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High-Frequency Conjugal Transfer of a Gonococcal Penicillinase Plasmid

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Gonococci containing a 24 × 10^6-dalton conjugal plasmid were able to mobilize for transfer a smaller, non-self-transmissible penicillinase (Pc^r) plasmid with high frequency under appropriate conditions. In some strains, over 10% of donor colony-forming units transferred the Pc^r plasmid in a mating of less than 2 h, which suggests that the conjugal system was naturally derepressed. Colony-opacity variants containing different quantities of an approximately 28,000-dalton outer membrane protein were altered in their ability to act as conjugal donors and recipients. Maximal transfer of the Pc^r plasmid was observed between transparent donors and recipients, lacking appreciable amounts of the 28,000-dalton protein. Under conditions of high-frequency Pc^r plasmid mobilization, no conjugal mobilization of chromosomal markers could be discerned.

Gonococci which contain a 24 × 10^6-dalton (24-Mdal) conjugative plasmid are able to mobilize a smaller, non-self-transferable penicillinase plasmid (Pc^r) for conjugal transfer to appropriate recipients (2, 6, 8, 16, 19). Frequencies of Pc^r plasmid transfer have been approximately 10^{-5} to 10^{-3} per donor colony-forming unit (CFU) (2, 6, 8, 16, 19). Roberts and Falkow (17) reported that the 24-Mdal conjugative plasmid mobilized for transfer several chromosomal markers as well, although at a lower frequency than the Pc^r plasmid. Studies from this laboratory, however, failed to find evidence for conjugal transfer of chromosomal markers and suggested that apparent chromosomal mobilization might be the result of transformation instead (19). Others have come to the same conclusion (14, 21).

Because of the uncertainty about conjugal transfer of gonococcal chromosomal markers, we have reexamined the problem. We first optimized conditions for conjugal mobilization of the Pc^r plasmid, on the assumption that similar conditions would be optimal for conjugal mobilization of chromosomal genes. We found that the gonococcal conjugational system for Pc^r plasmid transfer was much more efficient than previously demonstrated, but even under these conditions no chromosomal mobilization could be discerned.

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MATERIALS AND METHODS

Strains and materials. The bacterial strains used in these studies are listed in Table 1. The isogenic derivatives containing 4.7-Mdal Pc^r plasmids or 24-Mdal conjugal plasmids were constructed by conjugation using FA288 as the donor. Media and growth conditions were as described previously (19); GC base broth or agar (Difco) was used throughout. The presence of β-lactamase production was confirmed by a chromogenic cephalosporin indicator (Glaxo) (15).

Conjugation procedure. The conjugation method consisted of mixed incubation of genetically marked donor and recipient cells on Gelman Metricel GA6 membrane filters. The inocula were derived from either logarithmically growing cells in GC base liquid medium or 18-h-old GC base agar plates. Unless otherwise stated, about 10^7 CFU of the donor per ml and 10^8 CFU of the recipient per ml were suspended in GC base broth (19). One milliliter of the donor, recipient, or donor plus recipient cell suspension was collected on sterile filters by vacuum suction. The filters were then placed onto GC base agar medium (19). In all experiments, 0.05 ml of a solution containing 1 mg of pancreatic DNase per ml was added to the filter immediately before the cells, so as to inhibit transformation. In some experiments both cell suspensions were treated with 100 μg of pancreatic DNase I (Worthington) per ml for 10 min at 37°C before being placed on the filter, or DNase was added at intervals to the mating mixtures on the filters. Donor and recipient cells were used in their nonpiliated phase, which is essentially noncompetent for genetic transformation (20). Filters containing cells were incubated in the presence of 4% CO_2 for 90 min unless otherwise stated. After incubation, each filter was agitated in 1 ml of medium A of Davis and Mingioli (5) to suspend the cells. The suspended cells were then vigorously agitated to disrupt the mating pairs, diluted appropriately in medium A, and spread on GC base agar containing either streptomycin (Str) (200 μg/ml) or nalidixic acid (Nal) (10 μg/ml) to inhibit the Str^s or Nal^s donor but not the Str^r or Nal^r recipient. Unless otherwise stated, 4 to 6 h of incubation at 37°C in 4% CO_2 was allowed for phenotypic expression of newly introduced donor markers before plates were overlaid

TABLE 1. *Bacterial strains used*

Strains and plasmids[a]	Source and reference	Distinguishing characters[b]
N. gonorrhoeae		
F62	D. Kellogg	*pro-1*
FA288(pFA2)(pFA3)	C. Thornsberry, CDC6-7 (6)	β-Lactamase-producing (Pc^r) *str-12* prototrophic conjugative donor
FA306(pFA2)	From FA288 (6)	Like FA288 but Pc^s *nal-2 rif-1 fus-2*
FA583	From F62 (19)	*nal-1 arg-1 met-1 pro-1*
FA589(pFA2)(pFA3)	From F62 (19)	*rif-1 str-7 spc-3 leu-1* Pc^r, conjugative donor
FA616	From F62	*str-7 rif-1*
FA676	S. Falkow, KH7155	*nal-5 rif-3 arg-3 met-2*
FA698	From FA19	*arg-2 hyx-1 nal-3*
FA720(pFA2)(pFA3)	From F62	*str-7 spc-3 met-1,* Pc^r conjugative donor
FA721(pFA3)	From F62	*str-7 spc-3 met-1* Pc^r
FA722(pFA2)(pFA3)	From FA583	As FA583, but Pc^r, conjugative donor
FA573(pFA2)(pFA3)	From FA757	*str-11* Pc^r, opaque colony, conjugative donor
FA754(pFA2)(pFA3)	From FA753	As FA753, but transparent colony type
FA755	From FA757	*nal-4,* opaque colony type
FA756	From FA755	As FA755, but transparent colony type
FA757	Clinical isolate	Definite opaque and transparent colony types
E. coli		
C600.5	C. A. Hutchison	Restriction$^-$ modification$^-$

[a] All gonococcal strains also contain a 2.6-Mdal plasmid of uncertain function.

[b] Symbols: Pc^r, plasmid-encoded production of β-lactamase; *fus, nal, rif, spc, str,* chromosomally determined resistance to fusidic acid, nalidixic acid, rifampin, spectinomycin, or streptomycin, respectively; *arg, hyx, met, pro,* requirement for arginine, hypoxanthine, methionine, or proline, respectively.

with 4 ml of GC base medium containing drug(s) selective for the donor antibiotic resistance marker(s). To select for donor prototrophic nutritional markers, cell suspensions were washed once in medium A and plated immediately onto NEDA medium (4) lacking the amino acid required by the recipient, and containing streptomycin or nalidixic acid to which the recipient was resistant but the donor was sensitive. In each experiment, respective donor and recipient cells were incubated on separate filters as controls to check for spontaneous mutation. Conjugation frequencies were expressed as numbers of transcipients per input donor CFU.

Single-cell penicillin sensitivities. Approximately 100 CFU of individual gonococcal strains was spread onto the surface of GC base agar plates. The plates were air dried for about 15 min, and were overlaid with 4 ml of soft GC base agar containing sufficient penicillin to yield the desired concentration after diffusion throughout the medium. (This method was chosen to approximate most closely the conditions for selection of Pc^r transconjugants.) The lowest concentration that resulted in any decrease in number of colonies after 40 h of incubation at 37°C was taken as the single-cell minimum inhibitory concentration.

Membrane isolation and polyacrylamide gel electrophoresis. Membranes were prepared as previously described (7) from mid-log-phase cultures grown in GC base broth. Purity of the colony types of the harvested cells was >90%. The outer membrane cell wall complex was purified by treatment with 0.2% sodium lauryl sarcosinate (7). Outer membrane proteins were solubilized as described previously (7) and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (9), using a 15% polyacrylamide separating gel.

RESULTS

Variables affecting apparent frequency of Pc^r plasmid mobilization. (i) Concentration of penicillin. The results shown in Table 2 demonstrate that the apparent conjugation frequency started to decrease sharply with penicillin concentrations higher than 0.12 μg/ml in the selective medium. This presumably was due to the very low level of single-cell resistance (single-cell minimum inhibitory concentration, 0.12 μg of penicillin G per ml) resulting from acquisition of the Pc^r plasmid by the gonococcal strains used in these experiments (Fig. 1). With appropriately low concentrations of penicillin, very high frequencies of Pc^r transfer ($\geq 10^{-1}$) were consistently observed in crosses between FA589 and FA583.

Satellites of penicillin-sensitive colonies around penicillinase-producing colonies were observed when high cell densities of the undiluted mating mixture were applied to selective media containing low concentrations of penicillin. However, this was not a significant problem when 10^{-3} to10^{-4} dilutions of the mating mixture were applied to the same media. Further confirmation of the ability of media containing only 0.12 μg of penicillin per ml to select Pc^r transconjugants was obtained in two ways. (i) Twenty-seven of 30 clonally purified "transconjugant" colonies produced β-lactamase, as determined by the chromogenic cephalosporin method (15). (ii) Plating of suitable dilutions of

TABLE 2. *Effect of penicillin concentration on apparent conjugation efficiency*[a]

Penicillin concn (μg/ml)	Pc^r transconjugants per input donor CFU
0.06	1.2×10^{-1}
0.12	1.4×10^{-1}
0.25	7.0×10^{-3}
0.50	2.0×10^{-4}
1.0	2.5×10^{-4}

[a] FA589 served as the Pc^r donor, and FA583 was the Pc^s recipient. After a 90-min mating, cells were plated on medium containing 10 μg of nalidixic acid per ml. Penicillin was added after another 4 h of incubation. The MIC of the recipient was 0.004 μg of penicillin per ml.

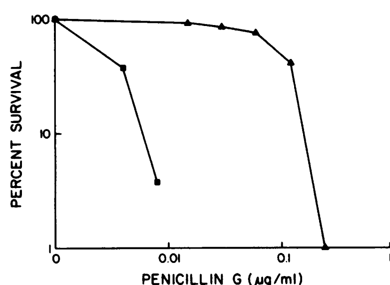

FIG. 1. *Single-cell sensitivities to penicillin G of the isogenic strains FA722 (Pc^r; ▲) and FA583 (Pc^s; ■).*

a mixture of 10^7 CFU of FA589 (Pc^r) and 10^8 CFU of FA583 (Pc^s) on media containing 0.12 μg of penicillin per ml resulted in growth of only 10^7 CFU; all of the survivors were also Str^r, as expected for FA589 but not FA583 (data not shown).

(ii) Concentrations of parental cells. The frequencies of Pc^r plasmid transfer were influenced both by the absolute numbers of parental cells and the ratio of donor to recipient cells (Table 3). When the recipient cell concentration was kept at a fixed but high level (300×10^6 CFU), about 10% of donors transferred the Pc^r plasmid to a recipient, over a large range of donor cell concentrations. With a fixed and relatively low number of donor cells, there was a progressive decline in frequencies of Pc^r plasmid transfer per donor cell as the number of recipients was reduced. When the total cell density was low, even a high ratio of recipient to donor cells did not result in maximal frequencies of Pc^r plasmid transfer.

(iii) Growth phase of parental cells. Efficiencies of Pc^r plasmid transfer appeared to be independent of the growth state of parental cells. Conjugation frequencies were unchanged when cells of either parent were taken from 18-h GC base agar plates or from aerated broth cultures in various phases of growth (lag phase, exponential, stationary). However, transfer frequencies decreased, often by 100-fold or more, when the inocula were derived from overnight, nonshaken cultures. Addition of 2 mM $CaCl_2$, 10 mM $MgCl_2$, or 50 mM NaCl to the broth medium had no appreciable effect on transfer frequencies (data not shown).

(iv) Effect of outer membrane structure. Dark-colored (opaque) colony types of *Neisseria gonorrhoeae* are known to have markedly increased amounts of one or more approximately 28,000-dalton outer membrane proteins, as compared with light-colored (transparent) colony types (10, 11, 23, 24). Presence of this protein results in increased cell clumping and increased colony opacity (22). Alterations of outer membrane proteins are known to affect mating efficiencies in *Escherichia coli* (1, 13). We used an isogenic set of opaque and transparent colonial variants to determine whether the increased content of the approximately 28,000-dalton protein in the opaque variant (Fig. 2) had an appreciable effect on conjugation efficiency. Although opacity variants of some gonococcal strains exhibit differences in sensitivity to penicillin (11), the penicillin sensitivity of the opaque and transparent derivatives used in these experiments was identical. We observed a 5- to 10-fold decrease in efficiencies of conjugal Pc^r transfer when either the donor or the recipient was of the opaque colony phenotype; slightly greater decreases were seen when both donor and recipient were opaque (Table 4). Nearly identical results were obtained using transparent and opaque variants of a second, unrelated strain in similar experiments (data not shown).

(v) Interstrain matings. Matings between

TABLE 3. *Optimal cell concentrations for conjugation on filters*[a]

Donor (10^6 CFU)	Recipient (10^6 CFU)	Transconjugants per ml	Conjugation efficiency[b]
39	300	1.0×10^7	0.26
5.2	300	1.0×10^6	0.19
0.66	300	9.0×10^4	0.14
0.067	300	2.0×10^4	0.30
0.001	300	2.5×10^2	0.25
0.43	300	7.1×10^4	0.16
0.43	22	9.5×10^3	0.022
0.43	3.7	8.2×10^2	0.002
0.43	0.32	6.6×10^2	0.002

[a] Nonpiliated, transparent colony forms of donor FA722 and recipient FA616 were mated as described in the text, and transconjugants were selected on plates containing 0.1 μg of penicillin G and 200 μg of streptomycin per ml.

[b] Conjugation efficiency = transconjugants per input donor CFU.

different strains of *N. gonorrhoeae* were investigated. For each recipient, the lowest possible concentration of penicillin was used to select Pc^r transconjugants. When FA288 was used as the donor, maximal conjugation efficiencies were approximately 2.5×10^{-2} with an isogenic strain and one unrelated strain as recipients, but were reduced by nearly 100-fold with two other unrelated gonococcal strains as recipients (Table 5). Maximal conjugation efficiencies with *E. coli* C600.5 as recipient were only 5×10^{-4}. When a derivative of gonococcal strain F62 (FA589, containing the conjugal plasmid pFA2 originally isolated in FA288) was used as donor in isogenic matings with an F62 derivative (FA583), frequencies of conjugal transfer of Pc^r of 10 to 30% per donor cell were consistently observed. Interstrain matings of FA589 with recipients derived from other strains showed modest reductions in conjugation efficiency (Table 5). Thus, there

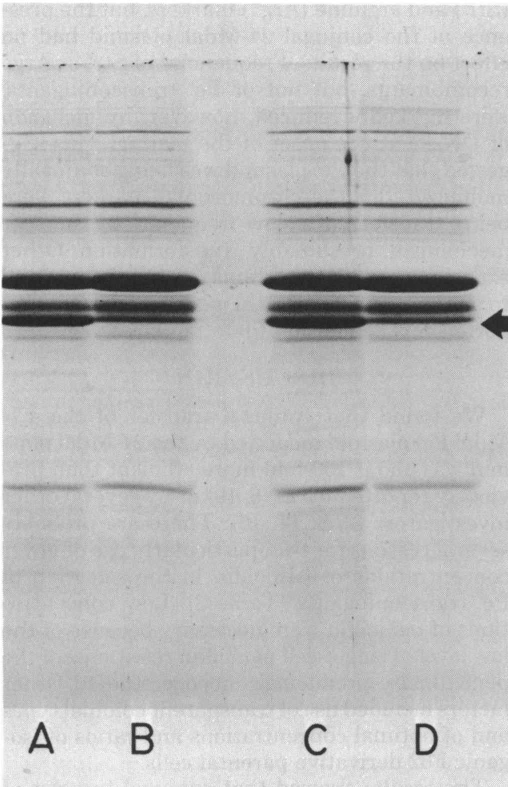

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins from strains used in experiments presented in Table 4. Membranes were prepared from nonpiliated opaque or transparent colonies. Arrow indicates 28,000-dalton opacity-associated protein. (A) FA753 (opaque, conjugal donor); (B) FA754 (transparent, conjugal donor); (C) FA755 (opaque, conjugal recipient); (D) FA756 (transparent, conjugal recipient).*

TABLE 4. *Effect of outer membrane structure on efficiency on conjugal Pc^r plasmid transfer*

Donor	Recipient	Conjugation efficiency[a]
FA753 (Op)[b]	FA755 (Op)	6.3×10^{-3}
FA753 (Op)	FA756 (Tr)	1.4×10^{-2}
FA754 (Tr)	FA755 (Op)	2.1×10^{-2}
FA754 (Tr)	FA756 (Tr)	1.0×10^{-1}

[a] Mean of three experiments. Matings were as described in the text, and transconjugants were selected on plates containing 0.1 μg of penicillin G and 10 μg of nalidixic acid per ml.

[b] Parentheses indicate colony type: Op, opaque; Tr, transparent. All strains were in their nonpiliated colony type.

TABLE 5. *Conjugal Pc^r plasmid transfer between unrelated gonococcal strains*

Donor strain[a]	Recipient	Conjugation efficiency[b]
FA288	FA306[c]	2.4×10^{-2}
	FA583[d]	2.5×10^{-2}
	FA698[d]	2.3×10^{-4}
	FA676[d]	6.8×10^{-4}
FA589	FA583[c]	3.0×10^{-1}
	FA306[d]	1.6×10^{-2}
	FA698[d]	5.5×10^{-2}
	FA676[d]	8.2×10^{-2}

[a] All strains were nonpiliated and predominantly in the transparent colony type.

[b] Pc^r transconjugants per input donor CFU. Matings were for 90 min, followed by 5 h for phenotypic expression of Pc^r, with selection on plates containing 0.1 μg of penicillin and 10 μg of nalidixic acid per ml.

[c] Isogenic mating.

[d] Interstrain mating.

were sometimes significant reductions in conjugation efficiencies in interstrain matings. Strain F62 derivatives were consistently the best donors. It was not clear whether these differences were due to differences in efficiency of formation of mating aggregates, or to other factors.

Conjugal plasmid pFA2 is not repressed. Experiments revealed that mating for 6 h, followed by plating directly onto Pc^r selective medium, yielded maximum conjugation frequencies. Six hours of mating presumably included both the time required for plasmid transfer as well as the time necessary for phenotypic expression of penicillin resistance. To determine the times required for plasmid transfer and for phenotypic expression, a time course experiment was undertaken. Cells were mated on filters for up to 120 min, and then mating pairs were disrupted. At hourly intervals thereafter, penicillin was applied to select transconjugants. The results showed that for any given time of mating, further incubation to a total of 6 h was required

for apparent full phenotypic expression of the newly introduced plasmid. However, transfer of the Pc^r plasmid was effected rapidly, reaching a maximum in about 1 h (Fig. 3 and data not shown). Observation of rapid high-frequency conjugational transfer of penicillin resistance suggested that the gonococcal conjugative system is not strongly repressed.

Basis for reported chromosomal gene transfer. Utilizing the conditions found optimal for Pc^r plasmid transfer (transparent variants; log-phase cells from broth culture; donor/recipient ratio of 1:10; total cell concentration on membrane filter, 10^8 CFU), we reinvestigated the possibility of chromosomal gene mobilization by the 24-Mdal conjugal plasmid.

If the conjugal plasmid truly mobilizes the chromosome, the number of recombinants for chromosomal genes should differ when strains varying only in presence of the conjugal plasmid are used as donors. The results shown in Table 6 demonstrated that this was not observed. Mixed-cell matings did result in apparent transfer of the chromosomal streptomycin resistance (Str^r) and arginine (Arg$^+$) markers, but the presence of the conjugal 24-Mdal plasmid had no effect on the results. Frequencies of Str^r or Arg$^+$ recombinants, but not of Pc^r transconjugants, were markedly reduced, however, by inclusion of DNase at the onset of the mating. This suggested that the Pc^r plasmid was being conjugally mobilized, but the chromosomal markers were being transferred at low frequency by another mechanism, presumably transformation. Other experiments with the strains previously reported to mobilize chromosomal genes (17) also supported this conclusion (data not shown)

TABLE 6. *Lack of effect of the 24-Mdal conjugal plasmid on transfer of chromosomal genes in mixed-cell matings*[a]

Donor strain	DNase (μg)	Frequency of donor marker transfer		
		Str^r	Arg$^+$	Pc^r
FA720	50	8.0×10^{-7}	2.0×10^{-7}	8.0×10^{-2}
FA720	0	1.6×10^{-5}	1.0×10^{-6}	1.3×10^{-1}
FA721	50	8.0×10^{-7}	1.8×10^{-6}	$<10^{-8}$
FA721	0	1.1×10^{-5}	2.0×10^{-6}	$<10^{-8}$

[a] Isogenic, noncompetent, nonpiliated, transparent colonies of donors FA720 (containing the conjugal plasmid pFA2) or FA721 (lacking pFA2) and recipient FA583 were used. FA720 and FA721 contain the Pc^r plasmid pFA3 and are Str^r and Arg$^+$, whereas FA583 is Str^s, Arg$^-$, and Pc^s. DNase was added as indicated before mixing of donor and recipient on membrane filters. Mating was for 6 h on the filters. Recombinants were selected on media containing 10 μg of nalidixic acid per ml (to counterselect the donor) and either 200 μg of streptomycin per ml, for defined medium lacking arginine, or 0.1 μg of penicillin per ml, as described in the text.

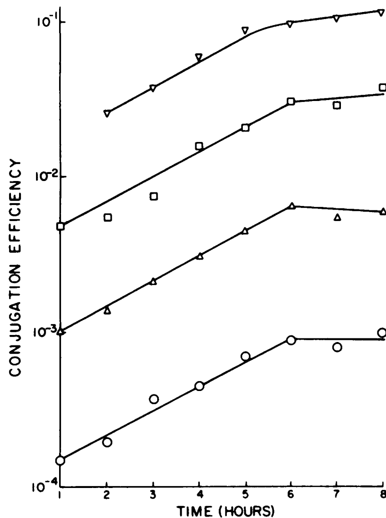

FIG. 3. *Time course for transfer and phenotypic expression of Pc^r plasmid. N. gonorrhoeae strains FA721 and FA616 were mated on a series of filters for times varying from 5 to 120 min (contact time). The mating mixtures were then suspended in broth, vigorously agitated, diluted, and incubated on agar medium containing 200 μg of streptomycin per ml to inhibit donor cells. At hourly intervals thereafter, penicillin (0.1-μg/ml final concentration) was applied in a soft agar overlay to select Pc^r transconjugants (time for phenotypic expression). The times plotted are the sum of the contact time and time for phenotypic expression.* (○) *5-min contact time;* (△) *15-min contact time;* (□) *30-min contact time;* (▽) *120-min contact time.*

DISCUSSION

We found that conjugal transfer of the 4.7-Mdal Pc^r plasmid mediated by the 24-Mdal plasmid was about 100-fold more efficient than previously reported by us (6, 19) or by several other investigators (2, 8, 14, 16). There are probably several reasons for this, particularly use of lower concentrations of penicillin in the selection of Pc^r transconjugants (Table 2). Low concentrations of penicillin were necessary because of the low level of single-cell penicillin resistance of the penicillinase-producing gonococci studied. Other factors included use of transparent colonial types and of optimal concentrations and ratios of isogenic F62 derivative parental cells.

The results showed that conjugal transfer of the Pc^r plasmid was rapid, being complete in less than 2 h (Fig. 3). This was probably too rapid for secondary rounds of derepressed transfer from new transconjugants (3), and suggests that the gonococcal conjugal system is not naturally repressed. Although earlier preliminary efforts to visualize conjugal pili in gonococci containing the 24-Mdal conjugal plasmid were unsuccessful

(S. To and C. C. Brinton, personal communication), further efforts are warranted on the basis of the results reported here.

Marked differences in Pc^r plasmid transfer efficiencies were noted in isogenic strains varying only in the 28,000-dalton outer membrane heat-modifiable opacity-associated protein. One might have expected that this protein would increase efficiency of conjugation rather than decrease it (Table 4), since it promotes increased adhesion between gonococcal cells (22) and therefore would be expected to increase formation and stability of mating aggregates. Presence of this or other outer membrane proteins was not influenced noticeably by the 24-Mdal conjugative plasmid (data not shown). An outer membrane protein of *E. coli* designated 3A, which is also heat modifiable (12), affects conjugation, but in the opposite direction: *E. coli* mutants lacking protein 3A are reduced in their ability to act as conjugal recipients (12). We presume that one or more of the approximately 28,000-dalton opacity-associated outer membrane gonococcal proteins reduces efficiency of effective mating pair formation. Further work is needed to clarify which of the closely related heat-modifiable proteins (11) is involved in conjugation, and also to delineate precisely how conjugation is influenced by these variations in gonococcal outer membrane structure.

We were unable to demonstrate conjugal chromosomal transfer due to the presence of the 24-Mdal plasmid. We have no evidence for the assumption that conditions optimal for mobilization of the non-self-transferable Pc^r plasmid by the 24-Mdal conjugal plasmid would also be optimal for mobilization of the chromosome. However, our failure to demonstrate detectable chromosome mobilization agrees with the results of Norlander et al. (14) and Steinberg and Goldberg (21). At present, since there still are no known gonococcal bacteriophage, and therefore no system for transduction, the only reproducible system for study of the genetics of the gonococcal chromosome remains transformation. It remains a reasonable goal to try to construct derivative strains with conjugal plasmids which will mobilize the chromosome with appreciable frequency, for this would significantly enhance capability for genetic studies in this organism.

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