Plasmid-Mediated Chromosomal Gene Transfer in *Neisseria gonorrhoeae*

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An indigenous *Neisseria gonorrhoeae* conjugative plasmid, pLE2450, was tested for its ability to mediate chromosomal gene transfer between gonococcal strains. Plasmid-mediated chromosomal transfer was detected at a low frequency and can be used to establish certain linkage relationships between amino acid and antibiotic resistance markers.

Transformation has historically been the only known mode of genetic exchange between strains of *Neisseria gonorrhoeae* (3, 30, 34). Competence in the gonococcus is limited, for all practical purposes, to T1 and T2 colony types (3, 31, 34). This technical restriction greatly reduces the number of strains and markers available for genetic analysis. In addition, transforming gonococcal DNA fragments are rarely larger than 5% of the genome (30, 32). Hence, genetic analysis of *N. gonorrhoeae* by transformation has identified only a relatively few linkage groups (16). In particular, Spangler and his associates have identified a cluster of linked antibiotic resistance loci comprising about 2% of the gonococcal chromosome (31, 32) which represents genes coding for ribosomal proteins (17) or associated with the cell envelope (11, 18, 29, 32). Little is known about the linkage relationships between recently identified gonococcal nutritional markers (4–6, 20, 34).

In 1976, penicillin-resistant β-lactamase-producing strains of *N. gonorrhoeae* were reported throughout the world (1, 24, 25, 33). β-Lactamase production by these strains was shown to be plasmid mediated (2, 8, 9). More recently, we (28) as well as others (2, 8, 15) have demonstrated the ability of a 24.5 × 10^6-dalton (24.5-Mdal) indigenous gonococcal plasmid to promote transfer of itself and a smaller nonconjugative R plasmid among gonococci and even from gonococci to *Escherichia coli*. In this paper, we report that the indigenous conjugative plasmid pLE2450 can mediate chromosomal gene transfer between gonococcal strains.

**MATERIALS AND METHODS**

Bacterial strains. A T4 penicillin-resistant (Pcr') and streptomycin-resistant (Str') strain of *N. gonorrhoeae* CDC67 which falls into the zero auxotype class of Carifo and Catlin (4) was used as a donor. CDC67 carries a 4.4-Mdal R plasmid, pMR0360, which codes for β-lactamase (26), a small 2.6-Mdal nonconjugative indigenous plasmid identical to pLE2600 (26), and a 24.5-Mdal conjugative plasmid, pLE2450. *N. gonorrhoeae* CDC66, which has the same phenotype as CDC67 but lacks the 24.5-Mdal plasmid, was used as a control donor. CDC66 has been shown to be unable to transfer its 4.4-Mdal R plasmid, whereas CDC67 is able to transfer the 4.4-Mdal R plasmid (27). A T4 gonococcal isolate, KH7155, which has been made nalidixic acid resistant (Nal') and rifampin resistant (Rif') and requires arginine (Arg'), uracil (Ura'), proline (Pro'), hypoxanthine (Hx'), and methionine (Met') for growth, was used as the recipient. Strain KH7155 carries a small nonconjugative indigenous plasmid identical to pLE2600. A second T4 strain, LP99, was also used, which has the same phenotype as KH7155 but carries the 24.5-Mdal conjugative plasmid identical to pLE2450 along with a 2.6-Mdal plasmid. Both KH7155 and LP99 are characteristic of strains isolated from cases of disseminated gonococcal infections; they show a high degree of susceptibility to penicillin G (less than 0.01 μg/ml) and are resistant to the bactericidal action of normal serum (20).

**Media.** Catlin and her associates have described a system for differentiating isolates of *N. gonorrhoeae* based on their ability to grow on chemically defined agar media (4, 5). A complete defined media contains all of the compounds required for gonococcal growth, whereas the differential media is lacking one or more essential growth supplements. For our purposes, Catlin complete medium and Catlin media lacking either L-proline, L-arginine, L-methionine, or uracil were employed for mating experiments and for auxotyping transconjugants. Agarose (0.085% Seakem) was substituted for agar in the formulation of these media. The differential plates used in the mating procedure to detect putative transconjugants were further supplemented with 5 μg of rifampin and 10 μg of nalidixic acid per ml to prevent the donor CDC67 from growing. Mating mixtures containing 10^7 to 10^8 recipients per ml (0.5 ml) were plated on two to four petri dishes (150 by 15 mm) containing 50 ml of the appropriate selective medium. Controls consisted of CDC67 donor cells and KH7155 recipient cells plated alone on a medium...
of the same composition. The total recipient cell pop-
ulation in the mating mixture was enumerated by
placing mixtures on complete Catlin medium contain-
ing 5 µg of rifampin and 10 µg of nalidixic acid per ml.
GC medium base (Difco) to which 1% defined supplement
was added as described by Mayer (19) was also
employed for colony counts and as a base for anti-
biotic-susceptibility testing. This medium containing 5 µg of rifampin and 10 µg of nalidixic acid per ml was
also used to detect transfer of streptomycin resistance
(100 µg of streptomycin per ml) from donor to recipient
cell as well as to detect transfer of the donor R plasmid
(1 µg of penicillin G per ml). Liquid GC medium base
(GCH broth) is similar in composition to GC medium
base except for the omission of agar and the use of N-
2-hydroxyethyl piperazine-N2-2-ethanesulfonic acid
(Sigma) in place of phosphate.
Preparation of transforming DNA. Deoxyribo-
nucleic acid (DNA) was extracted from strain CDC67
by the method described by Mayer (20). The ability of the
DNA preparation to transform competent T1 gono-
cocci was determined using the proline-requiring
strain F62 T1. Transformation was performed by a
modification of the transformation procedure de-
scribed by Sparling (30). Pro+ transformants were
obtained at a frequency of 10⁻³ with 1 µg of DNA per
ml.
Standard mating procedure. T4 donor cells,
CDC67, and T4 recipient gonococcal cells, KH7155,
were inoculated into 10 ml of GCH broth supple-
mented with 2 x 10⁻³ M CaCl₂ and 2 x 10⁻³ M MgCl₂
and allowed to grow overnight. The cells were col-
cected by passing 1.5 ml of each overnight culture
through a membrane filter (Biorad, 0.2 µm). To de-
termine whether transformation or reversion was occur-
cing, recipient and donor cells were placed on separate
filters. The recipient KH7155 was incubated with 100 µg
of purified DNA per ml from CDC67. CDC66 which
did not contain the 24.5-Mdal plasmid was used as a
donor in the matings with KH7155. The filters were
then placed on GC medium plates which had 2% defined supplement added and incubated for 6 h at
36.5°C in a CO₂ incubator. After incubation, the filters
were placed in 2 ml of buffered salt solution (4-6) and
vigorously agitated to remove the cells. A final cell
density of 10⁷ to 10⁹ recipients per ml was optimal.
Samples of the mating mixture were plated on Catlin
defined media which lacked one amino acid, whereas
other samples were diluted and plated on GC medium
or complete Catlin defined media supplemented with
nalidixic acid and rifampin to determine the colony
count of the recipient. Matting plates were incubated
at 36.5°C in a CO₂ incubator. Plates were held 7 days
before being discarded as "no growth."
Matings were also performed in the presence of
deoxyribonuclease (DNase). The cells were grown
overnight in 100 µg of DNase (Sigma) per ml, and
before the cells were placed on filters, 1,000 µg of
DNase per ml was added to the filter, and 600 µg of
DNase per ml was added to the buffered salt solution
before filters were added. No differences in frequencies
were found whether DNase was or was not used during
the mating procedures.
Auxotyping and antibiotic resistance testing.
Each colony appearing on the mating plates was re-
streaked two or three times on a medium with the
same composition. Incubula for testing were taken from
plates incubated overnight. Cells were suspended in a
buffered salt solution, and cell density was adjusted to
allow the Steers replicator to deliver between 10⁵ and
10⁶ colony-forming units to the plate. Twenty-nine
isolates and both parental strains were included on
each plate. The differential medium lacking one amino
acid was inoculated first, followed by a complete plate
containing all required compounds, and then the anti-
biotic plates. Each isolate was tested twice.
Agarose gel electrophoresis of DNA. Cleared
lysates of bacterial strains were prepared, and samples
were subjected to electrophoresis through a 0.7% agar-
ose gel as described by Meyers et al. (21).
Assay of β-lactamase activity. β-Lactamase ac-
activity was assayed by the rapid chromogenic cephal-
sophrin substrate method of Kammer et al. (12) and
O'Callahan (22).

RESULTS
Plasmid-mediated chromosomal gene transfer.
Matings were performed between T4 colony variants of
 donor strain CDC67 and recipient strain KH7155
with selection for transfer of the donor markers arg, ura, pro, and met. To
rule out transformation or reversion, the donor
CDC67 and recipient KH7155 were placed on
separate filters, and the recipient was also incu-
bated with 100 µg of purified DNA per ml from
the donor CDC67. Matings were also performed
using CDC66 as a donor. This strain is pheno-
typically identical to CDC67, except that it does
not carry the 24.5-Mdal conjugative plasmid and is
unable to transfer its 4.4-Mdal R plasmid by
conjugation (27). In addition, matings were per-
formed with CDC66 plus 100 µg of purified DNA
per ml from CDC67 and KH7155 to eliminate
the possibility that a cell-DNA interaction was
promoting T4 transformation. The results of the
three sets of experiments are summarized in
Table 1. No colonies appeared when the donor
CDC67 was plated alone or when KH7155 was
mated with CDC66 in the presence of 100 µg of
purified DNA per ml. One colony or less per 10⁷
colonies per ml was found when KH7155 was
plated alone, and similar results were obtained
when KH7155 was incubated with purified DNA
or mated with CDC66. The colonies appearing
on these controls were prototrophic for only one
marker and most likely represent reversions.
However, a significant number of colonies did
appear when KH7155 was mated with CDC67.
The presence of DNase during the mating pro-
cedure did not significantly alter the frequency;
therefore, it was not used in all subsequent ex-
periments.
The data indicate that reversion for the four
selective markers was very low and that trans-
formation did not occur to a significant extent
when saturating DNA concentrations were incubated with the T4 recipient. The data are consistent with the view that the 24.5-Mdal plasmid plays a significant role in the appearance of recombinants. We assume, therefore, that the 24.5-Mdal plasmid was mobilizing chromosomal genes.

Transconjugant colonies were picked and restreaked twice on the same selective medium prior to testing for auxotype and antibiotic resistance. Representative data pooled for several experiments for the inheritance of recombinant unselected donor traits in the transconjugants are shown in Table 2. Most transconjugant colonies tested showed a variety of recombinant phenotypes which differed from either parent by more than one marker. The data clearly show a high level of linkage between arg and ura. Other linkage relationships, met-pro and met-str, are obviously dependent upon the selected marker.

It may be noted that R-plasmid inheritance was not associated with coinheritance of any chromosomal trait (less than 1%).

In other chromosomal transfer systems mediated by autonomous conjugative plasmids, the conjugative plasmid is usually found in the transconjugants. Yet, previous studies by us (27) have shown that while pLE2450-mediated mobilization of R plasmids occurred at a high frequency during the 6-h mating, transconjugants receiving the R plasmid rarely showed the 24.5-Mdal plasmid, pLE2450. We selected twenty transconjugants isolated on a variety of selected media for different nutritional traits and possessing various recombinant phenotypes. DNA was extracted from these transconjugants and analyzed by agarose-gel electrophoresis. All twenty transconjugants examined that had inherited chromosomal genes had also inherited the conjugative plasmid pLE2450; three isolates are represented in Fig. 1. Although the 4.4-Mdal R plasmid is not visible in the transconjugants shown in Fig. 1, it should be noted that, in fact, most (more than 90%) of the transconjugants selected for the inheritance of chromosomal traits received the R plasmid (data not shown). No attempt was made to maintain the R plasmid through penicillin selection, and, as a consequence, the R plasmid was frequently lost, as has been previously noted (26).

To determine whether entry exclusion was operating in this mating system, we compared the chromosomal gene transfer and R-plasmid transfer using KH7155, which carries only the 2.6-Mdal plasmid, and LP99, which carries both the 24.5-Mdal conjugative plasmid and the 2.6-Mdal plasmid. Both these strains are phenotypically the same and are representative of DGI strains. As illustrated in Table 3, virtually no difference in frequency was observed for either chromosomal or R-plasmid transfer, and the frequency of transfer was similar: about $1 \times 10^{-6}$ for each of the four selected amino acid markers.

### Table 1. Chromosomal gene transfer

<table>
<thead>
<tr>
<th>Selected marker</th>
<th>Recipient KH7155</th>
<th>Donor CDC66</th>
<th>Donor CDC67</th>
</tr>
</thead>
<tbody>
<tr>
<td>arg</td>
<td>0</td>
<td>0</td>
<td>480</td>
</tr>
<tr>
<td>ura</td>
<td>0</td>
<td>0</td>
<td>250</td>
</tr>
<tr>
<td>met</td>
<td>0</td>
<td>0.8c</td>
<td>320</td>
</tr>
<tr>
<td>pro</td>
<td>0.3d</td>
<td>0</td>
<td>450</td>
</tr>
</tbody>
</table>

*a* The average number of colonies per 107 recipients (KH7155) in a 6-h mating for three experiments.

*b* Donor strain CDC66 contains the 4.4-Mdal R plasmid and the indigenous 2.6-Mdal plasmid. Donor strain CDC67 contains the 24.5-Mdal conjugative plasmid, the 4.4-Mdal R plasmid, and the indigenous 2.6-Mdal plasmid.

*c* Each of these colonies was prototrophic for only one marker; they most likely represent reversions, though transformation cannot be ruled out.

*d* Each of these colonies was prototrophic for only one marker; they represent reversions.

### Table 2. Linkage frequency

<table>
<thead>
<tr>
<th>Selected marker</th>
<th>No. of colonies tested</th>
<th>Frequency of unlinked marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>ura</td>
<td>154</td>
<td>0.98</td>
</tr>
<tr>
<td>arg</td>
<td>89</td>
<td>0.94</td>
</tr>
<tr>
<td>met</td>
<td>134</td>
<td>0.69</td>
</tr>
<tr>
<td>pro</td>
<td>99</td>
<td>0.27</td>
</tr>
<tr>
<td>pc</td>
<td>100</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*a* Each transconjugant colony appearing on the mating plates was restreaked two or three times on a medium with the same composition. Inocula for testing were taken from plates incubated overnight. Cells were suspended in a buffered salt solution, and cell density was adjusted to allow the Steers replicator to deliver between 105 and 106 colony-forming units to the plate. Twenty-nine isolates and both parental strains were included on each plate. The differential medium lacking one amino acid was inoculated first, followed by a complete plate containing all required compounds and then the antibiotic plates. Each isolate was tested twice.
The transfer of the R-plasmid pMR0360 was at least two orders of magnitude higher than seen with chromosomal transfer.

DISCUSSION

The four markers, arg, pro, ura, and met, were transferred at roughly the same frequency, $10^{-5}$ to $10^{-7}$ per recipient cell, whereas the gonococcal R-plasmid mobilization ranged from $10^{-3}$ to $10^{-5}$ per recipient cell. However, in any one experiment, R-plasmid transfer was at least 100 times more frequent than the corresponding chromosomal gene transfer.

Aside from the difference in frequency, there was striking difference between transconjugants selected solely for R-plasmid inheritance and those which were selected for the inheritance of chromosomal markers. Transconjugants receiving only the R-plasmid rarely received the conjugal plasmid pLE2450 in a 6-h mating, whereas 90% of the transconjugants which were selected for chromosomal markers were found to receive both the donor R plasmid and the conjugal plasmid. These data suggest that there may be different mechanisms operating by which the conjugal plasmid pLE2450 brings about mobilization of the chromosome and mobilization of the autonomous R plasmid.

The frequency of gene transfer for the four markers used was roughly equal, suggesting that there is no oriented gene transfer. Therefore, the system may be similar to the classical E. coli F' × F- mating, in which it has been well documented that there is no obvious preference for the transfer of markers from any one segment of the chromosome in crosses from normal rec+ strains mediated either by autonomous F' (7) or by Col I (10) and other plasmids (23). However, since only four markers were tested, it is still possible that subsequent studies will demonstrate that oriented gene transfer does occur.

Preliminary data suggest that transconjugants do not become more efficient donors in subsequent matings, as might be expected for F-prime elements. Similarly, we have not been able to isolate any donor derivatives analogous to an E. coli Hfr or a derepressed conjugal R plasmid with an increased capacity to transfer chromosomal genes.

The significance of conjugation in the natural ecology of N. gonorrhoeae is unknown. Epidemiological data do suggest that conjugation is the primary mode of transmission for the β-lactamase R plasmid. Does low-level chromosomal transfer among gonococci or between the gonococcus and related species occur in nature? At the present time we are examining a model in vivo system to gain insight into this question. Regardless of its role in nature, which may be inconsequential, we expect that plasmid-mediated chromosomal transfer will prove of considerable utility in analyzing and mapping the N. gonorrhoeae genome.

ACKNOWLEDGMENTS

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LITERATURE CITED