ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1992, p. 2562–2565
0066-4804/92/112562-04$02.00/0

Vol. 36, No. 11

Occurrence of the *nfxB* Type Mutation in Clinical Isolates of *Pseudomonas aeruginosa*

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Received 2 April 1992/Accepted 5 September 1992

Seven spontaneous norfloxacin (NFLX)-resistant mutants obtained in vitro from 20 NFLX-susceptible clinical isolates and 3 NFLX-resistant clinical isolates of *Pseudomonas aeruginosa* were transformed with the pNF111 plasmid, whose *Bam*HI fragment is responsible for conferring susceptibility to NFLX, by complementing the *nfxB* mutation. The resulting patterns of MICs of NFLX, β-lactams, aminoglycosides, and chloramphenicol and the observed increased accumulation of NFLX were consistent with the occurrence of the *nfxB* type mutation in these clinical isolates.

Because of the increasing number of infections caused by *Pseudomonas aeruginosa*, the accurate determination of resistance patterns associated with this organism has become an essential step in chemotherapy. There are numerous reports describing a high incidence of *P. aeruginosa* strains resistant to a variety of antimicrobial agents (2, 11, 15). New quinolones generally show potent antibacterial activities against *P. aeruginosa* strains (6), but the number of resistant clinical isolates has increased with the wide usage of these drugs in clinical practice (7, 9, 12).

Bacterial resistance to quinolones is due to chromosomal mutations that involve alterations in DNA gyrase and in the bacterial outer membrane. In *P. aeruginosa*, several established types of mutations are responsible for norfloxacin (NFLX) resistance. These mutation types, designated *nfxA*, *nfxB*, *nfxC*, and *nalB*, have been well characterized previously (4, 5, 13).

In our preliminary study, the chromosomal localization of the *nfxB* mutation in clinical isolates of *P. aeruginosa* was established (5). A wild-type DNA fragment complementing the *nfxB* mutation was cloned (10). Recently, NFLX-resistant mutants derived from NFLX-susceptible clinical isolates and NFLX-resistant clinical isolates were tested in order to determine whether the observed *nfxB* resistance phenotype is complementable by using the constructed recombinant plasmid; the results are presented in this paper.

P. aeruginosa strains were isolated and characterized by the Clinical Laboratory of Gunma University Hospital, Maebashi, Japan. A total of 20 randomly selected NFLX-susceptible and 11 NFLX-resistant clinical isolates were chosen to examine the NFLX resistance mechanism. The strains with different clinical origins belonged to various O serotypes. Serotyping was performed according to the Standards of the Japan *Pseudomonas aeruginosa* Society (1975).

The NFLX-susceptible parent strains were initially cultured on nalidixic acid-cetrimide (Eiken) plates. Ten single colonies from each plate were randomly chosen and inoculated into Mueller-Hinton broth, which was incubated for 18 h at 37°C. From the overnight cultures, 0.1-ml volumes were spread onto Sensitivity Disk Agar-N plates (Nissui) with 1.6 μg of NFLX per ml and then incubated for 18 h at 37°C. The concentration of 1.6 μg/ml was 2 to 4× the MIC for NFLX-susceptible strains of both laboratory PAO and clinical isolates of *P. aeruginosa* and corresponded to the concentration that inhibited the growth of about 10^8 CFU of the NFLX-susceptible strains and that allowed the complete growth of NFLX-resistant mutants of PAO strains. One or two single colonies from each plate were independently isolated and purified on the same medium. Spontaneous NFLX-resistant progenies were obtained at a frequency of 2×10^{-8} to 6×10^{-8}. The MICs of NFLX (Kyorin), imipenem (IPM) (Banyu), chloramphenicol (CP) (Sankyo), carbenicillin (CBPC) (Fujisawa), and gentamicin (GM) (Shering) for 10 to 15 mutants derived from each parent were determined by the agar dilution method in Sensitivity Disk Agar-N. The inoculum size of the diluted bacteria for the MIC assay was on the order of about 10^3 CFU (μl of diluted culture at about 10^5 CFU/ml). The MIC was defined as the lowest concentration of antibiotic that inhibited the development of visible growth on the plate after 18 h of incubation at 37°C. The *nfxA*, *nfxB*, *nfxC*, and *nalB* phenotypes were determined according to the susceptibility patterns of the isolate to β-lactams, aminoglycosides, and CP (4). The *nfxA* mutation is associated with an altered subunit A of DNA gyrase and is manifested as an increase in quinolone resistance only. The other mutations are characterized by a decrease in outer membrane permeability to quinolones. The *nfxB* mutation is manifested by hypersusceptibilities to β-lactam and aminoglycoside antibiotics. The *nfxC* mutation differs from the *nfxB* type by conferring resistance to IPM. The *nalB* mutation determines increased resistance to β-lactams (4). In all but two cases, *nfxB* type mutants were obtained from these resistance clones isolated in vitro. The total number of mutants obtained from the 20 cases was 224, 68 of which were of the *nfxB* type (Table 1).

One representative *nfxB* type mutant was chosen from each parent and was transformed with the recombinant plasmid pNF111. This plasmid was constructed from pME294, a high-copy-number plasmid vector containing a 4.4-kb *Bam*HI fragment of *P. aeruginosa* PAO1 chromosomal DNA which complements the *nfxB* mutation. The

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TABLE 1. Distribution of mutants by mutation type

Parent strain	Source	Serogroup	No. of mutants tested	No. of the following mutation type:				No. of nontypeable mutants
				nfxA	*nfxB*	*nfxC*	*nalB*	
GU1	Pus	D	10					10
GU2	Pus	D	10		1	3		6
GU3	Pus	G	10		3			7
GU4	Blood culture	I	10		1	1		8
GU5	Eye	C	10		3	6		1
GU6	Pus	G	10		8	1		1
GU9	Urine	G	10		2	2		6
GU11	Pus	A	10		2	4		4
GU12	Pus	B	10		1	7		2
GU13	Urine	B	10	2	5			3
GU15	Trachea	H	12		8			4
GU16	Nasopharynx	H	15		9			6
GU17	Pus	G	12		4	5		3
GU20	Trachea	H	12		7	2		3
GU22	Trachea	E	15		2	2		11
GU23	Sputum	G	10			7		3
GU24	Pus	A	12		4	8		
GU25	Pus	I	11		3	8		
GU28	Urine	C	15		3			12
GU29	Other	C	10		2			8
Total			224	2	68	56	0	98

*Bam*HI fragment is responsible for conferring susceptibility to NFLX in merodiploid strains (10). The plasmid contains a CBPC resistance gene as a selective marker. Clinical isolates of *P. aeruginosa* strains were transformed by a chemical method (8) or electroporation (3). Electroporation was carried out at a field strength of 10 kV/cm by using a resistance of 20 kΩ. The phenotype expression was performed for 2 h at 37°C in Mueller-Hinton broth containing 10% sucrose. The selection of transformants occurred on bromthymol blue agar containing CBPC. The transformation was successful with 15 out of 18 strains. The MICs for the mutants and transformants were found to be stable in seven cases upon storage; that is, the two mutant strains lost their resistance to NFLX and the transformants of the remaining six NFLX-resistant mutants lost the pNF111 plasmid. A comparison of the MICs of NFLX, GM, IPM, CBPC, and CP for the parent, mutant, and transformant strains is shown in Table 2. The mutants required an increased MIC of NFLX but decreased MICs of GM and β-lactams compared with those required by the parent strains. The MIC of CP was increased

TABLE 2. Comparative MICs for the parent, mutant, and transformant strains and their NFLX accumulation

Strain	MIC (μg/ml)					NFLX accumulation (μg/mg [dry wt] of cells)[a]
	NFLX	GM	CP	IPM	CBPC	
GU2	0.39	3.13	25	1.56	25	0.552 (100)
GU2M[b]	6.25	0.39	50	0.2	3.13	0.363 (66)
GU2M (pNF111)[c]	0.39	6.25	25	1.56	>50	0.537 (97)
GU4	0.39	3.13	25	1.56	12.5	0.444 (100)
GU4M[b]	3.13	<0.2	12.5	<0.2	0.78	0.288 (65)
GU4M (pNF111)[c]	0.2	3.13	12.5	0.39	>100	0.663 (149)
GU9	0.39	3.13	25	3.13	25	0.229 (100)
GU9M[b]	6.25	0.39	50	0.2	1.56	0.048 (19)
GU9M (pNF111)[c]	0.39	6.25	50	1.56	>50	0.257 (112)
GU13	0.39	6.25	12.5	1.56	12.5	0.307 (100)
GU13M[b]	3.13	1.56	50	0.39	1.56	0.045 (15)
GU13M (pNF111)[c]	0.2	12.5	12.5	0.78	>100	0.214 (70)
GU20	0.39	3.13	25	0.78	50	0.131 (100)
GU20M[b]	6.25	0.39	50	<0.2	1.56	0.008 (6)
GU20M (pNF111)[c]	0.39	3.13	25	0.78	>50	0.059 (45)
GU22	0.39	3.13	25	0.78	50	0.284 (100)
GU22M[b]	6.25	0.39	50	<0.2	1.56	0.000 (0)
GU22M (pNF111)[c]	0.39	3.13	25	0.78	>50	0.070 (25)
GU28	0.2	3.13	50	0.78	12.5	0.215 (100)
GU28M[b]	3.13	0.78	50	0.2	1.56	0.115 (54)
GU28M (pNF111)[c]	0.1	3.13	12.5	0.78	>100	0.262 (122)

[a] Values in parentheses are percents. Accumulation by the NFLX-susceptible wild-type strain is expressed as 100%.
[b] NFLX-resistant mutant of the wild-type strain.
[c] pNF111 transformant of the mutant strain.

TABLE 3. Identification of the *nfxB* mutation in NFLX-resistant strains and their NFLX accumulation

Strain	Source	Serotype	MIC (μg/ml)					NFLX accumulation (μg/mg [dry wt] of cells)[a]
			NFLX	GM	CP	IPM	CBPC	
GN10106	Urine	E	3.13	0.39	50	0.39	6.25	0.017 (41)
GN10106 (pNF111)			0.78	0.78	6.25	0.39	>100	0.041 (100)
GU35	Pus	G	3.13	0.78	25	0.39	6.25	0.137 (30)
GU35 (pNF111)			0.39	3.13	6.25	0.78	>100	0.459 (100)
GU59	Sputum	M	6.25	<0.2	25	0.39	1.56	0.019 (13)
GU59 (pNF111)			0.78	0.2	<6.25	<0.2	>100	0.141 (100)

[a] Values in parentheses are percents. Accumulation by transformants is expressed as 100%.

in most cases. The MICs for the transformants returned to approximately the levels found for the parents with the exception of that of CBPC (marker on the pNF111 plasmid). The presence of the correct plasmid in all transformants was confirmed by previously published (14) methods (data not shown).

The NFLX accumulation in the wild-type clinical isolates was compared with that in their respective NFLX-resistant mutants by a previously described method (4). The exposure time for the uptake of NFLX was 20 min at 37°C. The accumulation levels of NFLX were not the same among NFLX-susceptible clinical isolates. But in every group, the accumulation in the mutant was much lower than that in the parent or the transformant (Table 2). The relative levels of NFLX accumulation in the transformants were higher than those in the mutants and returned to the levels in the respective NFLX-susceptible parent strains in most cases. In the three cases in which NFLX accumulation was markedly decreased in the mutants (GU13M, GU20M, and GU22M), the increased accumulation did not return to the parental level. It is possible that in wild-type *P. aeruginosa* strains of clinical origins, the biochemical composition of the cell surface is different from strain to strain. This could reflect the differences in the levels of accumulation of NFLX observed in our study.

The NFLX-resistant clinical isolates were further examined in order to determine whether the purported *nfxB* type mutation was complementable by the pNF111 plasmid. From the 11 putative *nfxB* type strains among the 30 NFLX-resistant clinical isolates, successful transformation was observed with 3 (Table 3). Following transformation, the MIC of NFLX for the resistant clinical isolates decreased to the level for strains with the susceptible phenotype. The MIC of GM was increased and that of CP was decreased concomitantly. The MIC of IPM was decreased in one of the three transformants but remained unchanged in the others. The presence of the recombinant plasmid was confirmed in these transformants (data not shown). The NFLX accumulation of the NFLX-resistant clinical isolates and their transformants was also examined (Table 3). The resistant strains showed decreased NFLX accumulation compared with that by the transformants.

Bacterial resistance to nalidixic acid and other quinolones is due to chromosomal mutations (9). Plasmids or transposons that determine quinolone resistance in genes have not yet been found in bacteria (1, 9). In the present study, *nfxB* type mutants derived from NFLX-susceptible clinical strains were demonstrated via complementation with a wild-type gene. Yoshida et al. (16) reported spontaneous quinolone-resistant mutants from a laboratory strain of *P. aeruginosa*. Since IPM susceptibility was not studied, the *nfxB* and *nfxC* type mutations were not distinguishable. Eleven of 63 mutants tested (17%) selected with enoxacin were of the *nfxA* type and the remaining 52 mutants tested (83%) were of the *nfxB* or *nfxC* type in that study. In our study, from 224 spontaneous quinolone-resistant mutants, 2 (1%) were thought to have *nfxA*, 68 (30%) were thought to have *nfxB*, and 56 (25%) were thought to have *nfxC* type mutations. The permeability type mutation was more common than the gyrase (*nfxA*) type in both reports.

The *nfxB* mutation alters membrane permeability, resulting in a decreased rate of accumulation of the drug. NFLX uptake by NFLX-resistant clinical isolates as well as that by laboratory-derived mutants was much lower than those by the parents and transformants.

Our study confirms that the *nfxB* gene is responsible for conferring susceptibility to NFLX in *P. aeruginosa*. Examination of a randomly selected, NFLX-susceptible population demonstrated that the *nfxB* mutation occurred in *P. aeruginosa* from different clinical sources; serogroup dependence was not found. Our study also supports the occurrence of this mutation type in NFLX-resistant clinical isolates.

We thank Istvan Barcs for help with the manuscript and Ayako Takahashi and Yukie Yomoda for supplying clinical isolates of *P. aeruginosa*.

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