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Restriction of Plasmid DNA during Transformation But Not Conjugation in *Neisseria gonorrhoeae*

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***Neisseria gonorrhoeae* strains WR302 and PGH3-2 were characterized with respect to their restriction-modification phenotype. WR302 DNA was cleaved by *Hae*III, indicating the lack of methylation at the GGCC sequence. PGH3-2 produced *Ngo*SI (an isoschizomer of *Ngo*II). WR302 produced a restriction enzyme with a recognition sequence different from that of *Ngo*I, *Ngo*II, or *Ngo*III. Plasmid pFT180 isolated from WR302 was unable to transform PGH3-2, whereas plasmid pFT180 isolated from PGH3-2 was able to transform PGH3-2 at a very high frequency. When plasmid pFT180 isolated from WR302 was methylated in vitro with meth M · *Hae*III, this plasmid was able to transform PGH3-2. *Ngo*SI was able to restrict WR302 DNA in vitro, whereas it was incapable of restricting PGH3-2 DNA in vitro. When the self-transmissible R factor pFT6 was mobilized from WR302 to PGH3-2 by conjugation, a 1-order-of-magnitude difference in transfer frequencies was observed, as compared with an isogenic cross. The data indicate that host-mediated restriction can prevent the gonococcus from acquiring DNA via transformation but not via conjugation.**

Restriction and modification of DNA have been demonstrated in a wide variety of taxonomically unrelated bacteria. More than 200 restriction endonucleases have been discovered, and in many cases the corresponding methylases have been characterized (15). In *Escherichia coli*, host-mediated restriction has been shown to prevent the acquisition of plasmid DNA during conjugation, transformation, and transduction (1, 4, 10). However, there are several systems in which DNA methylases are present in the apparent absence of endonuclease activity (12). *Neisseria gonorrhoeae* is a bacterial species that produces at least five different restriction enzymes (3, 13, 15; A. Piekarowicz, unpublished observations; J. Davies, personal communication) and 11 different DNA methylases (8, 9; D. Stein, unpublished observations). The role that these enzymes play in cell physiology remains unclear.

Stein et al. (22) characterized a plasmid, pFT180, that was able to transform the gonococcus at high frequencies. This plasmid, even though it contained the uptake sequences necessary for transformation (5), was unable to transform certain strains of *N. gonorrhoeae* (Stein, unpublished). The inability of this plasmid to transform certain strains of *N. gonorrhoeae* led us to postulate that plasmid DNA can be restricted in vivo, thereby preventing the acquisition of plasmid DNA by transformation. This study was performed to determine the role that host-mediated restriction plays in genetic exchange in *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. gonorrhoeae* PGH3-2 was provided by J. Boslego, and *N. gonorrhoeae* WR302 was provided by H. Schneider, both from Walter Reed Army Institute of Research. Both strains have been extensively characterized (16, 17, 25). *E. coli* HB101 was obtained from H. Boyer. *N. gonorrhoeae* was propagated on standard gonococcal medium base agar (Difco Laboratories, Detroit, Mich.) plus growth supplements (7) (GCK). Broth cultures were supplemented with $NaHCO_3$ (420 mg/liter) before use. *E. coli* was grown in L broth (11) or on MacConkey agar. All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise indicated.

DNA isolation procedures. Plasmids pFT180 and pLES4 have been previously described (23, 24). Plasmid DNA was isolated by cesium chloride-ethidium bromide density centrifugation by a modification (24) of the method of Guerry et al. (6). DNA was suspended in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]) to give a final concentration of 100 μg of DNA per ml.

Transformation of *N. gonorrhoeae*. A single type 1 colony was streaked onto GCK agar and grown for 15 h. The cells were suspended in 10 ml of GCK broth–10 mM $MgCl_2$–0.042% $NaHCO_3$ to an optical density corresponding to 10^7 CFU/ml. DNA (100 ng) was added to 1 ml of cells and incubated with shaking for 30 min before the addition of DNase (50 μg/ml). Cells were given 6 h to express the β-lactamase gene present on the plasmid before plating on selective medium. Suspected β-lactamase-producing transformants were verified by their ability to cleave a chromogenic cephalosporin (14).

Conjugation procedure. Conjugations were performed by a modification of the filter mating technique developed by Sox et al. (18). Type 4 colonies were grown on GCK agar for 18 h, scraped from the agar medium with a sterile Dacron swab, and suspended in 10 ml of GCK broth plus growth supplements. Donor cells were suspended to a density of 10^8 CFU/ml, and recipient cells were suspended to a density of 10^7 CFU/ml. Equal volumes (0.1 ml) of donor and recipient cells were mixed and placed on a filter membrane (pore size, 0.2 μm) (Nuclepore Corp., Pleasanton, Calif.) that had been placed on a GCK agar plate. After 16 h of incubation, the filter was placed in 1 ml of GCK broth, the cells were suspended by vortexing, and transconjugants were selected by plating on GCK agar containing 1 μg of penicillin and 1 μg of rifampin per ml. Transconjugants were verified by their ability to cleave a chromogenic cephalosporin (14).

Restriction enzyme purification. Restriction enzymes were detected in crude lysates as follows. Overnight cultures (1 liter) were suspended in 8 ml of 10% sucrose (in 0.5 M Tris hydrochloride, pH 8.0) and frozen at −70°C. After freeze-thawing, lysozyme (2 mg) and NaCl (1 ml of a 1.0 M stock)

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TABLE 1. Transformation of *N. gonorrhoeae* with pFT180

Source of DNA[a]	Transformation frequency[b]	
	WR302	PGH3-2
N. gonorrhoeae PGH3-2	3.3×10^{-3}	2.8×10^{-3}
N. gonorrhoeae WR302	3.1×10^{-4}	$<3.5 \times 10^{-8}$
E. coli HB101	$<2.3 \times 10^{-8}$	$<7.1 \times 10^{-8}$

[a] Plasmid DNA was isolated from the various strains, and 1 μg was used to transform 5×10^7 cells. Expression time was 6 h.

[b] Transformation frequency is the number of transformants per number of CFU.

were added. After 60 min of incubation on ice, 1 ml of buffer (200 mM Tris hydrochloride, 100 mM $MgCl_2$, 2 mM EDTA, 76 mM 2-mercaptoethanol) was added and the cells were lysed by sonication (Biosonic sonifier [Bronwill Scientific, Rochester, N.Y.], 2 min, full power). Cellular debris was removed by centrifugation (48,000 × *g*, 60 min). Nucleic acids were removed by the dropwise addition of polyethyleneimine to a final concentration of 1%. After 45 min, the supernatant was collected by centrifugation (12,000 × *g*, 15 min). Proteins were precipitated by bringing the solution to 70% ammonium sulfate, and the precipitate was collected by centrifugation (28,000 × *g*, 30 min) and then dialyzed against buffer (20 mM Tris hydrochloride, 1 mM EDTA, 6 mM 2-mercaptoethanol). The presence and identity of restriction endonuclease activity was determined by digesting lambda DNA with dialysate and comparing the digestion pattern seen with those patterns that were obtained by using commercially available enzymes.

DNA manipulations. Restriction enzymes were purchased from IBI, New Haven, Conn., and meth M · *Hae*III was purchased from New England BioLabs, Inc., Beverly, Mass.; both were used according to manufacturer specifications. *Ngo*SI digestions were performed by using a low-salt buffer provided by IBI for use with *Hae*III. The presence of methylated DNA was determined by isolating plasmids pLES4 and pFT180 from strains of *N. gonorrhoeae* and digesting these DNAs with commercially available enzymes that recognize sequences corresponding to known gonococcal methylases and whose activity is blocked by the presence of methylcytosine or methyladenine in the recognition sequence.

RESULTS

Transformation of *N. gonorrhoeae* with pFT180. Plasmid pFT180, isolated from *N. gonorrhoeae* WR302 and PGH3-2 and *E. coli* HB101, was used to transform *N. gonorrhoeae* WR302 and PGH3-2. The transformation frequencies obtained are given in Table 1. The data indicate that plasmid pFT180, when isolated from PGH3-2, was able to transform both gonococcal strains at very high frequencies ($\sim 3 \times 10^{-3}$). However, when this plasmid was isolated from WR302, no transformants were detected when PGH3-2 was used as a recipient. Plasmid DNA isolated from HB101 was unable to transform either of the strains, even though it has been shown to transform other gonococcal strains at the same frequencies at which the PGH3-2-grown plasmid was able to transform PGH3-2 (24).

Restriction enzymes and modification activities present in WR302 and PGH3-2. The failure of pFT180 isolated from *E. coli* HB101 to transform *N. gonorrhoeae* led us to postulate that this plasmid was being restricted. We examined strains PGH3-2 and WR302 for their ability to produce restriction enzymes. Crude extracts of the two strains were prepared and incubated with lambda DNA. If the extract was able to digest lambda DNA into discrete fragments, it was presumed that this strain produced a restriction endonuclease. Both strains demonstrated restriction endonuclease activity, and analysis of the DNA banding patterns seen on agarose gels indicated that the enzymes produced were not the same (data not shown). To determine whether the restriction enzymes present in these strains possessed specificities similar to ones that have been previously described, lambda DNA was digested with isoschizomers of *Ngo*I (*Hae*II), *Ngo*II (*Hae*III), and *Ngo*III (*Sac*II). The DNA banding pattern obtained on agarose gels after digestion with these enzymes was compared with the digestion pattern seen with the gonococcal extracts. From this type of analysis, it was concluded that PGH3-2 produced an enzyme with the same recognition sequence as *Ngo*II (Fig. 1). Furthermore, this enzyme was able to digest pFT180 that was isolated from WR302. This enzyme has been called *Ngo*SI. WR302 produced a restriction endonuclease whose specificity was different from those of *Ngo*I, *Ngo*II, and *Ngo*III (Piekarowicz, unpublished).

For plasmid DNA to be restricted by PGH3-2, the plasmid must not be methylated at the *Ngo*SI restriction site. The presence or absence of methylation at this site was examined by digesting plasmid pFT180 isolated from HB101, PGH3-2, and WR302 with *Hae*III, an isoschizomer of *Ngo*SI. The data presented in Fig. 2 indicate that pFT180 isolated from WR302 and HB101 were digested with *Hae*III, indicating that the DNA was not methylated, whereas the same plasmid isolated from PGH3-2 was not cleaved by this enzyme.

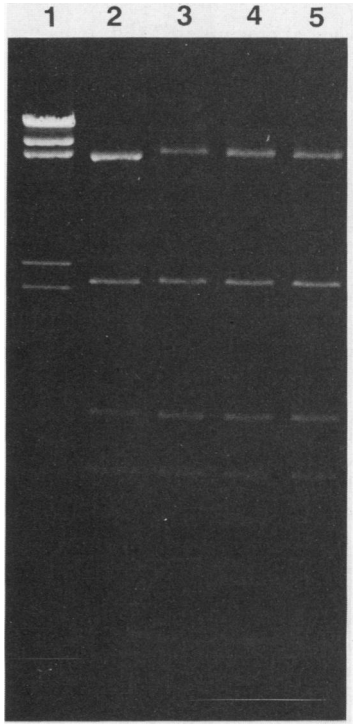

FIG. 1. In vitro susceptibility of pFT180 DNA to cleavage by extracts from PGH3-2. Lanes: 1, lambda cleaved with *Hin*dIII; 2, pFT180 isolated from *E. coli* HB101 digested with *Hae*III; 3, pFT180 isolated from *E. coli* HB101 digested with *Ngo*SI; 4, pFT180 isolated from *N. gonorrhoeae* WR302 digested with *Hae*III; 5, pFT180 isolated from *N. gonorrhoeae* WR302 digested with *Ngo*SI.

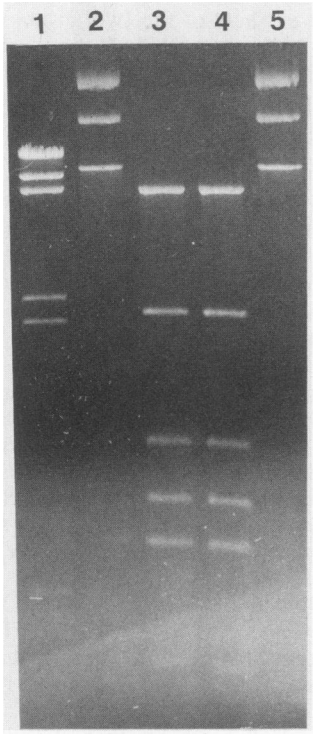

FIG. 2. Cleavage of plasmid pFT180 grown in *N. gonorrhoeae*. Lanes: 1, lambda digested with *Hin*dIII; 2, undigested pFT180; 3, pFT180 isolated from *E. coli* HB101; 4, pFT180 isolated from *N. gonorrhoeae* WR302; 5, pFT180 isolated from *N. gonorrhoeae* PGH3-2. Lanes 3 to 5 have been digested with *Hae*III.

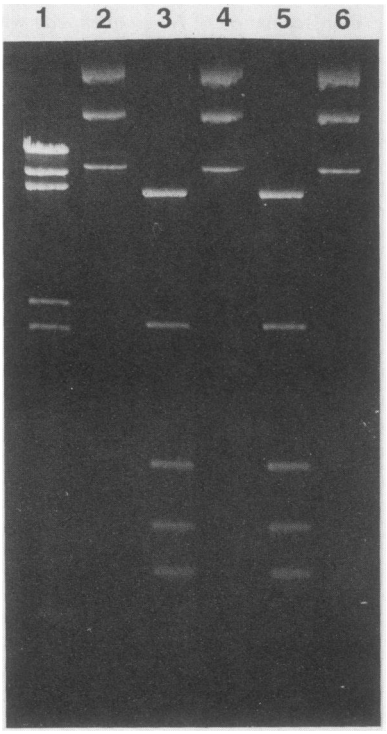

FIG. 3. Cleavage of m · *Hae*III-treated pFT180 with *Ngo*SI. Lanes: 1, lambda digested with *Hin*dIII; 2, undigested pFT180; 3, pFT180 isolated from *E. coli* HB101 digested with *Ngo*SI; 4, pFT180 isolated from *E. coli* HB101, methylated with meth M · *Hae*III, and then digested with *Ngo*SI; 5, pFT180 isolated from WR302 and digested with *Ngo*SI; 6, pFT180 isolated from WR302, methylated with meth M · *Hae*III, and digested with *Ngo*SI.

In vitro cleavage of plasmid pFT180. Isoschizomers such as *Mbo*I, *Sau*3A, and *Dpn*I recognize the same DNA sequence but differ markedly in their abilities to cleave at the recognition site because of differences in DNA methylation. To determine whether the restriction enzyme *Ngo*SI was able to restrict DNA in vitro, pFT180 isolated from WR302 and HB101 was incubated with *Ngo*SI, with subsequent analysis by gel electrophoresis. The data presented in Fig. 3 demonstrate that *Ngo*SI was able to restrict pFT180 when it was isolated from these strains. The cleavage of pFT180 by *Ngo*SI could be prevented if the DNA was methylated with meth M · *Hae*III before digestion. These results indicate that *Ngo*SI is able to restrict pFT180 in vitro, and this cleavage can be blocked by methylation with meth M · *Hae*III.

Transformation by using in vitro methylated DNA. The effect of in vitro methylation of pFT180 on the transformation of WR302 and PGH3-2 was examined. Plasmid pFT180 isolated from HB101, WR302, and PGH3-2 was methylated with meth M · *Hae*III and used to transform the two gonococcal strains. The data presented in Table 2 demonstrate that it was possible to inhibit the ability of PGH3-2 to restrict plasmid DNA isolated from WR302 and HB101 by first methylating this plasmid with meth M · *Hae*III. The in vitro methylation did not completely protect pFT180 from being restricted by PGH3-2. This can be explained by the fact that not all of the *Hae*III sites present on pFT180 were completely methylated, as evidenced by a small amount of digestion obtained after digesting the in vitro methylated DNA with *Hae*III (unpublished data). Likewise, it could be due to the presence of another restriction enzyme.

Conjugal transfer of plasmid pFT6. To determine whether DNA restriction has an effect on conjugation, a series of conjugations were performed using the 45-kilobase self-transmissible R factor pFT6, constructed by Fred Tenover (Ph.D. thesis, University of Rochester, N.Y., 1980). Plasmid pFT6 is a cointegrate of the 4.4-megadalton β-lactamase plasmid and the gonococcal conjugal plasmid. The data presented in Table 3 indicate that when WR302 contains pFT6, it is able to transfer this β-lactamase-encoding plasmid by conjugation into a nalidixic acid-resistant derivative of WR302 at a frequency of 2.3×10^{-4}. This compares with a transfer frequency of 2.7×10^{-5} obtained when

TABLE 2. Effect of in vitro methylation of plasmid pFT180 on transformation of *N. gonorrhoeae* PGH3-2

Source of DNA[a]	Transformation frequency[b]	
	Methylated[c]	Unmethylated[d]
N. gonorrhoeae PGH3-2[e]	2.0×10^{-4}	7.4×10^{-4}
N. gonorrhoeae WR302	2.1×10^{-5}	$<4.0 \times 10^{-8}$
E. coli HB101	5.4×10^{-5}	$<7.0 \times 10^{-8}$

[a] 100 ng of DNA per ml was used to transform 5×10^7 cells. Plasmid pFT180 was isolated from various strains.

[b] Transformation frequency is the number of transformants per number of CFU.

[c] Plasmid pFT180 was methylated with meth M · *Hae*III before use in transformation experiments.

[d] Plasmid pFT180 was exposed to methylation buffers in a mock methylation experiment before use in transformation experiments.

[e] This DNA is fully methylated at the recognition site for this enzyme. The treatment does not add any methyl groups to the DNA.

TABLE 3. Conjugations with plasmid pFT6

Recipient strain[a]	Transfer frequency for donor strain[b]	
	WR302(pFT6)	PGH3-2(pFT6)
WR302	2.3×10^{-4}	4.0×10^{-4}
PGH3-2	2.7×10^{-5}	ND[c]

[a] Recipient strains are spontaneous nalidixic acid-resistant derivatives.
[b] Transfer frequencies are the averages of three experiments and are expressed as the number of transconjugants per input donor cell. Donor gonococcal strains are spontaneous rifampin-resistant mutants containing plasmid pFT6.
[c] ND, Not determined.

WR302(pFT6) is mated with a nalidixic acid-resistant derivative of PGH3-2. These data indicate that host-mediated restriction plays a relatively minor role in preventing the acquisition of DNA by conjugation in the gonococcus.

DISCUSSION

Plasmid transformation in the gonococcus is inefficient, unless the plasmid contains gonococcal DNA (20, 22). The apparent increase in transformation efficiency is presumably due to the presence of uptake sequences (5). Sox et al. (19) have shown that when the gonococcus was transformed with plasmid DNA that lacks homology with resident plasmids, about 25% of the transformants contained plasmids of altered size. They postulated that the newly isolated plasmids were the result of endonuclease action during the uptake process. Our inability to introduce cloned genes into certain strains of *N. gonorrhoeae* even though the plasmids were able to transform other strains of *N. gonorrhoeae* led to the experiments demonstrating that host-mediated restriction was responsible for preventing transformation.

When PGH3-2, which produces *Ngo*SI, was transformed with plasmid DNA that was not methylated at the *Ngo*SI sites, the DNA was restricted during transformation. This restriction could be relieved if the transforming DNA was first methylated in vitro with meth M · *Hae*III. In the absence of methylation, we saw at least a 5-order-of-magnitude decrease in the transformation frequency, presumably the result of restriction. The data presented in Table 3 indicate that if restriction is occurring during conjugation, it is much less efficient than what is seen during transformation, since we saw only a 1-order-of-magnitude difference when we conjugated pFT6 from WR302 into PGH3-2.

In addition, strains of *N. gonorrhoeae* that produce restriction enzymes other than *Ngo*SI are able to inhibit the transformation of plasmid DNA (Table 1; Stein, unpublished). However, the extent of restriction is dependent on the amount of DNA used for the transformation, the form of the DNA (covalently closed circles versus linear DNA), the number of restriction sites present on the plasmid, and the amount of enzyme that the strain produces (unpublished observations). PGH3-2 produces at least 10^6 U of *Ngo*SI per g of cells (unpublished results). We have never been able to transform this strain with plasmids that have not been methylated at the *Ngo*SI recognition site.

Since the gonococcus is competent for transformation throughout its growth cycle, transformation may be a principal means by which the gonococcus acquires genetic information. Chromosomal DNA is taken up as double-stranded DNA and remains double stranded until shortly before recombination with the recipient chromosome (21). The data presented in this paper are consistent with a model in which the gonococcus acquires DNA by transformation as a double-stranded molecule (2) and acquires DNA by conjugation as a single-stranded molecule since most restriction endonucleases only act on double-stranded DNA.

The data presented in this paper indicate that researchers will have difficulty introducing genes cloned in *E. coli* back into the gonococcus unless special attention is paid to the restriction phenotype of the strain in use. We have found that most gonococcal strains produce at least one restriction endonuclease. The unavailability of the appropriate DNA methylases will make it difficult to introduce cloned genes into the gonococcus by transformation. The conjugation data indicate that conjugation should be the method of choice for introducing genes cloned in *E. coli* back into the gonococcus.

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