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 antibacterial activity against gram-positive and gram-negative bacteria including *Pseudomonas aeruginosa*.

NFLX strongly inhibited the conjugal transfer of R plasmids belonging to different incompatibility group in *P. aeruginosa*. Inhibitory activities of NFLX, pipemidic acid and nalidixic acid (NA) for conjugal transfer of R plasmids were correlated with their antibacterial 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, livestock, and cultured fish[1,2]. 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 bacteria[3~10].

Norfloxacin(NFLX, formerly known as AM-715),1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid, is a new quinolone carboxylic acid derivative that has shown potent antibacterial activity against gram-positive and gram-negative bacteria including *Pseudomonas aeruginosa*[11]. 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 targets[12,13].

DNA replication is prerequisite to the conjugal transfer of plasmids[6]. We have studied the inhibitory activity of NFLX against transfer of R plasmids belonging to different incompatibility groups in *P. aeruginosa*[14]. 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 Pharmaceutical 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

R plasmid	Incompatibility group	Resistance marker
RP4	P- 1	Tc Km Cb
Rms 139	P- 2	Tc Cm Sm Su Cb
Rms 159	P- 2	Tc Cm Sm Hg
Rms 164	P- 2	Sm Su Gm Hg
Rlb 151	P- 3	Sm Su Gm Cb
Rms 163	P- 5	Tc Cm Sm
Rms 148	P- 7	Sm
FP5	P- 8	Hg
R91	P-10	Cb

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

tants. 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% KNO_3 was diluted 100-fold with BSG, which consisted of 8.5 g of NaCl, 0.3 g of KH_2PO_4, 0.6 g of Na_2HPO_4, 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℃, minimal inhibitory concentration (MIC) was determined.

Inhibition of R plasmid transfer. The donor and recipient cells were grown at 37℃ in penassay broth to mid-exponential phase and then were mixed at a ratio of 1 : 4. The mixed culture was incubated at 37℃ 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, 4×10^2 to 8×10^6 transconjugants/ml were detected after 15 min of mating between donor (5×10^7 cells/ml) and recipient cells (2×10^8 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.

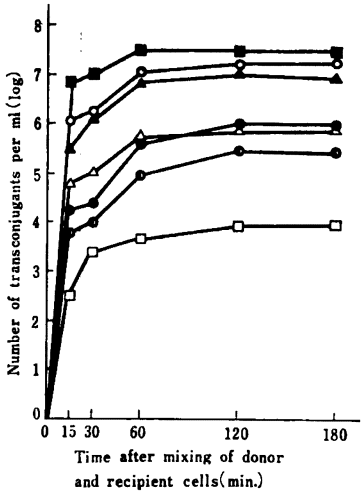
Inhibition of Rms 159 transfer by NFLX. The effect of NFLX on R plasmid transfer was examined using Rms 159 plasmid (Fig. 2). The number of donor (PAO 1836 Rms 159$^+$) and recipient (PAO 2142 *rif*) cells was slightly affected by the presence of 0.1 or 0.2 μg/ml of NFLX, but the number of transconjugants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

Table 2 *Pseudomonas aeruginosa* strains used

Strain	Genotype	MIC (μg/ml)		
		NFLX	PPA	NA
PAO 1836	*trp*	0.39	12.5	100
PAO 2142 *rif*	*ilv, lys, met, rif*[a]	0.39	12.5	100
PAO 2142 *rif nal*	*ilv, lys, met, rif, nal*[b]	3.13	50	1600
PAO 4009	*leu* (FP5$^+$)	0.39	12.5	100
PAO 4009 *nal*	*leu, nal* (FP5$^+$)	3.13	50	1600

[a] *rif* indicates resistance to rifampicin;
[b] *nal* indicates resistance to nalidixic acid.

Fig. 1 Kinetics of R plasmid transfer. Donor, *P. aeruginosa* PAO 1836 RP 4$^+$(◐), Rms 159$^+$(△), Rlb 151$^+$(□), Rms 163$^+$(●), Rms 148$^+$(○), FP 5$^+$(▲), and R 91$^+$(■); recipient, *P. aeruginosa* PAO 2142 *rif*.



centration 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 ID_{90} of NFLX, PPA and NA after 60 min of mating is shown in Table 3. ID_{90} 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

Fig. 2 Effect of NFLX on Rms 159 transfer. Donor, *P. aeruginosa* PAO 1836; recipient, *P. aeruginosa* PAO 2142 *rif*. Number of cells: donor(A), recipient(B), and transconjugants (C). NFLX concentrations (μg/ml): (●), 0; (△), 0.1; (▲), 0.2; (○), 0.39.

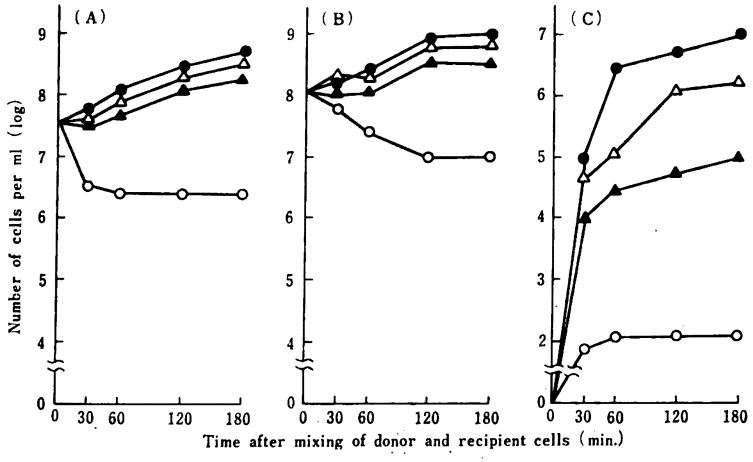


Table 3 Inhibition of R plasmid transfer by NFLX, PPA and NA

R plasmid	Selected marker	Transfer frequency*	ID_{90} (μg/ml)**		
			NFLX	PPA	NA
RP4	Tc	1.3×10^{-3}	0.17	11.5	150
Rms139	Tc	5.4×10^{-3}	0.27	11.5	135
Rms159	Tc	1.6×10^{-2}	0.20	11.0	70
Rms164	Hg	1.6×10^{-2}	0.13	9.5	85
Rlb151	Gm	3.1×10^{-4}	0.27	19.0	165
Rms163	Tc	3.1×10^{-3}	0.45	22.0	140
Rms148	Sm	3.8×10^{-1}	0.37	10.0	96
FP5	Hg	2.1×10^{-1}	0.30	13.5	88
R91	Cb	4.3×10^{-1}	0.28	17.5	120

*Transfer frequency: Number of transconjugants per number of donor cells
**ID_{90}: 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

Donor strain	Recipient strain	ID_{90} (μg/ml)a)		
		NFLX	PPA	NA
PAO 4009	PAO 2142	0.62	19	70
PAO 4009	PAO 2142 *nal*	1.1	40	150
PAO 4009 *nal*	PAO 2142	2.3	78	1000
PAO 4009 *nal*	PAO 2142 *nal*	5.6	160	>1600

a) ID_{90}: 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 gyrase[12,13,15,16,17] which introduces negative superhelical turns into duplex DNA and is considered to play an important role in DNA replication[18]. 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 system[19]. The *nalA* locus was mapped between *hex* and *leu*-10 in *P. aeruginosa* PAO chromosome[19]. NA-resistant mutants (PAO 1836*nal* and PAO 2142*nal*) 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 ID_{90} 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.

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Norfloxacin による緑膿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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緑膿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キノロンカルボン酸系薬剤 Norfloxacin のRプラスミド接合伝達阻害活性を緑膿菌由来の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。