INHIBITION OF CONJUGAL TRANSFER OF R PLASMIDS
BY NORFLOXACIN IN PSEUDOMONAS AERUGINOSA

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Norfloxacin (NFLX) is a new quinolone carboxylic acid derivative that has potent anti-
bacterial activity against gram-positive and gram-negative bacteria including Pseudo-
monas aeruginosa.

NFLX strongly inhibited the conjugal transfer of R plasmids belonging to different in-
compatibility group in P. aeruginosa. Inhibitory activities of NFLX, pipemidic acid and
nalidixic acid (NA) for conjugal transfer of R plasmids were correlated with their anti-
bacterial activities against donor and recipient strains. The correlation was especially
clear in the activity against donor strains. NFLX was also an effective inhibitor of
plasmid transfer between NA-resistant donor and NA-resistant recipient strains, whereas
NA showed no transfer inhibition.

INTRODUCTION

Drug resistance plasmids are widely distributed
in bacteria isolated from clinical materials, live-
stock, and cultured fish1,2). Since the spread of
drug resistance plasmids is a serious problem in
practical medicine, the development of new drugs
is needed which are effective against plasmid-
bearing bacteria, plasmid replication and plasmid
transfer.

It has been reported that pyridonecarboxylic acid
derivatives inhibited the conjugal transfer of R
plasmids in gram-negative bacteria3-10).

Norfloxacin (NFLX, formerly known as AM-
715), 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piper-
asinyl)-3-quinoline carboxylic acid, is a new quin-
olone carboxylic acid derivative that has shown
potent antibacterial activity against gram-positive
and gram-negative bacteria including Pseudomonas
aeruginosa11). NFLX was 2 to 4 times more active
than gentamicin against P. aeruginosa. It also
strongly inhibited DNA replication in Escherichia
coli, and it was considered that DNA gyrase might
be one of its main targets12,13).

DNA replication is prerequisite to the conjugal
transfer of plasmids8). We have studied the inhibi-
tory activity of NFLX against transfer of R
plasmids belonging to different incompatibility
groups in P. aeruginosa14). The activity of other
pyridonecarboxylic acid derivatives, nalidixic acid
(NA) and pipemidic acid (PPA), was also compared.

MATERIALS AND METHODS

R plasmids and Pseudomonas aeruginosa
strains. R plasmids and P. aeruginosa strains
used in this study are shown in Tables 1 and 2,
respectively.

Drugs. Norfloxacin was synthesized by the
Central Research Laboratories of Kyorin Pharma-
ceutical Co., Ltd.. Nalidixic acid and pipemidic
acid were obtained from Daiichi Pharmaceutical
Co., Ltd. and Dainippon Pharmaceutical Co. Ltd.,
respectively. Tetracycline (Tc), chloramphenicol
(Cm), streptomycin (Sm), Kanamycin (Km),
gentamicin (Gm), carbenicillin (Cb), sulfonamide
(Su), rifampicin (Rf), and mercuric chloride (Hg)
were obtained commercially.

Isolation of nalidixic acid-resistant mu-
Table 1 R plasmids used

<table>
<thead>
<tr>
<th>R plasmid</th>
<th>Incompatibility group</th>
<th>Resistance marker</th>
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<tbody>
<tr>
<td>RP 4</td>
<td>P - 1</td>
<td>Tc Km Cb</td>
</tr>
<tr>
<td>Rms 139</td>
<td>P - 2</td>
<td>Tc Cm Sm Su Cb</td>
</tr>
<tr>
<td>Rms 159</td>
<td>P - 2</td>
<td>Tc Cm Sm Hg</td>
</tr>
<tr>
<td>Rms 164</td>
<td>P - 2</td>
<td>Sm Su Gm Hg</td>
</tr>
<tr>
<td>Rlb 151</td>
<td>P - 3</td>
<td>Sm Su Gm Cb</td>
</tr>
<tr>
<td>Rms 163</td>
<td>P - 5</td>
<td>Tc Cm Sm</td>
</tr>
<tr>
<td>Rms 148</td>
<td>P - 7</td>
<td>Sm</td>
</tr>
<tr>
<td>FP 5</td>
<td>P - 8</td>
<td>Hg</td>
</tr>
<tr>
<td>R 91</td>
<td>P - 10</td>
<td>Cb</td>
</tr>
</tbody>
</table>

Abbreviations: Tc, tetracycline; Km, kanamycin; Sm, streptomycin; Gm, gentamicin; Cb, carbenicillin; Su, sulfonamide; Cm, chloramphenicol; Hg, mercuric chloride.

NA-resistant mutants were obtained by plating approximately $10^9$ bacterial cells at early stationary phase of growth on nutrient agar plates containing NA (800 μg/ml).

Determination of minimal inhibitory concentrations. An overnight culture in Mueller-Hinton broth containing 0.4% KNO₃ was diluted 100-fold with BSG, which consisted of 8.5 g of NaCl, 0.3 g of KH₂PO₄, 0.6 g of Na₂HPO₄, 0.1 g of gelatin and 1,000 ml of distilled water. One loopful (5 μl) of the diluted culture was spotted on Mueller-Hinton agar plates containing serial two-fold dilutions of a drug. After 18 hr of incubation at 37°C, minimal inhibitory concentration (MIC) was determined.

Inhibition of R plasmid transfer. The donor and recipient cells were grown at 37°C in penassay broth to mid-exponential phase and then were mixed at a ratio of 1 : 4. The mixed culture was incubated at 37°C without shaking, and then a portion of the appropriately diluted culture was spread on the selective plate. In the inhibitory experiments of R plasmid transfer, an appropriate amount of drug was added to the conjugation mixture immediately after mixing of donor and recipient cells. The transfer frequency was expressed as the number of transconjugants per input donor cells, and the inhibitory percentage was calculated by determining the transfer frequency in the presence or absence of a test compound. The percent inhibition was plotted against the concentration of drug expressed as the log base 2, and the concentration required to produce 90% inhibition was estimated. The selection plate for the transconjugants was nutrient agar containing rifampicin (100 μg/ml) and one of the selective drugs. The concentrations of drugs used for selection were Tc, 50 (μg/ml); Sm, 12.5; Gm, 0.78; Cb, 200; and Hg, 12.5.

RESULTS

Kinetics of R plasmid transfer. We investigated the time course of R plasmid-transfer between *P. aeruginosa* strains (donor: PAO 1836, recipient: PAO 2142). In the absence of drugs, $4 \times 10^2$ to $8 \times 10^6$ transconjugants/ml were detected after 15 min of mating between donor (5 × 10⁷ cells/ml) and recipient cells (2 × 10⁸ cells/ml) and the number of transconjugants varied with the R plasmids used. The number almost reached a plateau at about 60 min after mixing of donor and recipient cells.

Inhibition of Rms 159 transfer by NFLX. The effect of NFLX on R plasmid transfer was examined using Rms 159 plasmid (Fig. 2). The number of donor (PAO 1836 Rms 159+) and recipient (PAO 2142 rif) cells was slightly affected by the presence of 0.1 or 0.2 μg/ml of NFLX but the number of transconjugants decreased 10 to 100 times with the same addition. At a con-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>MIC (μg/ml)</th>
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<tr>
<td></td>
<td></td>
<td>NFLX</td>
</tr>
<tr>
<td>PAO 1836</td>
<td>trp</td>
<td>0.39</td>
</tr>
<tr>
<td>PAO 2142 rif</td>
<td>itv, lys, met, rif&lt;br&gt;</td>
<td>0.39</td>
</tr>
<tr>
<td>PAO 2142 rif nal</td>
<td>itv, lys, met, rif, nal&lt;br&gt;</td>
<td>3.13</td>
</tr>
<tr>
<td>PAO 4009 leu (FP5⁺)</td>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>PAO 4009 nal leu (FP5⁺)</td>
<td></td>
<td>3.13</td>
</tr>
</tbody>
</table>

a) rif indicates resistance to rifampicin;
b) nal indicates resistance to nalidixic acid.
centrations of 0.39 μg/ml of NFLX, which was MIC for PAO 1836 and PAO 2142 rif by agar dilution method (Table 2), the number of both donor and recipient cells in the mixture decreased about 10-fold. The ratio of transconjugants in mixtures with 0.39 μg/ml of NFLX to that without NFLX was about 10⁻⁵. Since NFLX showed potent antibacterial activity against donor and recipient cells, the percentage of inhibition was calculated by determining the transfer frequency in the presence and absence of NFLX.

Inhibition of the transfer of various R plasmids by NFLX, PPA or NA. The ID₉₀ of NFLX, PPA and NA after 60 min of mating is shown in Table 3. ID₉₀ values of NFLX for various R plasmids were 0.13 to 0.45 μg/ml, remarkably lower than those of PPA or NA. Furthermore, these values correlated with the MICs for donor and recipient strains. Transfer inhibition by these drugs did not vary with incompatibility groups of R plasmids.

Effect of NA sensitivity of donor and recipient on transfer inhibition. Effect of NA sensitivity on transfer inhibition was studied using NA-resistant mutants of both donor (PAO 4009) and recipient (PAO 2142). The inhibition of FP 5 plasmid transfer by NA was affected by the sensitivity of donor and recipient strains to the drug. Donor strain’s sensitivity strongly influenced the degree of transfer inhibition. NFLX inhibited the FP 5 transfer between NA-resistant donor and recipient cells at extremely low concentrations, whereas NA showed no inhibition in this combination.

DISCUSSION

The present paper has shown that NFLX inhibited the conjugal transfer of R plasmids in P. aeruginosa, and was more potent transfer inhibitor
Table 3 Inhibition of R plasmid transfer by NFLX, PPA and NA

<table>
<thead>
<tr>
<th>R plasmid</th>
<th>Selected marker</th>
<th>Transfer frequency*</th>
<th>ID₉₀ (µg/ml)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NFLX</td>
</tr>
<tr>
<td>RP4</td>
<td>Tc</td>
<td>1.3 x 10⁻³</td>
<td>0.17</td>
</tr>
<tr>
<td>Rms139</td>
<td>Tc</td>
<td>5.4 x 10⁻³</td>
<td>0.27</td>
</tr>
<tr>
<td>Rms159</td>
<td>Tc</td>
<td>1.6 x 10⁻²</td>
<td>0.20</td>
</tr>
<tr>
<td>Rms164</td>
<td>Hg</td>
<td>1.6 x 10⁻²</td>
<td>0.13</td>
</tr>
<tr>
<td>Rlb151</td>
<td>Gm</td>
<td>3.1 x 10⁻⁴</td>
<td>0.27</td>
</tr>
<tr>
<td>Rms163</td>
<td>Tc</td>
<td>3.1 x 10⁻³</td>
<td>0.45</td>
</tr>
<tr>
<td>Rms148</td>
<td>Sm</td>
<td>3.8 x 10⁻¹</td>
<td>0.37</td>
</tr>
<tr>
<td>FP5</td>
<td>Hg</td>
<td>2.1 x 10⁻¹</td>
<td>0.30</td>
</tr>
<tr>
<td>R91</td>
<td>Cb</td>
<td>4.3 x 10⁻¹</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Transfer frequency: Number of transconjugants per number of donor cells

**ID₉₀: Concentrations of drugs for 90% inhibition of transfer after 60 min of mating between donor and recipient cells.

Table 4 Relation of NA sensitivity of donor and recipient strains with the inhibition of FP5 plasmid transfer by NFLX, PPA, or NA

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Recipient strain</th>
<th>ID₉₀ (µg/ml)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NFLX</td>
</tr>
<tr>
<td>PAO 4009</td>
<td>PAO 2142</td>
<td>0.62</td>
</tr>
<tr>
<td>PAO 4009</td>
<td>PAO 2142 nal</td>
<td>1.1</td>
</tr>
<tr>
<td>PAO 4009 nal</td>
<td>PAO 2142</td>
<td>2.3</td>
</tr>
<tr>
<td>PAO 4009 nal</td>
<td>PAO 2142 nal</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*ID₉₀: See legend of Table 3.

than PPA and NA. NFLX showed effective inhibition in the conjugal transfer between both NA-resistant donor and recipient cells.

NFLX, like other pyridonecarboxylic acids, inhibits bacterial DNA synthesis in vivo and in vitro, and recently it has been shown that NFLX, PPA and NA inhibit the DNA gyrase12, 13, 15, 16, 17) which introduces negative superhelical turns into duplex DNA and is considered to play an important role in DNA replication18). DNA gyrase has not yet been identified in P. aeruginosa, but it has been reported that the DNA replication of highly NA-resistant mutants (termed nalA) was resistant to NA in a permeabilized cell system19). The nalA locus was mapped between hex and leu-10 in P. aeruginosa PAO chromosome19). NA-resistant mutants (PAO 1836nal and PAO 2142nal) used in this study were mapped between hex-9001 and leu-9005 by FP5-mediated conjugation system and transduction with phage F116L (data not shown). NFLX showed transfer inhibition of the plasmid between NA-resistant donor(nalA) and recipient (nalA) strains in P. aeruginosa, indicating that it could inhibit the conjugal transfer of R plasmid even in NA-resistant(nalA) strains whose DNA replication was resistant to NA.

Since most ID₉₀ values of NFLX, PPA and NA are correlated with their MICs against donor strains, these drugs might inhibit the replication of plasmids as well as chromosomal DNA. Transfer inhibition by NFLX or other pyridonecarboxylic acids was not influenced by the incompatibility type of R plasmid but was by the donor's sensitivity to the drug. Thus the inhibition of DNA replication by pyridonecarboxylic acids is not plasmid-specific.

Gram-negative bacteria resistant to antimicrobial agents, such as aminoglycoside antibiotics, tetracycline, chloramphenicol, β-lactam antibiotics, trimethoprim, and sulphonamide are known to be primarily mediated by the conjugative(R) and nonconjugative(r) resistance plasmids, which can
easily acquire resistance to newly introduced drugs and spread that resistance to many species of bacteria\(^1\), \(^2\). On the contrary, NA-resistance in gram-negative bacteria is known to be mediated by the chromosome, and no plasmid specifying NA or other pyridonecarboxylic acid resistance has been detected in NA-resistant bacteria\(^20\), \(^21\).

These results suggest that pyridonecarboxylic acid derivatives, and particularly NFLX, are effective agents against bacteria carrying drug resistance plasmids and plasmid-bearing bacteria.

Acknowledgements

We are grateful to Dr. MATSUMOTO for providing \(P.\ aeruginosa\) PAO strains.

REFERENCES

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Norfloxacin による細菌由来薬剤耐性プラスミドの
接合伝達阻害について

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伊予部志津子・三橋 進
熊本大学医学部微生物学教室

細菌を含むグラム陰性菌およびグラム陽性菌に対し強い抗菌力を示す新しいクロノカルボン系薬剤
Norfloxacin の R プラスミド接合伝達阻害活性を細菌由来の R プラスミドを用いて検討した。
NFLX は R プラスミドの不和合性に関係なく接合伝達を強く阻害した。
NFLX, PPA, NA の R プラスミド接合伝達阻害活性は、各薬剤の抗菌活性と相関しており、特に供与菌に対する
抗菌力に強い相関性を示した。
NFLX は、NA が伝達阻害を示さない NA 耐性供与菌と受容菌間での R プラスミドの伝達も強く阻害した。