only partially protected by *M.SssI* methylase when incubated with PGH 3-2 extract (Fig. 1, lane 14). This result may be due to the additional *R.NgoII* (*R.HaeIII*) enzyme expected to be present in PGH 3-2 (29). RSF1010-km DNA isolated from ER1821 containing both pCAL7 and pFnuDI was protected against restriction extracts from both F62 (Fig. 1, lane 12) and PGH 3-2 (Fig. 1, lane 15). This result suggests that *M.SssI* protects plasmid DNA against some but not all restriction enzymes produced by *N. gonorrhoeae*. Furthermore, the added protection provided by *M.FnuDI* should result in an increased rate of conjugal transfer in PGH 3-2R but not F62-RN. We also noted that DNA, presumably protected by methylation (Fig. 1, lanes 11, 12, 14, and 15), appeared nicked in comparison with uncut DNA (Fig. 1, lanes 1 to 3). This suggested that most, but not all, sites are protected by the methylating coresident plasmids.

**Can methylated RSF1010 be mobilized into *N. gonorrhoeae*?** We addressed whether the rate of RSF1010 conjugal transfer into *N. gonorrhoeae* correlated with the protective capacity of the *M.SssI* methylase against gonococcal restriction. Triparental matings were set up among *N. gonorrhoeae* F62-RN, *E. coli* LE392 with and without pRK2013 (to provide *tra* functions in *trans*), and ER1821 containing RSF1010 with or without pCAL7. Results of the triparental matings indicated that mobilization of RSF1010 into *N. gonorrhoeae* occurred at a rate of  $3.6 \times 10^{-8}$  in the absence of *in vivo* cytosine methylation. A plasmid profile of the single transconjugant revealed the presence of RSF1010 (Fig. 2A, lane 3). The frequency of conjugation was increased by greater than 4 orders of magnitude to  $2.4 \times 10^{-3}$  when RSF1010 was coresident with pCAL7. Plasmid profiles from two transconjugants are presented in Fig. 2A, lanes 4 and 5.

*N. gonorrhoeae* cells are naturally competent; thus, we wanted to eliminate transformation as a mechanism by which strain F62-RN could acquire RSF1010. We attempted triparental matings as described above except that pRK2013 was absent. Consequently, *tra* functions were not provided

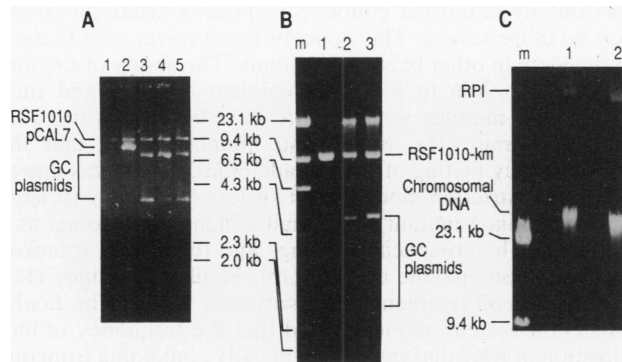


FIG. 2. Agarose gel containing plasmid DNA digested with *R.SacI* (A and B) or undigested (C) and stained with ethidium bromide. (There is a single *SacI* site in RSF1010, and this site is not protected by *M.SssI* or *M.FnuDI*.) (A) Lane 1, RSF1010 isolated from ER1821; lane 2, RSF1010 and pCAL7 isolated from ER1821; lanes 3 to 5, RSF1010 isolated from independent F62-RN transconjugants. (B) Lane 1, RSF1010-km isolated from ER1821; lanes 2 and 3, RSF1010-km isolated from single PGH 3-2R and F62-RN transconjugants, respectively. (C) Lane 1, undigested RP1 from F62-RN; lane 2, undigested RP1 from ER1821. Lane M in panels B and C represents lambda DNA digested with *R.HindIII*.

in *trans* from the IncP plasmid. We were unable to obtain any gonococcal transconjugants in the absence of *tra* functions (frequency of conjugation,  $<7.0 \times 10^{-7}$ ). Thus, we ruled out the possibility that the acquisition of RSF1010 was by transformation. Furthermore, in each F62-RN mating presented above, DNase was present in the mating mixture at a concentration of 100  $\mu\text{g/ml}$ .

Triparental matings between strain PGH 3-2R and *E. coli* ER1821 containing RSF1010-km did not result in any kanamycin- and sulfamethoxazole-resistant transconjugants (Table 3). When pCAL7 was coresident with RSF1010-km, a frequency of transfer of  $2.2 \times 10^{-6}$  was obtained (Table 3), which is approximately 2 orders of magnitude lower than the frequency obtained in an identical mating with strain F62-RN as a recipient (Table 4). As mentioned previously, one explanation for the reduced frequency could be the presence of an additional restriction barrier whose sequence specificity is not protected by *M.SssI* methylation. When ER1821 containing pCAL7 and pFnuDI was used as a donor, the frequency of RSF1010-km transfer was  $3.2 \times 10^{-3}$  (Table 3). A significant change in the frequency of transfer was not seen when the same donor was used in a mating with strain F62-RN, suggesting that methylation of *NgoII* (*HaeIII*) sites was not essential for the increased frequency of transfer in F62-RN but was essential when PGH 3-2R was the recipient.

TABLE 3. Triparental matings between *E. coli* ER1821 and *N. gonorrhoeae* F62-RN or PGH 3-2R<sup>a</sup>

Donors	Recipient	Frequency <sup>b</sup>
ER1821(RSF1010-km), LE392(pRK2013)	F62-RN	$<9.0 \times 10^{-8}$
	PGH 3-2R	$<5.5 \times 10^{-7}$
ER1821(RSF1010-km, pCAL7), LE392(pRK2013)	F62-RN	$4.9 \times 10^{-3}$
	PGH 3-2R	$2.2 \times 10^{-6}$
ER1821(RSF1010-km, pCAL7, pFnuDI), LE392(pRK2013)	F62-RN	$3.2 \times 10^{-3}$
	PGH 3-2R	$1.7 \times 10^{-3}$

<sup>a</sup> Performed as indicated previously.

<sup>b</sup> Determined by dividing the number of transconjugants by the total number of recipients.

TABLE 4. Biparental matings between *E. coli* ER1821 and *N. flava* as well as with *N. gonorrhoeae*<sup>a</sup>

Donor	Recipient	Frequency <sup>b</sup>
ER1821(RP1)	<i>N. flava</i> RSp	$<6.0 \times 10^{-9}$
ER1821(pAIT2, RP1)	<i>N. flava</i> RSp	$1.8 \times 10^{-5}$
<i>N. flava</i> RSp(RP1)	<i>N. flava</i> Sm	$1.9 \times 10^{-5}$
<i>N. flava</i> Sm(RP1)	ER1821-R	$7.6 \times 10^{-2}$
ER1821(RP1)	<i>N. gonorrhoeae</i> F62-RN	$<1.0 \times 10^{-9}$
ER1821(pAIT2, RP1)	<i>N. gonorrhoeae</i> F62-RN	$3.0 \times 10^{-7}$

<sup>a</sup> Performed as indicated in the text.

<sup>b</sup> Determined by dividing the number of transconjugants by the total number of recipients at the end of the mating.

Plasmids from F62-RN and PGH 3-2R transconjugants are observed in Fig. 2B, lanes 2 and 3.

**Can methylated RP1 (IncP) be mobilized into *N. gonorrhoeae*?** Previous investigations have suggested that the IncP broad-host-range vectors, such as pUB307, cannot be maintained in *N. gonorrhoeae* (18). This result may have been due to restriction of incoming DNA rather than to plasmid replication deficiencies. We tested this hypothesis, utilizing RP1 and placing it coresident with pAIT2 in *E. coli* ER1821. Initial bipartite matings with *N. flava* RSp revealed a frequency of transfer of  $<6.0 \times 10^{-9}$  in the absence of pAIT2 and  $1.83 \times 10^{-5}$  in the presence of pAIT2 (Table 4). Plasmid preparations from two of the transconjugants revealed the presence of RP1 (data not shown). The plasmid RP1 was mobilized from *N. flava* RSp to *N. flava* Sm at approximately the same rate as from *E. coli* ( $1.9 \times 10^{-5}$ ; Table 4). However, mobilization of RP1 from *N. flava* Sm to a R<sup>f</sup> derivative of *E. coli* ER1821 occurred at a rate of  $7.62 \times 10^{-2}$ , suggesting that mobilization (*mob*) and transfer (*tra*) functions were still intact. (The plasmid from a single transconjugant is observed in Fig. 2C, lane 2.) It is unclear why the rate between different derivatives of *N. flava* was lower than between *N. flava* and *E. coli*. Nevertheless, as with the IncQ plasmids, successful mobilization of RP1 into *N. flava* requires *M.SssI* methylation in the donor *E. coli*. Similarly, we were able to mobilize RP1 into *N. gonorrhoeae* F62-RN at a rate of  $3.0 \times 10^{-7}$  only if the plasmid was coresident with pAIT2 (Table 5). The plasmid from a single transconjugant is observed in Fig. 2C, lane 1. The low rate of transfer was unexpected, considering the high transfer frequencies obtained with RSF1010. Although transconjugants containing RP1 could become established in F62-RN, we noted that their rate of growth appeared markedly slower than that of wild-type F62-RN and that passage on nonselective media resulted in rapid loss of kanamycin resistance which correlated with plasmid loss. The frequency of RP1 mobilization may actually be several orders of magnitude higher, but the plasmid appears to be unstable. We have not attempted to resolve whether derivatives of RP1, lacking regions which may be deleterious to the gonococci, are able to be maintained in F62-RN.

## DISCUSSION

In nature, the acquisition of foreign DNA by *N. gonorrhoeae* probably occurs by two mechanisms: conjugation and transformation. To date, no phage has been found which can transduce these organisms. Transformation may play a role in the spread of plasmids into *N. gonorrhoeae*, but this mechanism would require that the DNA contain specific sequences in order to be taken up into competent organisms

(8, 10). The mechanism of transformation is relatively inefficient in comparison with conjugation, and deletions often appear in plasmids during transformation (28). This may be the result of host-mediated restriction (29). In contrast, it has been suggested that restriction is not a barrier to the conjugal transfer of plasmids between different strains of *N. gonorrhoeae* (29). Stein et al. demonstrated that transformation of the gonococcal plasmid pFT180 isolated from *E. coli* HB101 or *N. gonorrhoeae* WR302 into the gonococcal strain PGH 3-2 required in vitro methylation with the *M.HaeIII* methylase (29). In the absence of methylation, a 5-order-of-magnitude decrease in the transformation frequency was observed. These experiments illustrated the effect of a restriction enzyme (*R.NgoII*) produced by PGH 3-2 which acts on *HaeIII* sites. This enzyme was not apparent in strain WR302. However, only a 1-order-of-magnitude difference was observed when a different plasmid, pFT6, was conjugated from the gonococcal strain WR302 to the recipient PGH 3-2. These observations suggested that host-mediated restriction acted on DNA entering the cell by transformation but not by conjugation (29). Likewise, Piffaretti et al. (18), utilizing the RP1 derivative pUB307, were able to mobilize R plasmids as well as the gonococcal shuttle vector pLES2 from *E. coli* to a derivative of *N. gonorrhoeae* MS11 at high frequency ( $10^{-4}$ ). Not only could the potential natural dissemination of antibiotic-resistant plasmids be reproduced in the laboratory, but also this system promises the possibility of investigating other biological problems by using allelic exchange, transposon mutagenesis, and complementation as genetic tools.

Utilizing the same plasmids described by Piffaretti et al. (18), we were unable to obtain any transconjugants when a different gonococcal strain, F62-RN, was employed as a recipient (9). Clearly, not all *Neisseria* strains behave equally, as some maintain a barrier to efficient and successful conjugation. Attempts to mobilize RSF1010 into *N. gonorrhoeae* F62-RN from the *E. coli* donor SM10 were not only inconsistent but also inefficient. We hypothesized that the barrier to mobilization of RSF1010 into strain F62-RN was at the level of restriction in the new host. The gonococcus has a very complex restriction-modification system (3, 12, 13, 30). Restriction enzymes from strain F62 can digest RSF1010-km unless the plasmid is protected by *M.SssI* methylation. RSF1010-km is protected from digestion by PGH 3-2 restriction enzymes only if methylated with both *M.SssI* and *M.FnuDI* methylases. Protection from digestion against the gonococcal restriction enzymes in vitro correlates with a substantial increase in the conjugal frequency. Methylation of RSF1010 in *E. coli* by *M.SssI* results in a  $10^4$  increase in the conjugal frequency when F62-RN is the recipient and a fourfold increase when PGH 3-2R is the recipient. The addition of a plasmid producing *M.FnuDI* results in an additional  $10^3$ -fold increase when PGH 3-2R is the recipient but has no effect on the frequency when F62-RN is the recipient. Thus, methylation of *NgoII* (*HaeIII*) sites is essential for increased conjugal transfer in strain PGH 3-2R but not in strain F62-RN. The difference between the two strains appears to be in the presence or absence of *R.NgoII* (*R.HaeIII*) restriction enzyme. Though both gonococcal strains produce methylases which recognize the sequence 5'-GGCC-3', only PGH 3-2 produces a restriction enzyme that recognizes the same sequence (29). It appears that some gonococcal strains, for instance, F62, may be more susceptible to foreign DNA exchange than others (e.g., PGH 3-2). Once acquired, plasmids would be appropriately methylated and thus easily able to disseminate

horizontally into other gonococci whose restriction barriers may be more severe. This concept is not novel, and there is a precedent in other bacterial systems. The ability of *Legionella pneumophila* to act as a recipient of IncQ and IncP plasmids in matings with *E. coli* was shown to vary from strain to strain (15). Marra and Shuman found that the low-efficiency mating of the Philadelphia-1 strain was due to a type II restriction-modification system (15). They isolated a Philadelphia-1 mutant which had a high ability to act as a recipient in heterospecific matings and found that it lacked the restriction enzyme activity (15). Similarly, Guiney (11), utilizing *E. coli* recipients both with and without the *EcoRI* restriction system, demonstrated that the frequency of mobilization of RK2 derivatives (artificially containing from one to four *EcoRI* sites) into the recipient containing *R.EcoRI* was reduced from 1 to 5 orders of magnitude, respectively, compared with mating frequencies in a recipient devoid of *R.EcoRI* (11). The premise of these investigations and our own is that during conjugation, a single-stranded molecule would enter the host cell and not be methylated. After synthesis of its complementary strand, the double-stranded molecule would exist transiently as an unmethylated duplex subject to restriction. As DNA enters a recipient cell during mating, a race between the restriction enzyme and the modification enzyme commences. If the modification enzyme wins, the plasmid is protected and survives in the new host, assuming that it can replicate (11). However, it is not known whether some restriction enzymes produced by the gonococcus are capable of digesting single-stranded DNA. This is not unusual, considering that *R.HaeIII* (and perhaps its isoschizomer, *R.NgoII*) has been shown to digest single-stranded DNA in vitro (32).

Our data indicate that RP1 would be unlikely to be maintained in *Neisseria* spp. and would be lost upon subsequent replication. The inability of some organisms to maintain IncP-type plasmids is not unusual. Investigations by Schmidhauser and Helinski, on maintenance of RK2 plasmids, revealed that the 760-bp RK2 *oriT* segment can lead to replicon instability in *Pseudomonas aeruginosa* in the absence of a 3.1-kb maintenance region (25). This 3.1-kb maintenance region was shown to increase or decrease the stability of maintaining IncP derivatives depending on the host species. Perhaps the *oriT* segment and/or the maintenance region causes the IncP plasmid to be unstable in *N. gonorrhoeae* and thus is unable to segregate proficiently in this organism. We have not investigated the conjugal frequency into *N. gonorrhoeae* of IncP derivatives devoid of the 3.1-kb maintenance region.

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