Conjugal Deoxyribonucleic Acid Replication by 
*Escherichia coli* K-12: Effect of Nalidixic Acid

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During the conjugal transfer of the R64-11 plasmid at 42°C from donor cells thermosensitive for vegetative deoxyribonucleic acid (DNA) synthesis to recipient minicells, the plasmids are conjunctively replicated in the donor cells. This conjugal replication is inhibited by nalidixic acid, and the degree of inhibition is comparable to the reduction in the amount of plasmid DNA transferred to the recipient minicells in the presence of the drug. In addition, the size of DNA transferred to the minicells and the fraction of conjunctively replicated DNA in the donor cells that can be isolated as closed-circular plasmid DNA under alkaline conditions are both reduced by nalidixic acid. When the drug is added to a mating that is underway, the rate of conjugal replication is immediately reduced. This change is accompanied by a reduction in the amount of conjunctively replicated DNA in the donor cells that can be isolated as closed-circular plasmid DNA. Furthermore, conjunctively replicated plasmid DNA that is not associated with the donor cell membrane becomes membrane bound after the addition of nalidixic acid.

The bactericidal drug nalidixic acid (1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) specifically inhibits deoxyribonucleic acid (DNA) synthesis (18). Holom and Pritchard (21) used this drug to examine the postulated requirement for DNA synthesis during conjugation by *Escherichia coli* (22) and found that it did inhibit recombinant formation. Barbour (2) demonstrated that nalidixic acid inhibited conjugal transfer of DNA by acting on the donor cell. He isolated donor and recipient strains that were resistant to the drug and found that transfer of F' lac was only inhibited when the donor cells were sensitive. These findings were extended to conjugal transfer of DNA from Hfr (7, 12, 16) and F+ (9) donor cells. Holom and Pritchard (21) originally described the effect of nalidixic acid on conjugation as reversible. They found that the inhibited condition could be reversed by dilution of the mating mixture into medium that did not contain nalidixic acid. Bouck and Adelberg (4) and Hane (20), however, discovered that chromo-

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some transfer does not resume from the point of interruption after such a treatment, but reinitiates at the origin of chromosome transfer.

A variety of techniques have been used to demonstrate that only one strand of donor cell DNA synthesized before mating is transferred to the recipients (see 11). During the transfer of F (27) and R (26) factors, the transferred strand is replaced by DNA synthesis in the donor cells. This conjugal DNA synthesis is not inhibited in donor cells that are thermosensitive for vegetative DNA replication (7, 15, 23, 28). Bresler et al. (7) measured the incorporation of [14C]thymine during a mating between Hfr and F- strains that were both temperature sensitive for vegetative replication and found that nalidixic acid inhibited conjugal DNA replication only when the donor cells were sensitive to the drug. Thus, both the DNA synthesis that is stimulated during conjugation and the conjugal transfer of DNA are inhibited by the drug.

Although the mode of action of nalidixic acid is not known (6), it is thought to act at the growing point or replication fork of DNA molecules because it immediately inhibits synthesis (18) and DNA degradation after exposure to the drug proceeds from the most recently replicated DNA to that synthesized previously (24). Disruption of the DNA at the replication fork
during conjugation would also explain the simultaneous interruption of conjugal transfer and replication described above (4, 20). Furthermore, DNA molecules that are being conjuga-
gally transferred and replicated must have a gap or nick in at least one DNA strand so that a linear, single strand of DNA can be transferred to the recipient cells. If nalidixic acid stops replication and transfer of DNA by blocking movement of replication forks, it might stabilize these gaps in the DNA strands. Such discontinuities would be difficult to locate in large molecules such as the E. coli chromosome, but they might be detectable in smaller DNA molecules that can be isolated as covalently closed circles. One break in the DNA of such a molecule, whether it involves one or both strands, will radically change its sedimentation behavior in either alkaline or neutral solutions (29). Thus, if nalidixic acid either causes breaks in DNA molecules or stabilizes gaps that arise during conjugal transfer and replication, these changes can be detected as an alteration of the sedimentation behavior of affected molecules.

In the accompanying paper (15), we reported that the derepressed R factor R64-11 is transferred from donor cells thermonensitive for vegeta-
tive DNA synthesis to minicells at 42 °C. This transfer is accompanied by the conjugal replication of the plasmid which is not affected by the nonpermissive temperature. In this communication, we demonstrate that this transfer and replication is interrupted by nalidixic acid. Furthermore, we have found that the presence of the drug increases the number of single-strand breaks in plasmid DNA and temporarily binds the R-factor DNA to the cellular membrane.

MATERIALS AND METHODS

**Bacterial strains.** The donor strain used in this study was χ1284 (15). This strain possesses the derepressed R factor R64-11 and has the chromosomal dnaB(ts) mutation that causes vegetative DNA syn-
thesis to be temperature sensitive. Recipient minicells were isolated from strain χ925, which is a single-colony isolate from P678-54 described by Adler et al. (1).

**Methodology.** The methods for preparation of recipient minicells, preparation of donor cells, mating, separation of donors and recipients after mating, and analysis of DNA transferred to minicells were as described by Fenwick and Curtis (15).

**Nalidixic acid.** Nalidixic acid was purchased from Sigma Chemical Co. and was dissolved in 0.02 N NaOH to make a stock solution of 2 mg/ml. When nalidixic acid was added to a mating, control matings received the same volume of 0.02 N NaOH. The NaOH had no effect on either conjugal transfer or DNA replication. When nalidixic acid was added to donor cells before the start of a mating, the same concentration of the drug was maintained in the final mating mixture by adding the proper quantity of nalidixic acid when the recipient minicells were mixed with the donor cells.

**Analysis of donor cell DNA.** Donor cells purified after a mating were washed with cold buffered saline with gelatin (10), collected by centrifugation, and suspended in 3 ml of cold washing buffer (17). The radioactively labeled DNA was then analyzed by sedimentation through sucrose gradients at alkaline or neutral pH, or both. Alkaline sucrose gradient centrifugation of donor cell DNA is described in the accompanying paper (15). For neutral sucrose graden
t centrifugation, a portion of the donor cells was collected by centrifugation and suspended in 150 μl of ice-cold 0.01 M tris(hydroxymethyl)amino-
methane (Tris) buffer at pH 8.0. The samples were kept in an ice bath to reduce metabolic activity, and 25 μl of egg-white lysozyme (2.5 mg/ml; Wor-
thington Biochemical Corp.) in the same buffer was added. After 15 min at ice-bath temperatures, 25 μl of 0.25 M sodium ethylenediaminetetra-
ate (EDTA) at pH 8.0 was added, and, after an additional 15 min, the cells were layered over 5 to 20% neutral sucrose gradients that had been previously overlayered with 50 μl of 10% Brij 58 (Atlas Chemical Industries, Inc.). The sucrose solutions for the gradients were in 0.7 M NaCl, 0.01 M EDTA, and 0.05 M Tris buffer at pH 7.4. The gradients were 3.2 ml and were formed over a 0.35-ml shelf of 60% sucrose that had been adjusted to a density of 1.70 g/cm³ with CsCl. Centrifugation was at 40,000 rpm (20 C) for 100 min in an SW56 rotor of a Beckman L3-50 ultracentrifuge. About 28 fractions of 8 drops each were collected from the bottom of the tubes onto paper strips (8), and the trichloroacetic acid-insoluble radioactivity in each fraction was determined as described in the accompanying paper (15).

**RESULTS**

**Inhibition of conjugal DNA replication by nalidixic acid.** The temperature-sensitive mu-
tation carried by the χ1284 donor cells causes an immediate cessation of vegetative DNA synthe-
sis when the strain is shifted from 35 to 42 C (3). We have demonstrated, however, that these cells will conjugally replicate the R64-11 plas-
mid during matings with DNA-deficient mini-
cells at the restrictive temperature (15). The stimulation of [3H]thymidine incorporation into acid-insoluble material during such a mating is illustrated in Fig. 1. If nalidixic acid can prevent the conjugal transfer of R64-11, then it should also inhibit this stimulation of DNA synthesis. When we added 10 μg of nalidixic acid per ml to the donor cells 2 min before the mating was initiated and maintained that concentration during the mating, the stimulation of [3H]thymidine incorporation was reduced by 98% during a 30-min mating (Fig. 1). This result
preincubation at DNA tentative C strains shows alkaline DNA synthesis during replication nalidixic acid inhibited experiments that nary shown in thesis cited vegetative agrees with that at the effects of measure DNA cells received radioactivity acid-insoluble closed-circular plasmid concentration J. 1238 as isolated of portion 50-Aliter 106/ml) mixed with 109/ml; 0, and adding by

Another Fig. 0 ~ ~ ~ ~~~~~~0

Inhibition of conjugal DNA replication during transfer of R64-11 from dnaB(ts) donor cells to recipient minicells by nalidixic acid. After 15 min of preincubation at 42 C, 2 ml of χ1284 donor cells (3 x 10^9/ml) were mixed with 2 ml of χ925 minicells (2 x 10^9/ml; ○, □) or 2 ml of growth media (O). The donor cells received 20 μCi of [3H]thymidine (3 μCi dThd) per ml and 200 μg of adenosine per ml 3 min before being mixed with the recipient minicells. Nalidixic acid (10 μg/ml) was added to the donor cells of one of the matings 2 min before the mating began (O), and that concentration was maintained in the mating mixture by adding more of the drug with the minicells. The acid-insoluble radioactivity is plotted as counts/min in 50-μliter samples of the mating mixture.

agrees with that of Bresler et al. (7), who found that nalidixic acid inhibited conjugal DNA replication during matings between Hfr and F- strains that were both thermosensitive for vegetative DNA synthesis. We used the nalidixic acid at 10 μg/ml because we found in preliminary experiments that this concentration inhibited vegetative DNA replication in χ1284 at 35 C by 90% but permitted enough conjugal transfer and replication of DNA at 42 C for us to measure the effects of the drug on these activities.

Another effect of nalidixic acid on conjugal DNA synthesis is demonstrated in Fig. 2, which shows alkaline sucrose gradient profiles of donor cell DNA labeled with [3H]thymidine during a 30-min mating with minicells at 42 C. As we have shown (15), the stimulation of DNA synthesis in such matings is specific because a large portion of the newly synthesized DNA can be isolated as the covalently closed-circular form of the R64-11 plasmid. In Fig. 2, 27% of the labeled DNA from the control donor cells sedimented as closed-circular plasmid DNA molecules (fractions 10 to 13), whereas only 5% of the radioactive DNA from donor cells that had been mated in the presence of 10 μg of nalidixic acid per ml sedimented in this position. This large decrease in the portion of newly synthesized DNA that was in the form of closed-circular plasmid DNA is misleading because there was some background incorporation of [3H]thymidine by unmated χ1284 cells at 42 C (Fig. 1). That incorporation apparently represents either vegetative DNA synthesis which escapes the temperature block or DNA repair synthesis, because the DNA synthesized by unmated χ1284 cells at 42 C is 1 to 4% closed-circular plasmid DNA and that percentage is a reflection of the relative sizes of the R64-11 plasmid and the E. coli chromosome (15). For this reason, we have corrected the experimental values by subtracting the amount of background DNA synthesis (Table 1). In this experiment, 1% of the label incorporated by unmated cells was in the closed-circular region of the alkaline sucrose gradients. When the total radioactivity incorporated by the unmated cells is subtracted from that incorporated by the mated cells and the

![Alkaline sucrose gradient profiles of R64-11+ dnaB(ts) donor cell DNA labeled with [3H]thymidine (3 μCi dThd) during a 30-min mating with χ925 minicells at 42 C. The donor cells were purified from the matings described in the legend of Fig. 1, and the DNA from the cells was sedimented through 5 to 20% alkaline sucrose gradients by centrifugation for 35 min at 36,000 rpm (20 C) in a Beckman SW56 rotor. The rapidly sedimenting component (fractions 10 to 13) contains covalently closed-circular R64-11 DNA. The donor cells were from matings conducted in the absence (●) or presence (O) of 10 μg of nalidixic acid per ml.](http://jb.asm.org/)

![Inhibition of conjugal DNA replication during transfer of R64-11 from dnaB(ts) donor cells to recipient minicells by nalidixic acid. After 15 min of preincubation at 42 C, 2 ml of χ1284 donor cells (3 x 10^9/ml) were mixed with 2 ml of χ925 minicells (2 x 10^9/ml; ○, □) or 2 ml of growth media (O). The donor cells received 20 μCi of [3H]thymidine (3 μCi dThd) per ml and 200 μg of adenosine per ml 3 min before being mixed with the recipient minicells. Nalidixic acid (10 μg/ml) was added to the donor cells of one of the matings 2 min before the mating began (O), and that concentration was maintained in the mating mixture by adding more of the drug with the minicells. The acid-insoluble radioactivity is plotted as counts/min in 50-μliter samples of the mating mixture.](http://jb.asm.org/)
same is done for the radioactivity in the closed-circular region of the gradients, we get corrected values of 38% of the mated donor cell DNA in the form of closed plasmid molecules and 24% when the mating was conducted in the presence of 10 μg of nalidixic acid per ml. The low levels of radioactivity incorporated in the presence of nalidixic acid make precise conclusions difficult, but from these values it appears that the drug may not only have caused a reduction in the amount of conjugation replication, but may have also increased the number of plasmid molecules having single-strand gaps or nicks in the DNA strands. This conclusion will be supported below by experiments in which nalidixic acid was added after the mating was initiated.

**Effect of nalidixic acid concentration on conjugal DNA transfer and replication.** Cohen et al. (9) found that nalidixic acid decreased the transfer of DNA to recipient minicells from F+ donor cells that had been labeled with [3H]thymidine before conjugation. Furthermore, by using several concentrations of the drug and resistant as well as sensitive donor cells, they demonstrated that the inhibition of transfer by nalidixic acid closely paralleled its ability to inhibit vegetative DNA replication in the donor cells. One would expect that this sort of relationship would also exist between conjugal DNA synthesis and transfer of DNA to recipient minicells. To test the effect of various concentrations of nalidixic acid, we performed a series of 30-min matings at 42°C in which we measured incorporation of [3H]thymidine by the mating mixture and the amount of acid-insoluble radioactivity in the minicells at the end of the mating. The donor cells were exposed to the nalidixic acid 2 min before and during the mating, and the experimental values are plotted in Fig. 3 as percentages of the values from a control mating done in the absence of nalidixic acid. Nalidixic acid did not decrease the amount of [3H]thymidine incorporation in the

\[ \text{counts/min in CC-DNA} \times \% \text{CC-DNA} \]

### TABLE 1. Effect of nalidixic acid on DNA conjugally replicated by donor cells

<table>
<thead>
<tr>
<th>Source of donor cells</th>
<th>Total counts/min in donor cells</th>
<th>Counts/min in CC-DNA*</th>
<th>% CC-DNA</th>
<th>Corrected CC-DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mated in the absence of nalidixic acid</td>
<td>9,573</td>
<td>2,579</td>
<td>27</td>
<td>38</td>
</tr>
<tr>
<td>Mated in the presence of nalidixic acid</td>
<td>3,398</td>
<td>168</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>Unmated</td>
<td>2,811</td>
<td>28</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Donor cells were purified from the matings described in the legend of Fig. 1, and the DNA from the cells was sedimented through alkaline sucrose gradients as described in the legend of Fig. 2.

* CC-DNA, covalently closed R64-11 DNA.

* Calculated as: (counts in mated cell CC-DNA – counts in unmated cell CC-DNA)/(total counts in mated cells – total counts in unmated cells).
mating mixtures as much as it decreased the appearance of acid-insoluble radioactivity in the minicells. This difference was caused by the fact that the drug has little effect on the background incorporation of \(^{3}H\)thymidine by donor cells at restrictive temperatures. We found that the addition of 10 \(\mu\)g of nalidixic acid per ml to unmated \(\chi\)1284 cells that were being incubated at 42 \(C\) did not reduce the incorporation of \(^{3}H\)thymidine, and Bresler et al. (7) have reported similar results with higher concentrations of the drug. We therefore show values in Fig. 3 (open circles) for \(^{3}H\)thymidine incorporation by the mating mixtures that have been corrected by subtracting the amount of background synthesis by unmated donor cells. This curve is more closely related to the curve for labeled DNA in minicells. Thus, nalidixic acid reduced conjugal replication and conjugal transfer of DNA to similar extents.

Size of DNA transferred to minicells. In matings of the type discussed in the preceding sections, the DNA being conjugally replicated in the donor cells is the strand of the plasmid which replaces the one being transferred to the minicells (28). The appearance of acid-insoluble radioactivity in the minicells is due to both transfer of plasmid DNA that has been labeled in the donor cells (i.e., second-round transfer of a plasmid that was labeled during the preceding cycle) and synthesis in the minicells of DNA strands complementary to those received in the mating (15). As mentioned above, Cohen et al. (9) found that nalidixic acid inhibited the transfer to minicells of DNA labeled in F* donor cells before mating. Since the drug can halt Hfr matings that are in progress and prevent reinitiation of transfer from the point of interruption, it has been concluded that its presence can cause breaks in the DNA strand that is being transferred (4, 20). To see if we could detect such damage to DNA, we labeled \(\chi\)1284 donor cell DNA before mating with \(^{3}H\)thymidine and conducted matings as described above but with the addition of 50 \(\mu\)g of unlabeled thymidine per ml to prevent any additional incorporation of radioactive thymidine during the mating. One mating was done with donor cells that were exposed to 10 \(\mu\)g of nalidixic acid per ml 5 min before and during the mating, and a second mating was done with untreated donor cells. After 30 min of mating, the minicells were purified and the size distribution of the labeled DNA transferred to the minicells was examined by alkaline sucrose gradient centrifugation. The profiles obtained from these gradients are shown in Fig. 4. The DNA from minicells mated with untreated donor cells had a prominent band at approximately the position in the gradient where full-length single strands of the R64-11 plasmid were expected to sediment (marked by the arrow). The expected position was determined by reference to the sedimentation behavior of T4 bacteriophage DNA in similar gradients (25) and by using \(38 \times 10^6\) as the molecular weight of a single strand of R64-11 DNA (26). The amount of labeled DNA transferred from donor cells in the presence of nalidixic acid was 28% of the amount transferred from the untreated donors. This DNA did

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**Fig. 4.** Alkaline sucrose gradient profiles of DNA transferred to recipient minicells from R64-11* donor cells. \(\chi\)1284 donor cell DNA was labeled before mating by incubating the cells at 35 \(C\) in the presence of \(^{3}H\)thymidine (\(^{3}H\)dTd; 10 \(\mu\)Ci/ml). Fifteen minutes before the matings were to begin, 50 \(\mu\)g of unlabeled thymidine per ml was added to the donor cell culture to prevent additional incorporation, and 3-ml samples of the culture were shifted to 42 \(C\). After 10 min, 10 \(\mu\)g of nalidixic acid per ml was added to one sample of the donor cells. The matings were initiated 5 min later by adding 3 ml of prewarmed minicells and additional nalidixic acid to maintain the drug concentration of 10 \(\mu\)g/ml. After 30 min of mating, the recipient minicells were purified, and the size of the transferred DNA was determined by sedimenting the DNA through 5 to 20% alkaline sucrose gradients. Centrifugation was for 131 min at 35,000 rpm (20 \(C\)) in a Beckman SW56 rotor. Symbols: O, labeled DNA transferred in the presence of nalidixic acid; , labeled DNA transferred in the absence of nalidixic acid; the arrow marks the expected position of complete single strands of R64-11 DNA.
not have a prominent band of unit-length plasmid DNA, but otherwise was very similar to that transferred in the untreated mating. Since nalidixic acid caused a 72% inhibition of conjugal transfer but not a comparable reduction in the average size of the DNA transferred, the drug must have completely prevented the conjugal transfer of most plasmids. However, the low amount of unit-length plasmid DNA that was transferred in the presence of nalidixic acid indicates that the drug did inhibit the transfer of complete plasmid molecules or increased the number of plasmid molecules that were broken before the entire molecule was transferred.

**Interrupting of conjugal replication by nalidixic acid.** To increase our understanding of the effect of nalidixic acid on conjugal transfer and to examine its effect on conjugally replicating DNA, we conducted two matings in the presence of [3H]thymidine and, after 15 min of conjugation, added 10 μg of nalidixic acid per ml to one of the matings (Fig. 5). The drug greatly reduced the rate of incorporation of the [3H]thymidine into acid-insoluble material. The donor cells from these matings were purified from samples withdrawn after 15, 20, and 30 min of mating, and the percentage of donor cell DNA in the form of covalently closed plasmid molecules was determined by sedimentation of the DNA through alkaline sucrose gradients. From the results of those analyses (Table 2), it can be seen that after the addition of the drug the percentage of closed-circular DNA decreased in the donor cells, whereas there was a slight increase in the untreated cells.

The observed decrease in the fraction of closed-circular plasmid DNA after the addition of nalidixic acid could represent an increase in the number of plasmid molecules that have breaks in either one or both DNA strands. To separate the plasmid molecules in donor cells that were not covalently closed circles, we examined the DNA synthesized in donor cells during conjugation by sedimentation on 5 to 20% neutral sucrose gradients that were formed over shelves of 60% sucrose plus CsCl. When cells are lysed with the nonionic detergent Brij 58, DNA that is associated with the cellular membrane, including most chromosomal DNA, will sediment rapidly in such a gradient and come to rest on the shelf, but most open- and closed-circular molecules of R-factor DNA do not appear to be bound to the membrane because they sediment more slowly (13). In addition, since covalently closed, open-circular, and linear forms of plasmid DNA all have distinctive sedimentation velocities at neutral pH (29), they can also be distinguished on such a gradient. If nalidixic acid causes an increase in the number of R64-11 molecules that have gaps in either one or both DNA strands, we should be able to detect this as a change in the sedimentation behavior of the plasmid DNA.

We conducted two matings between χ1284 cells and minicells at 42 C in the presence of [3H]thymidine. After 15 min of mating, one mixture was brought to a final concentration of 10 μg of nalidixic acid per ml, and the second remained as an untreated control. After 5 more min or a total mating time of 20 min, the matings were interrupted and the donor cells were purified. The radioactive profiles obtained after Brij 58 lysis and neutral sucrose gradient centrifugation are illustrated in Fig. 6. Rather than a shift of the radioactivity from the sedimentation position of covalently closed plasmid DNA decreased more in the presence of nalidixic acid.
TABLE 2. Reduction of covalently closed (CC) DNA in donor cells after addition of nalidixic acid

<table>
<thead>
<tr>
<th>Mating time (min)</th>
<th>No nalidixic acid added</th>
<th>Nalidixic acid added at 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total counts/min in donor cells</td>
<td>% CC-DNA*</td>
</tr>
<tr>
<td>15</td>
<td>5,100</td>
<td>27</td>
</tr>
<tr>
<td>20</td>
<td>6,100</td>
<td>29</td>
</tr>
<tr>
<td>30</td>
<td>6,962</td>
<td>31</td>
</tr>
</tbody>
</table>

* Samples (3 ml) were withdrawn from the matings described in the legend of Fig. 5. The donor cells were purified and the cell DNA was analyzed by alkaline sucrose gradient centrifugation as described in the legend of Fig. 2. The figures given have not been corrected for background DNA synthesis by un-mated donor cells.

We also found that there was little change in the alkaline gradient profiles through 1 h of mating time. The neutral gradient of the 20-min sample again showed a loss of the cytoplasmic forms of plasmid DNA and an increase in radioactivity in the membrane fraction. With additional time, however, we found a reappearance of the cytoplasmic forms of circular plasmid DNA. This was accompanied by a decrease in the amount of labeled DNA in the membrane fraction. Furthermore, the amount of DNA that sedimented as covalently closed plasmid molecules on the neutral gradients was only 15% lower in the 60-min sample than in the 15-min sample. This contrasted sharply with the amount of covalently closed plasmid DNA on the alkaline gradients of these samples, which was 45% lower in the 60-min sample than in the 15-min sample. Thus, unlike the results from the alkaline gradients, nalidixic acid did not cause a major loss of plasmid DNA that sedimented as covalently closed molecules of R-factor DNA on the neutral gradients. The significance of these results will be discussed below.

DISCUSSION

In this report we have sought to describe the effects of nalidixic acid on the molecular events involved in conjugal transfer of plasmid DNA. By conducting matings at 42°C between donor
cells that are unable to vegetatively replicate DNA at that temperature and recipient minicells, we have demonstrated that nalidixic acid decreased all of the molecular indexes of conjugation that we measured. The drug reduced the stimulation of DNA synthesis caused by the presence of recipient minicells and the synthesis of covalently closed R64-11 plasmid DNA within the donor cells. Thus, it inhibited conjugal DNA synthesis. Furthermore, it inhibited the transfer of plasmid DNA synthesized either before or during a mating to the minicells. These results are in agreement with previous genetic observations that nalidixic acid prevents the transfer of DNA from sensitive donor cells (2, 4, 7, 12, 16, 20, 21) and molecular observations that it inhibits transfer of donor cell DNA to recipients (9) and conjugal DNA replication in Hfr donor cells (7). Our finding that nalidixic acid reduced both conjugal replication and conjugal transfer to a similar degree supports the hypothesis of Jacob et al. (22) that conjugal transfer is dependent on DNA synthesis in the donor cell, but the possibility still exists that transfer and replication are independent processes that are both affected by nalidixic acid.

Recently, Bouck and Adelberg (4) and Hane (20) have demonstrated that the nalidixic acid-induced interruption of Hfr matings does not result from the dissociation of mating pairs but rather from a break in the DNA strand being transferred to the recipient cells. This conclusion was supported by the fact that donor cells are unable to resume transfer from the point of interruption when the drug is removed, but can reinitiate transfer from the Hfr origin under conditions that prevent new mating pair formation. These authors have argued that a disruption at the replication fork of the Hfr chromosome could simultaneously interrupt chromosome transfer and prevent resumption of transfer from the point of interruption. Our finding that nalidixic acid not only reduced the transfer of DNA to minicells but also caused a relative decrease in the amount of unit-length plasmid DNA that was transferred supports the idea that the drug increases the number of breaks in the DNA strands being transferred. In addition, we found that the drug decreased the amount of donor cell DNA that could be isolated as covalently closed plasmid molecules on alkaline sucrose gradients. Such an observation is compatible with the hypothesis that nalidixic acid disrupts replication forks because plasmid molecules that are being transferred must have a discontinuity in at least one DNA strand and any damage that prevents completion of conjugal transfer and replication could also prevent the covalent closure of the plasmid DNA.

We previously concluded that R64-11 DNA is transferred to recipient minicells as monomeric, single-stranded units and not by a continuous transfer mechanism (15). This conclusion was strengthened by our finding that both RNA and protein synthesis are required before an R factor that has been transferred once can be transferred again (14). These findings indicate that conjugal transfer of R64-11 is a cyclic process in the system that we have used. We believe that after a donor cell has formed a mating pair with a suitable recipient, the R factor enters a round of conjugal DNA transfer and replication, and,

![Figure 7. Effect of nalidixic acid on membrane-bound and circular forms of R64-11 DNA labeled in donor cells during conjugation. A mating between R64-11+ dnaB(ts) donor cells and recipient minicells was conducted at 42°C in the presence of 10 μCi of [3H]thymidine ([3H]dThd) per ml as described in the legend of Fig. 1. Nalidixic acid was added at 15 min to a concentration of 10 μg/ml. Donor cells were purified from samples withdrawn at the indicated times, and the cell DNA was analyzed by alkaline (as in Fig. 2) and neutral (as in Fig. 6) sucrose gradient centrifugation. The figure shows the percentage of the donor cell DNA that sedimented as closed-circular R64-11 DNA on the alkaline gradients (●) and as closed-circular (∆), open-circular (○), and membrane-bound (○) DNA on the neutral gradients.](http://jb.asm.org/Downloaded from http://jb.asm.org)
when transfer is complete, the plasmid then enters a "resting" state while the donor cell synthesizes the products needed for the next round of transfer and conjugal replication.

Since nalidixic acid specifically inhibits DNA synthesis (18) and only exerts its lethal effect on cells that are replicating DNA (31), it should affect conjugation by acting on the portion of the R-factor population that is being conjugally transferred and replicated when the drug is added. If nalidixic acid does not simultaneously affect other steps in the conjugal process and if conjugal transfer of R factors is a cyclic process as described above, then the drug should cause an accumulation of the R factors in the stage of the conjugal cycle that is blocked or in the step of the cycle that immediately precedes the point of the block, or both. Because nalidixic acid inhibits the continuation of DNA synthesis, we might expect the drug to cause an accumulation of R factors at the point of initiation of conjugal replication and transfer. Data from studies on the effect of nalidixic acid on vegetative replication lend support to this hypothesis because Boyle et al. (5) have found that treatment of vegetatively growing cells with nalidixic acid induced additional sites of DNA synthesis when the drug was removed, and Ward et al. (30) have shown that these sites are at the origin of chromosome replication. Thus, in the presence of nalidixic acid, cells can either initiate vegetative replication or generate the potential for this initiation.

In addition to the inhibition of conjugal replication, we noted two effects on the donor cell DNA when we added nalidixic acid to a mating that was in progress: (i) the amount of conjugally replicated DNA that could be isolated as covalently closed circles under alkaline conditions decreased (Table 2 and Fig. 7), and (ii) cytoplasmic forms of conjugally replicated plasmid DNA became membrane bound (Fig. 6 and 7). The loss of covalently closed plasmid DNA is consistent with our hypothesis that nalidixic acid should cause an accumulation of R-factor DNA in the initiation or continuation stages of conjugal transfer and replication, or both. Plasmid molecules that are being conjugally transferred must have a gap in at least one DNA strand so that a linear, single strand of DNA can be transferred to the recipient. Thus, an accumulation of R factors in this stage of the conjugation cycle would decrease the amount of covalently closed plasmid DNA in the donor cells. This effect may be accentuated if the gaps in the R factors are normally repaired when matings are interrupted before the purification of the donor cells but can not be closed in the presence of nalidixic acid. Since one step in the initiation of conjugal transfer must involve the induction of the gap or nick in the plasmid DNA, the initiation of conjugal transfer by plasmids that are not being transferred at the time of drug addition would also decrease the amount of covalently closed R-factor DNA in the donor cells. In a study of the conjugal replication of R-factor DNA in recipient cells, Falkow et al. (13) found that transferred DNA replicated as linear molecules that were attached to the cell membrane. The replicated plasmid DNA then left the membrane as open-circular molecules, and these were covalently closed in the cytoplasm of the cell. Using this information and data obtained from studies on R strains growing vegetatively, they suggested that the "resting" state of R factors between rounds of replication is a closed-circular structure that is not attached to the cell membrane.

In the mating system we used, we found that up to 75% of the [3H]thymidine incorporated by R64-11 donor cells during the first 5 min of a mating is associated with the membrane fraction (unpublished data). At later times in the mating, the percentage of conjugally replicated DNA in the membrane fraction decreased, and the amount of cytoplasmic DNA became a significant portion of the DNA synthesized during the mating (Fig. 6). Because of these facts and the considerable amount of evidence that DNA replication is associated with the cell membrane in bacterial systems (see 19), we believe that plasmid DNA is associated with the cell membrane while it is being conjugally replicated in the donor cell, and that the cytoplasmic forms of the R factor are molecules that have completed a round of conjugal replication and transfer. Thus, the initial membrane association of cytoplasmic plasmid DNA after the addition of nalidixic acid to the mating media (Fig. 6 and 7) probably represents an accumulation of the R factors in the initiation and continuation stages of the conjugal replication cycle.

At this time we can not determine whether the cytoplasmic forms of R64-11 DNA that appeared after prolonged incubation of the mating mixtures in the presence of nalidixic acid were the same population of molecules that became associated with the membrane fraction when we added the drug (Fig. 7). Furthermore, we do not know whether the reappearance reflected a temporal change in the effect of the drug on the cell or whether the change might have been caused by the prolonged incubation of the cells at a temperature that is nonpermissive for vegetative DNA synthesis and which
might itself alter membrane DNA interactions. The fact that the loss of covalently closed plasmid DNA detected with the alkaline gradients was permanent rules out the possibility that the reappearance of the cytoplasmic DNA was caused by the synthesis of H-labeled plasmid DNA in the presence of nalidixic acid.

One can also conclude from the results shown in Fig. 7 that the population of covalently closed plasmids isolated on the alkaline gradients was different from that isolated on the neutral gradients, although the two populations probably overlapped. We have isolated conjugal replicated DNA from the membrane fraction of the donor cells not treated with nalidixic acid and then have sedimented that DNA through alkaline sucrose gradients. We found that as much as 10% of that DNA is covalently closed plasmid DNA (unpublished data). Thus, the closed-circular DNA isolated directly on alkaline gradients probably included both covalently closed plasmid DNA that was associated with the cell membrane and that which was free in the cytoplasm.

In this discussion, we have made the assumption that nalidixic acid inhibits the synthesis of DNA by somehow preventing movement of the replication fork, and because of this we have suggested that the increase in membrane-bound plasmid DNA caused by nalidixic acid is a secondary effect of the drug and a result of a block in the conjugal cycle. However, it is equally as reasonable to speculate that nalidixic acid induces an aberrant association between DNA and the cell membrane. In this frame of reference, the inhibition of DNA synthesis could be caused by physical constraints on the DNA and would be a secondary effect of the drug. Regardless of which, if either, of these hypotheses is correct, the important points of our results are that we detected an increase in the amount of membrane-bound DNA and an increase in the number of breaks or gaps in DNA strands after we treated the donor cells with nalidixic acid.

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LITERATURE CITED


