Mechanisms of High-Level Resistance to Quinolones in Urinary Tract Isolates of Pseudomonas aeruginosa

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Twenty-eight strains of Pseudomonas aeruginosa with various degrees of norfloxacin resistance were isolated from patients with urinary tract infections. P. aeruginosa strains (norfloxacin MICs, 3.13 to 200 µg/ml) were transformed by either pPAW207 or pNF111 plasmid DNA, which included either the gyrA or nfxB gene, respectively. For transformants with pPAW207, norfloxacin MICs decreased 8- to 128-fold. It was suggested that moderate and high degrees of resistance to norfloxacin were expressed as a result of alterations in gyrA. No strain manifesting only an alteration in nfxB permeability was observed. The MICs of norfloxacin (200 µg/ml) for two P. aeruginosa strains, GN17605 and GN17434-2, were decreased following transformation not only by pPAW207 but also by pNF111. Analysis of outer membrane proteins disclosed the presence of a 54,000-Da protein in these parent strains that was not expressed in the pNF111 transformants. The level of accumulation of norfloxacin by the pNF111 transformant of GN17605 was higher than that by the parent strain. The norfloxacin susceptibility of DNA gyrase subunit A purified from GN17605 was only 1/35th that of the gyrase containing a subunit A from P. aeruginosa PAO1. These findings suggest that GN17605 is a gyrA-nfxB double mutant and that strain GN17434-2 possesses double mutations in both nfxB and some unknown gene.

Quinolones have a broad spectrum of antibacterial activity against gram-positive and -negative bacteria and have strong bactericidal activities (2, 11, 16, 17). These quinolones are therefore in wide use and have proven to be clinically valuable for the treatment of patients with a variety of types of infections. Recently, however, strains of bacteria resistant to the quinolones, especially Staphylococcus aureus and Pseudomonas aeruginosa, have frequently been isolated and have become a serious clinical problem.

The mechanisms of resistance to quinolones in P. aeruginosa include reductions in the susceptibilities of intracellular targets to the drugs, DNA gyrase, and alterations in outer membrane permeability (6, 7). Many findings relating an alteration in an outer membrane protein and its effect on resistance to quinolones have been presented by Hirai et al. (6), Chamberland et al. (1), and Fukuda et al. (3). In addition, mechanisms of resistance, including alterations in both DNA gyrase and outer membrane permeability, have been reported recently in studies of clinical isolates of P. aeruginosa (8, 19).

In the study described here, P. aeruginosa strains isolated from patients with urinary tract infections were analyzed by transformation with plasmids carrying either a gyrA or a nfxB gene in order to determine the mechanisms of resistance to quinolones.

MATERIALS AND METHODS

Bacterial strains. Twenty-eight strains of norfloxacin-resistant (norfloxacin MIC, >1.56 µg/ml) P. aeruginosa originating from patients with urinary tract infections were used in the study. These strains were isolated between 1988 and 1991 from various hospitals in Japan and were maintained in glycerol at −80°C. P. aeruginosa PA04009 and KH4013E, the nfxB type mutant derived from PA04009 (6), were also used in the study.

Antimicrobial agents. Norfloxacin (Kyorin Seiyaku Co., Ltd.), ciprofloxacin (Bayer Yakuhin, Ltd.), imipenem (Banyu Seiyaku Co., Ltd.), gentamicin (Schering-Plough Co., Ltd.), carbencillin (Fujiwara Yakuhin Kogyo Co., Ltd.), and ceftazidime (Nihon Glaxo Co., Ltd.) were used.

Antimicrobial susceptibility tests. Antimicrobial susceptibility was determined by the twofold agar dilution method with Sensitivity Disk Agar-N (Nissui Pharmaceutical, Tokyo, Japan) by using a final inoculum of 10⁴ CFU per spot. For preculture of transformant strains, Sensitivity Test Broth (Nissui) that included 400 µg of carbenicillin per ml was used. The MIC was defined as the lowest drug concentration that inhibited the visible growth of P. aeruginosa after 20 h at 37°C.

Preparation of plasmid DNA and transformation. Plasmid pPAW207 containing the wild-type Escherichia coli gyrA gene and plasmid pNF111 containing the wild-type P. aeruginosa nfxB gene, which mediates the effects related to membrane permeability, were used for transformation (12, 19). These plasmids were prepared by CsCl density gradient centrifugation. Competent cells of norfloxacin-resistant (norfloxacin MIC, >1.56 µg/ml) P. aeruginosa strains were prepared by using early-log-phase cells in L broth. Transformation was carried out with 100 mM MgCl₂-treated P. aeruginosa (9). The transformants were selected on bromothymol blue agar containing 400 µg of carbenicillin per ml, which was the selective marker for pPAW207 and pNF111 plasmids.

Characterization of outer membrane proteins. Outer membrane proteins of two clinical strains of P. aeruginosa, P. aeruginosa KH4013E, and pNF111 transformants were prepared by the method of Poxton et al. (13). Fifty-microgram samples of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gel was stained with Coomassie brilliant blue.

Norfloxacin accumulation by cells. The accumulation of norfloxacin by P. aeruginosa GN17605 and the pNF111 transformant were measured by a previously described method (6),

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TABLE 1. Comparative MICs for the parent and transformant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NFLX</td>
</tr>
<tr>
<td>GN17554</td>
<td>200</td>
</tr>
<tr>
<td>GN17554(pPAW207)</td>
<td>1.56</td>
</tr>
<tr>
<td>GN17436</td>
<td>100</td>
</tr>
<tr>
<td>GN17436(pPAW207)</td>
<td>1.56</td>
</tr>
<tr>
<td>GN17544</td>
<td>100</td>
</tr>
<tr>
<td>GN17544(pPAW207)</td>
<td>1.56</td>
</tr>
<tr>
<td>GN17418</td>
<td>50</td>
</tr>
<tr>
<td>GN17418(pPAW207)</td>
<td>0.78</td>
</tr>
<tr>
<td>GN17564</td>
<td>12.5</td>
</tr>
<tr>
<td>GN17564(pPAW207)</td>
<td>1.56</td>
</tr>
<tr>
<td>GN17585</td>
<td>6.25</td>
</tr>
<tr>
<td>GN17585(pPAW207)</td>
<td>0.78</td>
</tr>
<tr>
<td>GN17582</td>
<td>3.13</td>
</tr>
<tr>
<td>GN17582(pPAW207)</td>
<td>0.39</td>
</tr>
<tr>
<td>GN17605</td>
<td>200</td>
</tr>
<tr>
<td>GN17605(pPAW207)</td>
<td>6.25</td>
</tr>
<tr>
<td>GN17605(pNF111)c</td>
<td>25</td>
</tr>
<tr>
<td>GN17434-2</td>
<td>200</td>
</tr>
<tr>
<td>GN17434-2(pPAW207)</td>
<td>12.5</td>
</tr>
<tr>
<td>GN17434-2(pNF111)</td>
<td>100</td>
</tr>
</tbody>
</table>

* MICs were determined by the agar dilution method. Abbreviations: NFLX, norfloxacin; CPFX, ciprofloxacin; CBPC, carbenicillin; GM, gentamicin; IPM, imipenem; CAZ, ceftazidime.

b pPAW207 transormant of the norfloxacin-resistant strain.
c pNF111 transformant of the norfloxacin-resistant strain.

with a modification; that is, cells suspended in 5% acetic acid were boiled for 7 min, and the norfloxacin concentration in the eluted samples was measured by high-pressure liquid chromatography.

Preparation of DNA gyrase and supercoiling assay. The subunit A and B proteins of DNA gyrase were purified from P. aeruginosa GN17605 and PAO1 by a previously reported method (7). In that study, the specific activities of purified subunit were from 102 to 442 U/mg of protein. Subunit A purified from PAO1 (As) and GN17605 (Ar) and subunit B purified from PAO1 (Bs) and GN17605 (Br) were mixed, respectively. The reaction mixture, which included DNA gyrase and norfloxacin, was incubated for 1 h at 37°C, and then the reaction was stopped by the addition of 1% proteinase K (Sigma Chemical, St. Louis, Mo.) and the reaction mixture was subjected to 0.8% agarose gel electrophoresis. An ethidium bromide-stained gel was photographed during UV transillumination, and the photographic negatives were analyzed with a densiometer.

RESULTS

Antimicrobial susceptibilities of parents and transformants. Transformation of 28 P. aeruginosa strains resistant to norfloxacin (norfloxacin MIC, >1.56 µg/ml) was performed with two plasmids. In that study, successful transformation was observed for 9 of 28 strains. The susceptibilities of seven parent strains and pPAW207 transformants to quinolones and other antimicrobial agents are given in Table 1. No change in susceptibility to norfloxacin or ciprofloxacin was observed for the pNF111 (nfxB) transformants of these strains. The parent strains with a moderate degree of resistance to norfloxacin (norfloxacin MICs, 3.13 to 12.5 µg/ml) or a high degree of resistance to it (norfloxacin MICs, 50 to 200 µg/ml) became susceptible to norfloxacin (norfloxacin MICs, 0.39 to 1.56 µg/ml) after transformation with the pPAW207 ( gyrA) plasmid. The increase in susceptibility to norfloxacin between parents and transformants was 8- to 128-fold. Similar changes in susceptibility were found for ciprofloxacin; in the case of strain GN17544, however, the increase in susceptibility to ciprofloxacin was fourfold greater than that to norfloxacin. No significant differences in the MICs of any of the agents other than carbenicillin (marker for resistance on the pPAW207 and pNF111 plasmids) were observed between parents and transformants.

Following transformation, the MICs of norfloxacin for both the pPAW207 and pNF111 transformants were decreased in the case of strains GN17605 and GN17434-2 (Table 1). With the exception of these two strains, the MICs of norfloxacin for the pNF111 transformants were not decreased. For strain GN17605, susceptibility to norfloxacin increased 32-fold for the pPAW207 transformant and 8-fold for the pNF111 transformant. For the GN17434-2 strain, susceptibility to norfloxacin increased 16-fold for the pPAW207 transformant and 2-fold for the pNF111 transformant. Susceptibilities to ciprofloxacin for each pPAW207 and pNF111 transformant of GN17605 and GN17434-2 increased 128-, 8-, 64-, and 4-fold, respectively. In the case of both transformants, the MIC of carbenicillin was increased to >400 µg/ml, while susceptibil-
ties to gentamicin and β-lactams (except for carbenicillin) were decreased in pNF111 transformants.

Outer membrane proteins of the pNF111 transformant. The outer membrane protein profiles of P. aeruginosa GN17605, GN17434-2, and KH4013E and the pNF111 transformants of these strains are shown in Fig. 1. An outer membrane protein (54,000 Da) which was clearly detected in the GN17605 and GN17434-2 parent strains was not present after transformation by the pNF111 plasmid (arrow in Fig. 1). The amount of the 54,000-Da protein visualized in KH4013E (nfxB type mutant of PAO4009) was decreased in the pNF111 transformant.

Norfloxacin accumulation by cells. The accumulation of norfloxacin by the pNF111 transformant of P. aeruginosa GN17605 was compared with that by the parent strain (Fig. 2). The parent strains exhibited almost no accumulation of norfloxacin after incubation for 20 min. On the other hand, accumulation by the transformant was increased to 23 ng/mg (dry weight) of the cells after incubation.

Susceptibility of reconstituted DNA gyrase. The degrees of inhibition (as 50% inhibitory concentration) of the supercoiling activities of the reconstituted DNA gyrase purified from P. aeruginosa PA01 and GN17605 by norfloxacin were 0.95, 0.53, 32.2, and 18.6 µg/ml for subunits A (As) and B (Bs) from PAO1, subunit A from PAO1 and subunit B (Br) from GN17605, subunit A from GN17605 (Ar) and subunit B from PAO1, and subunits A and B from GN17605, respectively. The 50% inhibitory concentrations of norfloxacin for AsBs and ArBr gyrase were approximately 35-fold greater than those for AsBs and AsBr, respectively. This finding indicates that susceptibility to norfloxacin of the subunit A protein of GN17605 decreased to 1/35th of that of PAO1. This is comparable to the fact that the MIC of norfloxacin for the parent GN17605 strain was also decreased approximately 35-fold by transformation with the pPAW207 plasmid (Table 1).

**DISCUSSION**

In members of the family Enterobacteriaceae, resistance to quinolones is often due to a reduction in drug permeation associated with a decrease in OmpF, the principal porin protein (4, 5, 15). On the other hand, many mechanisms of resistance have been reported for P. aeruginosa (1, 3, 6, 10, 19). Yoshida et al. (19) studied the proportion of gyr mutations in quinolone-resistant clinical isolates of P. aeruginosa and found that the gyrA mutation conferred various degrees of quinolone resistance on susceptible strains. Also in our study, strains of P. aeruginosa obtained various degrees (8- to 128-fold) of norfloxacin resistance by alteration of gyrA alone. Yoshida et al. (18) also reported that the degree of resistance to quinolones seems to be related to the site of mutation on the E. coli gyrA gene. Variation in the degree of resistance to norfloxacin by the P. aeruginosa clinical isolates used in the present study might be related to the site of mutation of the gyrA gene, as was the case for the E. coli gene noted above. It was observed that the rate of reduction in MICs by transformation with the 35-fold pPAW207 differed among norfloxacin, ciprofloxacin, and the other quinolone antibiotics (data not shown). This finding suggests that the effect of the gyrA mutation differed between compounds.

In addition to mutations in DNA gyrase, which appears to be the major factor for the determination of resistance to quinolones, decreases in drug permeation can also result in resistance to quinolones by P. aeruginosa. Chamberland et al. (1) reported the finding of a correlation between altered cell permeability on the one hand and a reduction in outer membrane protein G (25,500 Da) and the loss of a 40,000-Da outer membrane protein on the other. Fukuda et al. (3) recently characterized a new norfloxacin resistance gene (nfxC) in P. aeruginosa PAO. The nfxC gene was mapped near 46 min on the chromosome, and a decrease in a 46,000-Da outer membrane protein and an increase in a 50,000-Da protein were observed in the nfxC mutant. Hira et al. (6) isolated a nfxB mutant of P. aeruginosa PAO1 which showed alterations of outer membrane permeability to norfloxacin. Their analysis of the outer membrane proteins demonstrated the appearance of a 54,000-Da protein in the nfxB mutant. Furthermore, resistant strains of the nfxB mutant type have been isolated clinically (8).

In our study, no strain with only nfxB mutations, such as that reported by Jakies et al. (8), was observed. We assumed that no strain had an nfxB mutation because most strains tested in the present study showed high-level resistance to norfloxacin, which differed from the case in the study of Jakies et al. (8). However, for two strains, P. aeruginosa GN17605 and GN17434-2, the MICs of norfloxacin and ciprofloxacin were decreased by transformation not only with pPAW207 but also...
with pNF11. This finding suggests the possibility of a double mutation in these two strains. Thus, they had a high degree of resistance to norfloxacin and ciprofloxacin by two different mechanisms, a decrease in the susceptibility to DNA gyrase subunit A (gyrA alteration) and a decrease in cell permeability (alteration of the outer membrane because of an nfxB mutation). Robillard (14) has reported clinical isolates of P. aeruginosa which featured a gyrA mutation coupled with a nongyrase A mechanism of resistance. However, no in-depth analysis was done on the strains that had two mutations. In the present study, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane proteins of GN17605 and GN17434-2 demonstrated the presence of a 54,000-Da protein. This protein was not present following transformation by the pNF111 plasmid. In addition, in P. aeruginosa GN17605, the accumulation of norfloxacin by the parent strain was less than that by the pNF111 transformant. These findings were in agreement with those of Hirai et al. (6), and accordingly, the occurrence of a mutation typed for nfxB was proven for GN17605. We determined the susceptibility to norfloxacin of the DNA gyrase purified from GN17605 and found a reduction in the susceptibility of DNA gyrase subunit A to this antibiotic. Therefore, the alteration of the gyrA gene occurred along with that of the nfxB gene in P. aeruginosa GN17605. It is suggested that the results obtained for GN17434-2 will be the same as those obtained for GN17605.

In the present study, we demonstrated the presence among quinolone-resistant P. aeruginosa strains not only of strains with single mutations showing various degrees of resistance but also strains with double mutations. High degrees of resistance to quinolones in P. aeruginosa (norfloxacin MICs, 100 to 200 μg/ml) were caused by either a single mutation (gyrA) or a double mutations (gyrA-nfxB or another mutation) in the present study. These findings suggest the possibility that the mechanisms of resistance to quinolones in P. aeruginosa will be quite complex in the future.

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REFERENCES