R-Plasmid Transfer and Its Response to Nalidixic Acid

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The conjugational transfer efficiency of 41 wild-type R-plasmids was studied in Escherichia coli K-12. Type I R-plasmids were transferred at comparatively high and rather uniform frequencies, whereas type F R-plasmids showed less uniform and, on average, somewhat lower transfer frequencies. R-plasmids not mediating sensitivity to F-, I-, or N-specific phages showed moderate transfer frequencies, and type N R-plasmids showed very low transfer frequencies. Various lines of evidence suggest that a well-expressed, but functionally inefficient, conjugation apparatus is the cause of the poor transfer of type N R-plasmids in liquid medium. Nalidixic acid efficiently inhibited transfer of type I and particularly type F R-plasmids, whereas the transfer of type N plasmids was resistant to the drug. Type F and type I plasmids appear to depend on at least one host function for their transfer, namely, the nalidixic acid-sensitive reaction in vegetative chromosome replication, whereas type N plasmids are independent of this function.

Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) is a synthetic compound bactericidal against most gram-negative bacteria. Its chief target of action is bacterial deoxyribonucleic acid (DNA) synthesis. Although the precise mechanism of its action is still not known, a recent study suggests that, in Escherichia coli, nalidixic acid interferes with the conversion of intermediate-sized DNA fragments into high-molecular-weight DNA. The inhibitory effect of nalidixic acid was first studied in vegetative chromosome replication in E. coli and later in chromosomal DNA synthesis during conjugation, as well as in transfer DNA replication of the plasmid F' /lac in E. coli. During studies of the bacteriology of infected burns, nalidixic acid was shown to suppress the transfer of an R-plasmid between two strains of gram-negative bacteria in mice with mixed wound infection. The drug also inhibited the in vitro transfer of a type I R-plasmid.

Conjugative plasmids in gram-negative bacteria can be divided into three main groups, F, I, and N on the basis of their sensitivity to the various fertility-specific phages mediated. The aim of this investigation was to look for further differences between sex factor types by comparing the individual transfer efficiencies of 41 different wild-type R-plasmids in an E. coli K-12 host and to study the influence of nalidixic acid on their conjugational transfer. It was found that R-plasmids belonging to different fertility groups differ in transfer efficiency and in the degree of transfer inhibition caused by nalidixic acid. Type N R-plasmids were unusual and showed very low transfer frequencies in liquid conjugation experiments. In addition, the transfer of type N R-plasmids was found to be resistant to a considerable level of nalidixic acid (100 μg per ml, which is 50 times the minimum inhibitory concentration [MIC] for the host cell). The transfer of type I and, in particular, type F R-plasmids was strongly inhibited by nalidixic acid.

The transfer of type N R-plasmids was studied in greater detail. The results obtained indicate that the low transfer frequency of type N R-plasmids is not due to strong repression of their fertility functions but, instead, a poor ability of their conjugation apparatus to establish mating pairs in liquid medium.

MATERIALS AND METHODS

Organisms and R-factors. Most of the different R-plasmids studied were isolated from gram-negative bacteria in clinical specimens and characterized with regard to sex pilus type and /fi ability as described in the accompanying paper (9). The R-plasmids R1, R1drd-19, R15, R46, R64, R128, R64drd-11, and N3 were kindly provided by Naomi Datta, London. The strains of bacteria used were all E. coli K-12 (8). Bacteria and phages were from the stock collection of this laboratory.

Media and growth conditions. The liquid medium routinely used was LB of Bertani (2), and most plates contained PDM agar (AB Biodisk, Stockholm), especially designed for antibiotic sensitivity.
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...testing of bacteria. The minimal medium used was medium E of Vogel and Bonner (28). It was supplemented with 0.2% glucose plus 25 μg of L-amino acids per ml, as required, and solidified by the addition of 1.5% agar. All incubations were performed at 37°C, and growth was recorded by using a Klett-Summerson colorimeter with filter W66. One hundred Klett units corresponded to about 4 x 10^4 bacteria per ml of LB medium. Plating of phases was performed as previously described (8).

Mating conditions. Two different mutants of strain D1 were used as parents in most of the R-plasmid transfer experiments. D1 rif' (chromosomally resistant to 100 μg of rifampin per ml) was the donor, and D1 nal (chromosomally resistant to 100 μg of nalidixic acid per ml) was the recipient. Overnight cultures of the parental bacteria were diluted into 37°C LB to 120 Klett units and mixed 1:1, and conjugation without agitation was then allowed for 30 min at 37°C (method A). An alternative conjugation method that used 25-Klett unit cultures, 1:1 mixing, and 120 min of mating was also employed (method B). The mating mixtures had a total volume of 2 ml and were incubated in 100-ml flasks. In some experiments, 100 μg of nalidixic acid per ml was present throughout the conjugation period. Other experiments were performed with strains X925 rif' (F-), KL16 rif' (Hfr), and G11 nal (Hfr Cavalli) as R-plasmid donors. R-plasmid-carrying recipients were selected by the use of 100 μg of oxytetracycline per ml, except in the case of R1 and Rtdr-13: 15 μg of chloramphenicol per ml was used. Donor bacteria were usually counterselected with 25 μg of nalidixic acid per ml. In the experiments with X925 rif', G11 nal, or KL16 rif' as R-plasmid donors, T6 phage or 50 μg of rifampin per ml (against G11 nal) was used for donor counterselection.

Chemicals. The various antibacterial agents employed were those in reference 9.

RESULTS

Transfer frequencies of different R-plasmids. Figure 1A shows the transfer frequencies obtained with 41 wild-type R-plasmids (having repressed fertility functions) in the 30-min conjugation experiment. The R-plasmids are here grouped according to the sensitivity to the donor-specific phage mediated: F, I, N, and unknown. R-plasmids with "unknown" fertility type did not enable propagation of the F, I, or N donor-specific phages employed, i.e., phages MS2 (14), IF1 (24), and IKe (23), respectively (see reference 9).

It can be seen that the 12 type I R-plasmids are transferred more efficiently than the others. Their transfer frequencies are rather uniform: on average, 1.4 x 10^6 R+ recipients per ml (2.8 per 1,000 input donors). The mutant R-plasmid R64tdr-11, which is derepressed with regard to fertility, gave 2 x 10^6 R+ recipients per ml and was thereby transferred 10 times more efficiently than its wild-type (fertility-repressed) parent R64.

The 10 type F R-plasmids were transferred at somewhat lower and less uniform frequencies than the type I plasmids, giving an average frequency of 7 x 10^4 R+ recipients per ml (1.4 per 1,000 input donors). The I group contained two f+ plasmids, and the F group had one f+ plasmid, but the transfer frequencies of these unusual R-plasmids did not differ significantly from those of the other R-plasmids in their respective group.

It is also evident from Fig. 1A that the type N plasmids differ considerably from the I and F plasmids, having a much less efficient conjugational transfer. However, two R-plasmids (T30 and T31) were outstanding in this group, showing high transfer frequencies (4 x 10^4 and 10^5 R+ recipients per ml, respectively) compared with the rest of the N group, whose average was 2 x 10^4 R+ recipients per ml (0.04 per 1,000 input donors). The sex pilus phages specific for type F or type I plasmids do not form plaques on hosts carrying a wild-type sex factor of the corresponding type because of repression of sex pilus formation in most host cells (24, 26), whereas R-plasmid mutants derepressed with regard to fertility, e.g., those isolated by Meynell and Datta (25), are transferred very efficiently and allow plaque formation by their respective sex pilus phage. However, the inefficient transfer of type N R-plasmids appears not to be due to unusually strong repression of their fertility functions, since the type N-specific phage IKe was able to form plaques on hosts carrying the type N R-plasmids studied here, except for T30 and T31, in which the efficiency of plaque formation (EOP) by IKe was 0. The latter R-plasmids are also aberrant in their response to nalidixic acid (see below). Other experiments with Hfr strains as donors of type N R-plasmids also indicate that the latter do not have strongly repressed fertility functions (see below).

A fourth group of R-plasmids did not enable propagation of either F-, I-, or N-specific phages. As seen in Fig. 1A, these R-plasmids were transferred at frequencies between those of type N and type F R-plasmids, with an average of 1.6 x 10^4 R+ recipients per ml (0.3 per 1,000 input donors). Except for two inefficient plasmids (T18 and T28), further discussed below, the transfer frequencies of these untypable R-plasmids were rather uniform, suggesting that they do not comprise a heterogeneous group. Figure 1B depicts the result of repeated conjugation experiments using method B, and the transfer frequency in 86 experiments is given. The prolonged mating period (2 h) did
not bring the transfer frequencies of the type N R-plasmids up to those of the F or I plasmids.

Influence of nalidixic acid on R-plasmid transfer frequencies. Table 1 summarizes the results of 30-min R-plasmid transfer experiments performed in parallel with those in Fig. 1A but in the presence of nalidixic acid (100 µg per ml).

Nalidixic acid strongly inhibited the conjuga-

tional transfer of a majority of the R-plasmids and, again, differences among the R-plasmid types were observed. Type F R-plasmids were the most sensitive, with an average of 0.16% nalidixic acid-resistant transfer, relative to their respective transfer rates in the absence of the drug. The conjugal transfer of type I R-plasmids was less sensitive to nalidixic acid than was that of the type F plasmids (average of 2.5% resistant transfer). The R-plasmids of unknown type(s) showed some variation in their responses to nalidixic acid. One R-plasmid (T28) had nearly 10% resistant transfer and that of T18 was almost completely resistant to nalidixic acid in repeated experiments. In the absence of nalidixic acid, these two R-plasmids showed a transfer efficiency that was about 10-fold lower than those of the other R-plasmids of this group (2 × 10^4 R+ recipients per ml) and similar to those of type N R-plasmids (see above).

In addition to T18 and T28, mentioned above, the members of the N group showed a high degree of nalidixic acid-resistant transfer (30 to 90%, Table 1) except for T30 and T31, which were also unusual in several other respects (see above). However, 100% resistance was usually not the case. When a donor strain highly resistant to nalidixic acid due to a chromosomal mutation was used, the drug still caused some reduction of R-plasmid transfer. This phenomenon was also observed by Hane during studies of chromosome transfer and was ascribed to a slightly negative effect of nalidixic acid on mating pair formation (20). It thus appears that the conjugal transfer of typical type N R-plasmids is completely resistant to nalidixic acid.

The fact that, in the same host strain, certain R-plasmids show a nalidixic acid-resistant transfer DNA replication, whereas that of other R-plasmids differs in sensitivity, suggests that the target of nalidixic acid in this process is a

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**Table 1. Influence of nalidixic acid on R-plasmid transfer**

<table>
<thead>
<tr>
<th>Fertility type</th>
<th>Degree of nalidixic acid-resistant transfer† of R-plasmid (% NaI)</th>
<th>Avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>R64 (1.5) R64drd-11 (1.1) T2 (0.9) T9 (1.8) T9 (4.0) T11 (0.9) T12 (3.3) T14 (1.3)</td>
<td>2.5%</td>
</tr>
<tr>
<td>F</td>
<td>T3 (0.13) T6 (0.30) T13 (0.12) T22 (0.10) T29 (0.04) T35 (0.02) T37 (0.29) T38 (0.32)</td>
<td>0.16%</td>
</tr>
<tr>
<td>N</td>
<td>T1 (80) R15 (30) R46 (50) N3 (80) T36 (90) T46 (60) R128 (50) T30 (0.5) T31 (0.2)</td>
<td>55%</td>
</tr>
<tr>
<td>?†</td>
<td>T10 (0.6) T15 (1.5) T16 (0.9) T18 (90) T24 (2.7) T25 (0.4) T26 (0.7) T27 (0.4) T28 (7)</td>
<td>12%</td>
</tr>
</tbody>
</table>

† Expressed as percent R-transfer in the presence of nalidixic acid (100 µg/ml) as compared with a parallel drug-free control experiment. Conjugation method A was used. Strain D1 rif was the donor and D1 nal was the recipient.

‡ Excluding the unusual plasmids T30 and T31 (see text).

§ Fertility type(s) unknown (not F, I, or N) for these R-plasmids.
plasmid function rather than a host function. When several different type F and type I R-plasmids were introduced into a nalidixic acid-resistant host (G11 nal), their transfer became resistant (60 to 90%) to the drug. This result suggests a link between the conjugal transfer of I and F plasmids and host DNA synthesis, since high levels of nalidixic acid resistance (as in G11 nal) are due to a drug-resistant vegetative chromosome replication apparatus and not due to decreased permeability or inactivation of the drug (4, 21). The nalidixic acid-sensitive step in transfer DNA replication of type F and type I R-plasmids thus seems to be carried out by the host cell DNA replication machinery, whereas type N R-plasmids appear to be independent of this host function (also, see below).

Further studies of type N R-plasmids. In most of the following experiments, the R-plasmid N3 was used for further studies of the inefficient and nalidixic acid-resistant conjugal transfer of type N R-plasmids. One possible explanation for their nalidixic acid-resistant transmission is that type N R-plasmids could mediate a general host cell resistance to the drug. This alternative appears to be ruled out by the fact that none of the R-plasmids studied here influenced the MIC of nalidixic acid for its host strain. Nor is a temporary tolerance to the drug (not revealed by MIC determinations) induced during the conjugal transfer of type N R-plasmids because, irrespective of which R-plasmid was carried, as well as in the absence of plasmids, 70 to 90% of the D1 rif cells were killed by the action of 100 µg of nalidixic acid per ml during the standard 30-min mating experiment. This observation also disfavors an inactivation of the drug due to type N R-plasmids as the explanation for their nalidixic acid-resistant transfer. It can, therefore, be concluded that, in a nalidixic acid-sensitive host cell, transfer DNA synthesis of type N R-plasmids is resistant to the drug. It also follows that for type N plasmids this process is independent of the host chromosome replication function that is the lethal target of nalidixic acid in growing cells.

Since the conjugal transfer of N3 is resistant to nalidixic acid, it is possible that this process continues on the selective plates used here (containing nalidixic acid for donor counterselection; see Materials and Methods), resulting in extra R+ recipients in addition to those present after 30 min of mating in liquid. The low transfer frequencies of type N and certain other R-plasmids that have a nalidixic acid-resistant transfer apparatus may, therefore, still represent an overestimation. N3 was therefore transferred to the T6-sensitive strain X925 rif, which was the donor in matings interrupted by violent mixing followed by addition of T6 phage (multiplicity of 100 plaque-forming phage particles per bacterium) to prevent additional conjugation on the agar surface. This procedure did not reduce the transfer of N3, indicating that it mainly occurs in the mating liquid and not on the selective plates.

The low transfer frequency observed with N3 and other type N R-plasmids could be due to an unusually slow conjugal transfer of their DNA, a slow or inefficient expression of their tetracycline resistance, or difficulties in establishing a stable contact or communication between donor and recipient cells. The first alternative is ruled out by the results in Fig. 1B and by the fact that matings interrupted early in conjugation by violent mixing plus the addition of phage T6 yield N3-carrying recipients after only a few minutes, as normally occurs in R-plasmid transfer. The second alternative is also highly unlikely since a low transfer frequency is strongly correlated with type N plasmids but not with particular tetracycline resistance determinants. Moreover, the MIC of tetracycline for hosts carrying type N R-plasmids is not lower than that due to other R-plasmids (always greater than 1,000 µg per ml). The third alternative was therefore investigated further. Loss of motility can be thought to decrease the cell collision frequency and, thereby, mating pair formation in a liquid medium. However, none of the type N R-plasmids here studied had any influence on the motility of its E. coli K-12 host cell in conventional motility tests in soft agar (11).

However, by using Hfr strains as donors of N3, its transfer was found to be increased by 100- to 1,000-fold as compared with F− donors (Table 2). The presence in the donor, of the mutant type F R-plasmid R1drd-19 that is dere-

### Table 2. Stimulation of N3 transfer due to the presence of other sex factors

<table>
<thead>
<tr>
<th>Donor of N3</th>
<th>Sex of donor</th>
<th>N3+ recipients per ml</th>
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</thead>
<tbody>
<tr>
<td>D1 rif</td>
<td>F−</td>
<td>7 × 10^4</td>
</tr>
<tr>
<td>X925 rif</td>
<td>F−</td>
<td>8 × 10^4</td>
</tr>
<tr>
<td>G11 nal</td>
<td>Hfr</td>
<td>8 × 10^4</td>
</tr>
<tr>
<td>KL16 rif</td>
<td>Hfr</td>
<td>10^4</td>
</tr>
<tr>
<td>D1 rif (R1drd-19)</td>
<td>R+</td>
<td>4 × 10^6</td>
</tr>
</tbody>
</table>

* Conjugation method A (see Materials and Methods) was used.

* R1drd-19 is a mutant of the type F R-plasmid R1, derepressed with regard to fertility (reference 25).
pressed with regard to fertility also increased the transfer of N3 considerably (Table 2). The high frequency transfer of N3 due to the presence of other sex factors was, however, always only partially resistant (about 10%) to nalidixic acid, suggesting that this transfer of N3 is not entirely N specific. With Hfr G11 as the donor of N3, the fate of an early-transferred chromosomal gene (proA) was compared with that of N3 in conjugations. The total numbers of N3+ and Pro+ recipients were equal (10 per 1,000 input donors) and, in 15 to 20% of the N3+ recipients, proA had also become transferred and vice versa. Thus, N3 and proA were not transferred jointly, although the transfer of one increased the probability of transfer of the other within the same mating pair by 15- to 20-fold. When nalidixic acid was present during conjugation, the frequency of Pro+ recombinants dropped more than 1000-fold, and none out of 100 N3+ recipients tested was Pro+. These results can be interpreted to mean that the N-specific transfer is brought to the same level as the F-specific transfer, mainly because of improved mating pair formation due to the F factor. All type N, but no other R-plasmids tested, were transferred at elevated frequencies by the Hfr donor G11nal. A stimulation of N3 transfer by about 10-fold was also obtained when Hfr strains served as recipients of N3, i.e., despite the ongoing F-mediated transfer of chromosomal DNA with a polarity opposite that of N3 DNA.

**DISCUSSION**

The present paper is focused on two aspects of the conjugational behavior of R-plasmids, the efficiency of their transfer in liquid matings and the influence of the DNA synthesis inhibitor nalidixic acid on their transfer.

Type I R-plasmids were transferred at frequencies that were higher and more uniform than those of type F R-plasmids. Seven typical type N R-plasmids studied were all transferred at very low frequencies. The present data could therefore reflect the presence of identical transfer units in type I R-plasmids and different transfer units among type F R-plasmids. This situation apparently parallels the one in compatibility grouping of type I (one group) and type F (four subgroups) R-plasmids (13), suggesting a correlation between compatibility (vegetative plasmid replication?) and transfer replication of plasmids.

The highly inefficient transfer of type N R-plasmids found in liquid matings is apparently not due to strong repression of fertility. In contrast to wild-type I and F plasmids, all the typical type N R-plasmids studied enabled plaque formation by the appropriate donor-specific phage, indicating the presence of receptors for phage IKE on a large proportion of the host cells of type N R-plasmids. It is, of course, possible that two different N-specific surface structures are involved in N plasmid transfer and in adsorption of phage IKE, respectively. However, the fact that N3 showed the highest transfer frequency among the typical N plasmids (7 × 10^3 R+ recipients per ml, Fig. 1A) and also enabled a considerably higher EOP of phage IKE (EOP = 1.0) than the other typical type N R-plasmids (EOP = 0.1) suggests a correlation between conjugational transfer of type N plasmids and adsorption of phage IKE.

Furthermore, the experiments in which other sex factors were used to stimulate the transfer of N3 indicate that a maximum of 0.1 to 1% of the cells that have a fully developed N-specific transfer apparatus are able to donate N3 in liquid matings. The formation of stable mating pairs due to the other sex factors enabled a strong enhancement of a largely N-specific transfer of N3.

Taken together, the present data suggest that a poor ability to establish stable mating pairs and not an infrequent expression of fertility genes is the main reason for the inefficient transfer of type N R-plasmids in liquid matings. Recently, Dennison and Baumbarg (15) also arrived at this conclusion when they were able to increase the transfer frequency of two type N R-plasmids by using prolonged mating on an agar surface before applying antibiotic selection. These authors, therefore, suggested that type N plasmids have evolved to fit soil bacteria conditions rather than those of water organisms. So far, typical sex pilus-like surface structures have not been demonstrated on bacteria carrying type N R-plasmids, and phage IKE has been seen to attach directly to the envelope of such cells (7). The poor ability of type N R-plasmids to establish mating pairs may thus be due to very short N-specific conjugation appendages.

The typical N plasmids were unusual also in that their conjugational transfer was resistant to nalidixic acid, despite the fact that their host cells retained their original sensitivity to the drug. Thus, the transfer of N plasmids appears to be independent of the host chromosomal DNA replication function that is the lethal target of nalidixic acid (see Introduction). The opposite seems to be true for type F and type I R-plasmids whose transfer was resistant to nalidixic acid only in mutant host cells that were highly resistant to the drug.
Two R-plasmids, T30 (fi+) and T31 (fi−), included in the N group because they enabled propagation of phage IKE on their host cells, were different from the typical N plasmids in all other respects. The EOP by phage IKE here was 0, indicating repression of its receptor. The transfer of T30 and T31 was efficient and sensitive to nalidixic acid. These R-plasmids might be hybrid plasmids, consisting of genes involved in the formation of the N-specific surface structure incorporated into an unrelated transfer factor.

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