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Mutations Producing Resistance to Norfloxacin in *Pseudomonas aeruginosa*

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Two genetically distinct classes of norfloxacin-resistant *Pseudomonas aeruginosa* PAO4009 mutants were isolated spontaneously. Two norfloxacin resistance genes, *nfxA* and *nfxB*, were mapped between *hex-9001* and *leu-9005* and between *pro-9031* and *ilv-9023*, respectively, on the *P. aeruginosa* PAO chromosome. The *nfxA* gene was shown to be an allele of *nalA* by transductional analysis with bacteriophage F116L. The *nfxB* mutant showed a 16-fold increase in resistance to norfloxacin and a slight increase in resistance to nalidixic acid. The *nfxB* mutant was unique in that it showed hypersusceptibility to beta-lactam and aminoglycoside antibiotics. This mutant had about a threefold-lower rate of norfloxacin uptake than that of the wild-type strain or *nfxA* mutant. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins demonstrated the appearance of a 54,000-dalton protein in the *nfxB* mutant. These findings suggested that the norfloxacin resistance mechanism in the *nfxB* mutant might be an alteration in outer membrane permeability to norfloxacin.

Norfloxacin and other new 4-quinolones show potent antibacterial activity against gram-negative and gram-positive bacteria (11, 14, 20). They also have high in vitro and in vivo antibacterial activity against *Pseudomonas aeruginosa* strains that show a strong intrinsic resistance to various antimicrobial agents including older quinolones such as nalidixic acid (11, 20, 42). The high antibacterial activity of new quinolones might be due to their strong inhibitory action against DNA gyrase, a target enzyme of quinolones, which has been isolated from various bacteria including *Pseudomonas aeruginosa* (5, 16, 26, 28, 42).

Drug resistance mediated by plasmids or transposons is a serious clinical problem. However, resistance to nalidixic acid and other quinolones in bacteria is due to chromosomal mutations (10, 13, 16–18, 32). Plasmids or transposons that carry quinolone resistance genes have not been found in bacteria (4). Several chromosomal mutations, *gyrA*, *nalB*, *nalC* (*gyrB*), and *nalD*, conferring nalidixic acid resistance were identified and mapped on the *Escherichia coli* K-12 chromosome (10, 17, 18). Recently, we (13) and Hooper et al. (16) identified norfloxacin resistance mutations (*norA*, *norB*, *norC*, *nfxA*, and *nfxB*) in *E. coli* K-12. *norA* and *nfxA* were alleles of *gyrA* encoding the A subunit of DNA gyrase, while *norB*, *norC*, and *nfxB* determined outer membrane permeability resistance to norfloxacin, were associated with a decrease in OmpF porin protein, and were mapped at 34 min, near 8 min, and at 20 to 22 min, respectively (13, 16). Two loci coding for resistance to nalidixic acid, *nalA* and *nalB*, have also been mapped on the *P. aeruginosa* PAO chromosome (32). It has been reported that DNA replication is resistant to nalidixic acid in permeabilized cells of *nalA* mutants and that *nalB* mutants cause a decrease of cell permeability to nalidixic acid and carbenicillin (32).

To gain information on the resistance mechanisms to norfloxacin in *P. aeruginosa*, we isolated spontaneous norfloxacin-resistant mutants of *P. aeruginosa* PAO and studied their properties. Two types of norfloxacin-resistant mutants were obtained from *P. aeruginosa* PAO4009 by spontaneous single-step mutation. One type appeared to result from mutation at the *nalA* locus, while the other was a novel mutant that showed alteration in norfloxacin uptake. In this report, we describe the genetic and biochemical properties of their novel mutant in detail.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. Bacterial strains used in this study are listed in Table 1. We isolated spontaneous norfloxacin-resistant *P. aeruginosa* PAO4009 mutants by plating on nutrient agar plates containing norfloxacin. *P. aeruginosa* PAF41 was used as a producer strain for aeruginocin AP41 (35). Phages F116L and G101 were used for transduction (21, 23), and phages E79tv-1 and aeruginocin AP41 were used for susceptibility tests (25). Plasmid FP5 with chromosome mobilization ability was used for conjugation (24).

Media. Antibiotic medium 3, Mueller-Hinton agar, Mueller-Hinton broth, Proteose Peptone, and nutrient agar were purchased from Difco Laboratories, Detroit, Mich. L broth, L agar, and minimal medium (citrate-free minimal A medium) were as described previously (13, 30). Minimal A medium contained either 0.4% glucose or 20 mM pyruvate as a carbon source and was supplemented with 1 mM amino acids and purine, if necessary. Proteose Peptone no. 2–0.5% NaCl (PP medium) was used for preparation of outer membrane proteins (9).

Antimicrobial agents. AM-833 (11), ciprofloxacin, norfloxacin, ofloxacin, and pipemidic acid were synthesized by the Central Research Laboratories of Kyorin Pharmaceutical Co., Ltd. Carbenicillin, cefsulodin, chloramphenicol, gentamicin, kanamycin, moxalactam, and tetracycline were obtained from commercial sources.

Antimicrobial susceptibility tests. Antimicrobial susceptibility was measured by an agar dilution method with Mueller-Hinton agar (20). The MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth after 18 h at 37°C.

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TABLE 1. Strains of *P. aeruginosa* used

Strain	Genotype or description[a]	Source
PAO4009	FP5$^+$ *leu-9018 nir-9006*	H. Matsumoto
KH4023	FP5$^+$ *nfx-23* derivative of PAO4009	This study
KH4013E	FP5$^+$ *nfx-13E* derivative of PAO4009	This study
PAO2142	FP$^-$ *ilv-9001 lys-12 met-9011 tyr-9009*	H. Matsumoto
PAO1840	FP$^-$ *hex-9001 leu-9005 met-9020*	H. Matsumoto
PAO4031	FP$^-$ *arg-9040 catA1 his-9015 ilv-9023 met-9020 mtu-9001 nad-9003 nar-9011 pro-9031*	H. Matsumoto
PAO4293	FP$^-$ *car-9003 catA1 ilv-9059 met-9020 nar-9011 pig-9001 pur-9047*	H. Matsumoto
PAO1052	FP$^-$ *cys-59 nalA proB pru-70 thr-48*	B. W. Holloway
PAO963	FP$^-$ *hex-9001 leu-9005 met-9020 nalA16*	D. Haas
PAO477	FP$^-$ *argB1 ilv-202 met-28 nalA12 str-1*	D. Haas
PAF41	Prototrophic (pyocinogenic for aeruginocin AP41)	M. Kageyama

[a] The genotype symbols are those of Royle et al. (33). *nfx* designates resistance to norfloxacin.

Genetic analysis. Plasmid FP5-mediated conjugation was carried out as described by Matsumoto et al. (23) and Okii et al. (30). Transductions with phage F116L were performed by the method of Krishnapillai (21).

Uptake of norfloxacin by bacterial cells. The uptake of norfloxacin by *P. aeruginosa* cells was measured by the method described previously (12, 15). Bacterial cells were grown at 37°C in antibiotic medium 3 (Difco), and norfloxacin was added to the bacterial culture (A_{570} = 0.7) to a final concentration of 10 μg/ml. At various times, 10 ml of the culture was chilled and the cells were sedimented by centrifugation and washed once in 2 ml of saline. The cells were then suspended in 1 ml of saline, and the suspension was immersed in boiling water for 7 min to elute norfloxacin. The concentration of norfloxacin in the supernatants was measured by bioassay with *E. coli* NIHJ JC-2. The uptake of norfloxacin was calculated by subtracting the absorbed norfloxacin at zero time from the total norfloxacin eluted.

Characterization of outer membrane proteins. Outer membrane proteins of *P. aeruginosa* PAO were prepared by the method of Poxton et al. (31), and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Sawai et al. (36). The proteins (about 40 μg) were run, and gels were stained with Coomassie brilliant blue.

Characterization of lipopolysaccharide. Lipopolysaccharide (LPS) was extracted from logarithmic-phase culture by the phenol extraction technique of Westphal and Jann (39). Analysis of urea gels (15% polyacrylamide) for LPS and silver staining was performed as described by Tsai and Frasch (38).

Phage and aeruginocin AP41 sensitivity test. The susceptibility of mutants to bacteriophages E79tv-1 and AP41 was determined by spot test (25). Crude AP41 was prepared by the method described by Meadow and Wells (25).

RESULTS

Susceptibility of norfloxacin-resistant mutants to antimicrobial agents. We isolated spontaneous norfloxacin-resistant *P. aeruginosa* PAO4009 mutants by plating approximately 10^{10} CFU of a late-logarithmic-phase culture on a nutrient agar plate containing norfloxacin. Mutants showing resistance to norfloxacin were obtained spontaneously at frequencies of about 10^{-9} by selection on nutrient agar plates containing 1.56 or 3.13 μg of norfloxacin per ml, but mutants highly resistant to norfloxacin (MIC, 12.5 μg/ml) could not be obtained by single-step mutations.

Mutants showing resistance to norfloxacin fell into two types according to their susceptibility to quinolones and other antimicrobial agents. The susceptibility of two representative mutants (KH4023 and KH4013E) to antimicrobial agents is shown in Table 2. KH4023 showed resistance to a high concentration (>1,600 μg/ml) of nalidixic acid. This mutant was four to eight times more resistant to norfloxacin and other new quinolones such as ciprofloxacin, ofloxacin, and AM-833 than was the parent strain (PAO4009). The *nalA* mutants, PAO1052, PAO963, and PAO477, also showed a susceptibility pattern similar to that of KH4023 (data not shown). These mutants showed no changes in susceptibility to other antimicrobial agents.

The second type of mutant, KH4013E, also showed a 4- to 16-fold increase in resistance to norfloxacin and other new quinolones; however, it showed only a slight (2-fold) increase in resistance to nalidixic acid and pipemidic acid. The mutant was unique in that it was hypersusceptible to carbenicillin, moxalactam, kanamycin, and gentamicin. This type of mutant demonstrated no significant change in susceptibility to other antimicrobial agents including tetracycline and chloramphenicol.

Mapping of norfloxacin resistance genes. To determine the approximate location of norfloxacin resistance genes, we carried out FP5-mediated conjugations with PAO2142 as the recipient strain. The results suggest that the norfloxacin resistance gene in KH4013E is located near *ilv-9001* (*ilvBC*), while that in KH4023 is located close to *met-9011*. The resistance genes of KH4023 and KH4013E were designated as *nfxA* and *nfxB*, respectively.

The susceptibility of KH4023 to quinolones and chromosomal mapping by FP5-mediated conjugation suggest that KH4023 results from a mutation in the *nalA* locus. *nfxA* in KH4023 was mapped more precisely by transduction with phage F116L and with PAO1840 (*hex-9001 leu-9005 met-9020*) as the recipient strain. Selection was made for these markers, and 250 transductants for each marker were scored for susceptibility to norfloxacin by being streaked on

TABLE 2. Susceptibility of norfloxacin-resistant mutants of PAO4009 to quinolones and other agents

Compound	MIC (μg/ml)[a] for:		
	PAO4009 (wild type)	KH4023 (*nfxA*)	KH4013E (*nfxB*)
Norfloxacin	0.39	3.13	6.25
Ciprofloxacin	0.10	0.78	0.78
Ofloxacin	1.56	6.25	6.25
AM-833	0.78	6.25	3.13
Pipemidic acid	12.5	50	25
Nalidixic acid	100	>1,600	200
Gentamicin	1.56	1.56	0.39
Kanamycin	100	100	25
Carbenicillin	50	50	12.5
Cefsulodine	1.56	1.56	0.78
Moxalactam	12.5	12.5	3.13
Tetracycline	25	25	25
Chloramphenicol	50	50	50

[a] MICs were determined by the agar dilution method with Meuller-Hinton agar.

nutrient agar containing 1.56 μg of norfloxacin per ml. *nfxA* was cotransducible with *leu-9005* (0.8% linkage) and *hex-9001* (38% linkage) but not with *met-9020*. The *nalA* loci of PAO1052 and PAO477 examined as controls gave similar cotransduction values with *hex-9001* (37 to 44% linkage) and *leu-9005* (0.8% linkage), suggesting that *nfxA* is an allele of *nalA*.

To map the *nfxB* locus more precisely with respect to other markers near *ilvBC*, we crossed PAO4031 and KH4013E(FP5). Selection was made for each auxotrophic marker, and 100 recombinants for each marker were scored for coinheritance of *nfxB* by being streaked on nutrient agar containing 3.13 μg of norfloxacin per ml. Recombinant analysis revealed that the *nfxB* marker was highly linked to *ilv-9023* (87% linkage), *pro-9031* (79%), and *his-9015* (66%), with selection for *ilv-9023*$^+$, *pro-9031*$^+$, and *his-9015*$^+$, respectively.

The highest linkage (97%) was seen when *pro-9031*$^+$ and *ilv-9023*$^+$ were coselected, suggesting that the *nfxB* mutation might be located between *pro-9031* and *ilv-9023*. *pro-9031* and *ilv-9023* are at about 4 and 8 min on the PAO chromosome, respectively (H. Matsumoto, personal communication). All of the *nfxB* recombinants tested had the same susceptibility to antimicrobial agents as that of the donor strain (KH4013E). However, attempts to cotransduce *nfxA* by F116L, G101, or E79tv-1 with *proB* (in PAO1052), *pro-9031* and *ilv-9023* (in PAO4031), *ilv-9059* and *car-9003* (in PAO4293), and *ilv-9001* (in PAO2142) were unsuccessful.

Uptake of norfloxacin by the *nfxB* mutant. Its susceptibility to antimicrobial agents suggested that the *nfxB* mutant might be altered in membrane permeability. To check this possibility, we compared the uptake of norfloxacin by the *nfxB* mutant with the uptake by the parent strain and the *nfxA* mutant (KH4023) by a previously described method (12) (Fig. 1).

The *nfxB* mutant (KH4013E) showed about a threefold-lower norfloxacin uptake than the parent strain and *nfxA* mutant did, indicating that decreased norfloxacin uptake may be the resistance mechanism in the *nfxB* mutant. As expected, the *nfxA* mutant showed the same uptake rate of norfloxacin as the parent strain did. It was thus considered that the resistance mechanism of the *nfxA* mutant was not alteration in the cell permeability of quinolones.

Outer membrane proteins and LPS of *nfxA* mutants. We previously found (13) that resistance to norfloxacin in *E. coli* K-12 was associated with decreased outer membrane protein

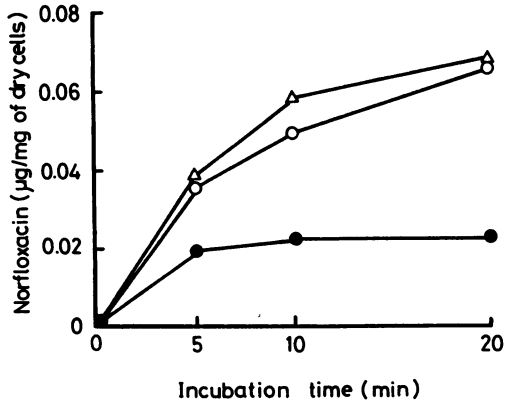

FIG. 1. Uptake of norfloxacin by mutants of *P. aeruginosa* PAO4009. Details are given in the text. Symbols: ○, PAO4009 (wild type); △, KH4023 (*nfxA* mutant); ●, KH4013E (*nfxB* mutant).

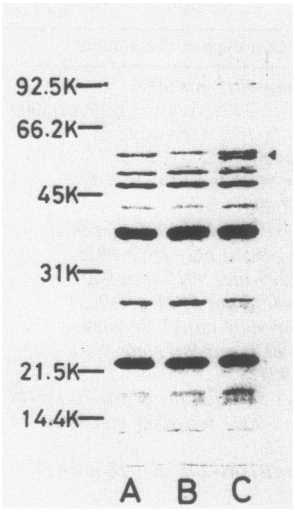

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of norfloxacin-resistant mutants of *P. aeruginosa* PAO. Lanes: A, PAO4009; B, KH4023 (*nfxA* mutant of PAO4009); C, KH4013E (*nfxB* mutant of PAO4009). Molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were phosphorylase B (92,500) bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,500). The arrow designates the 54K protein that is visualized in the *nfxB* mutant.

OmpF. The outer membrane proteins of the *nfxB* mutant (KH4013E), the *nfxA* mutant (KH4023), and the wild-type strain (PAO4009) were prepared by the method of Poxton et al. (31) and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A new outer membrane protein with an apparent molecular weight of 54,000 (the 54K protein), which was not visualized in the parent strain or the *nfxA* mutant, appeared in the *nfxB* mutant (Fig. 2). *nfxB* recombinants of PAO4031 and PAO2142 obtained by mating with KH4013E also possessed the 54K outer membrane protein which was not seen in the parent strain (data not shown).

To gain further information regarding the state of the outer membrane, we checked the susceptibility of the mutant to the smooth-specific phage E79tv-1 and to aeruginocin AP41 and analyzed the LPS of the mutant and parent strains by the method of Tsai and Frasch (38). No differences in susceptibility to E79tv-1 and aeruginocin AP41 or in LPS banding patterns between *nfxB* mutants and the parent strain were observed (data not shown).

DISCUSSION

There are a few reports of norfloxacin resistance mechanisms in members of the family *Enterobacteriaceae* (13, 16, 34); however, mechanisms of resistance to norfloxacin and other new quinolones by *P. aeruginosa* have not been studied in detail. The results presented here provide the first information on the genetic and biochemical properties of norfloxacin-resistant mutants of *P. aeruginosa* PAO and suggest particularly that alteration in cell permeability to norfloxacin is involved.

It has been reported that mechanisms of resistance to nalidixic acid in *P. aeruginosa* involve alterations in replicative DNA synthesis in permeabilized cells and alterations in cell permeability as a result of mutations in the *nalA* and

nalB loci, respectively (32). One of the norfloxacin resistance genes, *nfxA*, mapped between *hex-9005* and *leu-9001* and appeared to be an allele of *nalA*, suggesting that resistance encoded by the *nalA* gene is a mechanism common to quinolones in *P. aeruginosa*. More recently, Inoue and co-workers found that the A subunit of DNA gyrase isolated from *nalA* mutants of *P. aeruginosa* PAO1 was resistant to nalidixic acid and other quinolones including norfloxacin (Y. Inoue, K. Sato, T. Fujii, K. Hirai, M. Inoue, S. Iyobe, and S. Mitsuhashi, submitted for publication). Therefore, the *nalA* gene must encode the A subunit of DNA gyrase that is a target of quinolone action in *P. aeruginosa* PAO.

Alteration in outer membrane permeability is also a potential mechanism of resistance to quinolones (2, 8, 32, 34). Resistance to nalidixic acid is associated with pleiotropic resistance to other antimicrobial agents and with changes in outer membrane proteins in *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Serratia marcescens* (8, 34). We and Hooper et al. reported three kinds of norfloxacin-resistant mutants of *E. coli* KL-16 that show a decrease in OmpF protein, which functions as a porin for the penetration of various antimicrobial agents including quinolones (13, 16). In members of the family *Enterobacteriaceae*, most alterations in the permeability to quinolones have been associated with the decrease of specific outer proteins (8, 34). However, we have reported here that the altered outer membrane permeability to norfloxacin in *P. aeruginosa* is associated with the appearance of a new 54K outer membrane protein. Furthermore, we could not obtain any norfloxacin-resistant mutants with a decrease in specific outer membrane proteins such as F-porin proteins (data not shown). These findings suggest that the mechanism of resistance to norfloxacin involving outer membrane permeability may be different from that found in cell permeability mutants in *E. coli*. At present, the function of the 54K protein in the *nfxB* mutant of *P. aeruginosa* PAO is not clear, but it may act as a permeability barrier to norfloxacin.

It has been suggested that permeability to aminoglycoside and beta-lactam antibiotics is influenced by changes in the LPS structure and outer membrane proteins in *P. aeruginosa* (1, 3, 6, 7, 19, 22, 27, 29, 37). No differences in the LPS banding pattern and susceptibility to E79tv-1 (an LPS-specific phage) and aeruginocin AP41 were observed for *nfxB* mutants relative to wild-type strains and *nfxA* mutants, suggesting that alteration in the LPS structure may not have occurred in the *nfxB* mutant. Moreover, significant differences between *nfxB* mutants and wild-type strains could not be found in the outer membrane profile except with the 54K protein. It is possible that the 54K outer membrane protein in *nfxB* mutants interacts with either LPS or other membrane proteins and thus alters the cell permeability to beta-lactam and aminoglycoside antibiotics or that these antibiotics penetrate into the cell membrane by using 54K protein as a porin channel.

The *nfxB* mutation was located between *proB* and *ilvBC* on the *P. aeruginosa* PAO chromosome. The *tolA* locus in mutants showing hypersusceptibility to aminoglycoside antibiotics is located at 10 min (near *ilvBC*) and is closely linked to *carA* (27). The properties of a *tolA* mutant showing hypersusceptibility to aminoglycoside resemble those of the *nfxA* mutant, but the susceptibility of *tolA* and *nfxB* mutants to aeruginocin AP41 and beta-lactam antibiotics is distinct (27). The *tolA* mutant showed 90 to 95% cotransduction with the *carA* marker (27), but attempts to cotransduce *nfxB* with *car-9003* (*carA*) in PAO4293 by using phage F116L or G101 were unsuccessful, and a selected *ilv-9059* marker coinherited *nfxB* with higher frequency than did a selected *car-9003* marker (data not shown). These considerations make it likely that the *nfxB* mutation is distinct genetically from the *tolA* mutation. Genes *blsA1* and *tpsA1*, which are associated with hypersusceptibility to beta-lactam antibiotics, have also been reported (6). These markers were closely linked to the *nalB* locus (32 min in the PAO chromosome), encoding an alteration in cell permeability to nalidixic acid and carbenicillin (6). However, these genes mapped at a different location from the *nfxB* locus on the *P. aeruginosa* PAO chromosome (33). These results suggest that the *nfxB* gene might be a novel gene in *P. aeruginosa* PAO; however, further genetic studies are in progress.

We found previously that the outer membrane permeability to new quinolones such as norfloxacin in *E. coli* and *Salmonella typhimurium* differed from that to nalidixic acid (12). The incomplete cross-resistance among quinolones in *E. coli* is known (18, 41), and this phenomenon was also observed in *P. aeruginosa* (32). The *nfxB* mutation resulted in a 4- to 16-fold increase in the MIC of new quinolones but only a slight (2-fold) increase in the MIC of nalidixic acid and pipemidic acid. The results of this study demonstrate that mechanisms involving outer membrane permeability to new quinolones in *P. aeruginosa* may differ from those to nalidixic acid and pipemidic acid. Further studies of the *nfxB* mutant will provide additional information on the mechanisms of outer membrane permeability to norfloxacin and other new quinolones and on the intrinsic permeability barrier to beta-lactam and aminoglycoside antibiotics in *P. aeruginosa*.

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